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Protein surface display and virus like particle generation in insect cells

Master Thesis

submitted by

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Abstract

Protein surface display in insect cells offers manifold possibilities in the fields of library generation and the establishment of screening platforms. Thereby, insect cells provide benefits like easy handling and fast production, compared to mammalian cell lines, and the capability of eukaryotic protein processing. The generation of virus like particles (VLPs) emerged in recent years because of their application as possible vaccines, featuring strong immune responses, and as a tool in the domain of diagnostics.

In this study the baculovirus expression vector system (BEVS) was used to recombinantly express different model proteins on surface of insect cells. The same proteins, in combination with the influenza A matrix protein M1, were further applied to induce budding of virus like particles. The aim was to prove, both, the display of proteins and the generation of VLPs, together with a comparison of the suitability of two different insect cell lines.

First, expression constructs were cloned and recombinant baculoviruses derived thereof were used for infection of *Spodoptera frugiperda Sf9* cells and *Trichopulsia ni* BTI-TN5B1-4 “HighFive™” cells. Subsequently SDS-PAGE and Western Blot analysis assured the successful expression of these constructs. The display of selected proteins could be successfully demonstrated by fluorescent activated cell sorting (FACS) analysis. Ultracentrifugation combined with a discontinuous sucrose gradient was performed in order to separate baculoviruses from VLPs. After pooling of the fractions, in which the model proteins could be found, another round of ultracentrifugation was conducted, enabling a high degree of purity necessary for subsequent transmission electron microscopy (TEM). The images obtained from TEM showed baculoviruses as well as spherical virus like particle structures.

Zusammenfassung

Das Protein Oberflächen-Display in Insektenzellen eröffnet vielfältige Möglichkeiten im Bereich der Generation von Libraries und der Etablierung von Screening Plattformen. Dabei bieten Insektenzellen Vorteile wie eine einfache Handhabung und schnelle Produktion, im Vergleich zu Säugetierzellen, und die Fähigkeit, eukaryotische posttranslationale Modifikationen durchzuführen. In den letzten Jahren ist die Produktion von virusartigen Partikeln (VLPs) bedeutender geworden, wegen der Möglichkeit, diese als Vakzin zu verwenden, welche zu starken Immunantworten führen, und diese als Werkzeug im Bereich der Diagnostik einzusetzen.

In dieser Arbeit wurde das Baculovirus Expressionssystem (BEVS) verwendet, um rekombinant verschiedene Modellproteine an der Oberfläche von Insektenzellen zu exprimieren. Die Modellproteine wurden gemeinsam mit dem Influenza Matrixprotein M1 weiters dafür eingesetzt, um die Knospung von VLPs zu erreichen. Das Ziel war es, sowohl die Modellproteine an der Oberfläche der Zellen als auch die Generierung von VLPs nachzuweisen, sowie die Eignung zweier verschiedener Insektenzelllinien dafür zu vergleichen.

Zuerst wurden Expressionskonstrukte kloniert und rekombinante Baculoviren daraus erzeugt, um damit die Insektenzelllinien *Spodoptera frugiperda Sf9* und *Trichopulsia ni* BTI-TN5B1-4 “HighFive™“ zu infizieren. Darauffolgende SDS-PAGE und Western Blot Analyse zeigten die erfolgreiche Expression dieser Konstrukte. Das Display der gewählten Proteine konnte mittels Durchflusszytometrie erfolgreich bewiesen werden.

Eine Ultrazentrifugation in Kombination mit einem diskontinuierlichen Sucrose Gradienten wurde durchgeführt, um Baculoviren von VLPs zu trennen. Danach wurde eine erneute Ultrazentrifugation ausgeführt, um einen hohen Grad an Reinheit zu erreichen, welcher für die anschließende Transmissionselektronenmikroskopie (TEM) notwendig war. Die erhaltenen Bilder von der TEM zeigten Baculoviren, sowie auch Strukturen von kugelförmigen virusartigen Partikeln.

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1 Introduction

1.1 Recombinant protein production in insect cells

In recent years the use of insect cells, in combination with different baculoviral expression vector systems, for recombinant protein production has become increasingly popular (Palmberger et al., 2012; Altmann et al., 1999). The first continuous insect cell lines have been successfully established in 1962 (Vlak et al., 2006). Since then, several cell lines have been developed. Nowadays the most used cell lines for recombinant protein production are the *Sf9* and *Sf*21 cell line, derived from the fall armyworm *Spodoptera frugiperda* (Vaughn et al., 1977; Liu et al., 2013) and the *Trichoplusia ni* BTI-TN5B1-4 “High Five” (Granados et al. 1994; Wickham and Nemerow 1993) cell line, isolated from the American cabbage looper *Trichoplusia ni* (Durocher et al., 2009; Murhammer, 2007).

Insect cells are gaining popularity because of the relative ease and speed, compared to mammalian cells, with which heterologous proteins can be expressed (Altmann et al., 1999). In addition, they possess eukaryotic protein processing capabilities (Yin et al., 2007), like the cleavage of signal peptides, acetylation, and the formation of glycans (Altmann et al., 1999) and disulfide bonds (Vlak et al., 2006), oligomerization, folding, phosphorylation and glycosylation (Liu et al., 2013).
Insect cells are able to grow on serum free media, are safe, easy to handle and can be grown to cell densities higher than those of mammalian cells (Kollewe et al., 2013)

1.2 Baculovirus

The baculovirus expression vector system (BEVS) allows the expression of recombinant proteins and recombinant protein complexes with many subunits, like it is the case for most eukaryotic proteins (Trowitzsch et al., 2010), the expression of large proteins and the expression of multiple proteins required for virus like particle generation. The production is fast, provides high yields and allows efficient protein secretion (Kost el al., 2005).

Comparing the effects of baculoviral infection on the cell lines *Trichoplusia ni* BTI-TN5B1-4 and *Spodoptera frugiperda* (*Sf*) showed that the “High Five” cell line exhibits higher susceptibility to baculovirus infection than *Sf9* cells, whereas *Sf9* cells show a higher capacity for production of infectious virus particles. High Five cells show higher product yields, respective recombinant protein expression, as compared to *Sf9* cells. Overall, the High Five cell line is more efficient in terms of secreting proteins (Wilde et al., 2013;

Krammer et al., 2010). Nevertheless, *Sf9* is the standard cell line for virus generation, virus amplification and performing plaque assay for titer determination.

1.2.1 Structure and classification of baculovirus

Baculoviruses are a family of enveloped, large double-stranded DNA viruses that predominantly infect insects. The size of the genome of baculoviruses ranges from 80 to 180 kb and contains between 90 and 180 genes (Herniou et al., 2012). The viral genome is packed into rod-shaped nucleocapsids (NC) of 30-70 nm in diameter and 200-400 nm in length (Jehle et al., 2006).The major capsid protein is vp39, which forms a spiral structure sheathing the double-stranded viral genome (see figure 1-1).
The family of Baculoviridae consists of four genera: Alphabaculovirus, Betabaculovirus, Gammabaculovirus and Deltabaculovirus on the basis of genome phylogeny (Herniou et al., 2012). The Alphabaculovirus and Betabaculovirus contain lepidoperan-specific (moths and butterflies) nucleopolyhedroviruses (NPVs) and granuloviruses (GVs), while Gammabaculovirus and Deltabaculovirus encompass dipteran (mosquitoes and flies) and hymenopteran (wasps and bees) NPVs, respectively (Yin et al., 2013). The Alphabaculoviruses are further divided into group I and group II (Jehle et al., 2006; Zanotto et al., 1993). These two groups differ in the occurrence of two different budded virus (BV) envelope glycoproteins, gp64 (group I) and F protein (group II). These proteins are required for virus-cell fusion, receptor binding and viral propagation (Lung et al., 2002). Recently it has been shown that, besides gp64 and the F protein, another BV structural protein, namely ubiquitin, plays an important role for the BV structure (see figure 1-1) by extending an inner layer on the viral envelope (Wang et al., 2016).
In the past baculoviruses were characterized based on their occlusion body morphology: the nuleopolyhedroviruses and granuloviruses. These occlusion bodies (see figure 1-2) are highly stable and can resist most normal environmental conditions, allowing the virions to remain indefinitely infectious (Rohrmann, 2013). NPVs consist of a crystalline matrix composed of a protein called polyhedrin, whereas GVs are composed of the closely related protein granulin.

The most well studied baculovirus is the *Autographa californica* multicapsid nucleopolyhedrovirus (*Ac*MNPV). The size of its genome is about 134 kb and contains up to

150 genes (Kool et al., 1995). *Ac*MNPV is the most widely used expression vector for the generation of recombinant baculovirus (Murhammer, 2007; Rohrmann, 2013).

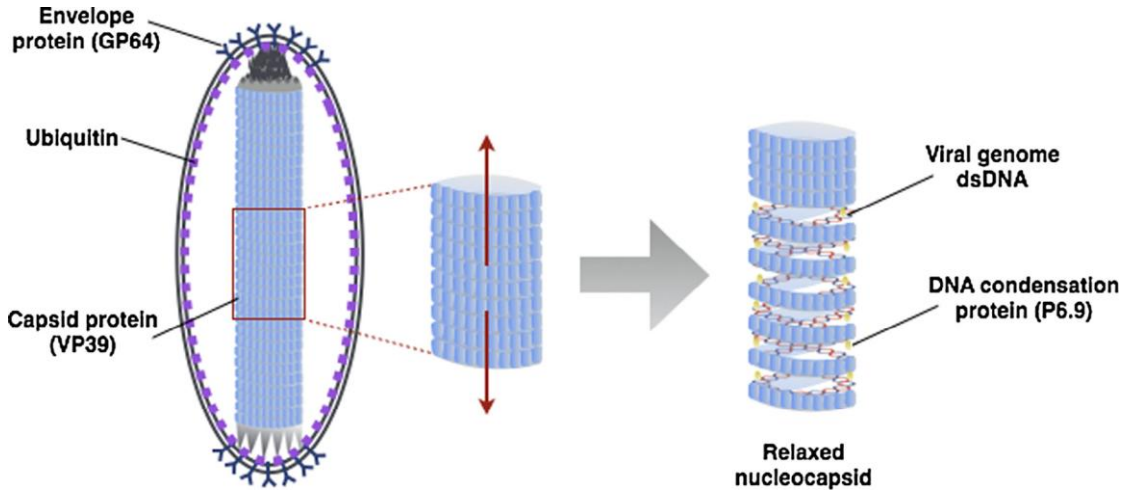


Figure 1-1: Structural model of the baculovirus budded virus (BV) represented by AcMNPV. (Wang et al., 2016)

1.2.2 Baculovirus gene expression and replication

Baculovirus genes are expressed in a transcriptional cascade in which each phase is dependent on the expression of genes from the previous phase (Kool et al., 1995). Baculoviruses begin their infection cycle by employing enhancers and transcriptional activators to exploit the host transcriptional apparatus. This early phase aims for establishing the infection and initiating viral DNA replication as well as other early functions. The genes for the early events are transcribed by the host RNA polymerase, whereas later genes are transcribed by a baculovirus-encoded RNA polymerase. A feature of baculovirus is that they occupy a combination of cellular and viral polymerases (Rohrmann, 2013).
In the late phase of infection viral DNA is replicated, structural proteins for BV and ODV are produced and the virus is assembled. In the very late stage of infection polyhedrin and p10 are expressed. These two proteins are necessary for virion occlusion. (Miller, 1997; Murhammer, 2007)

1.2.3 Baculovirus life cycle

Two phenotypes of infectious enveloped virions are produced during the infection cycle: the occlusion-derived viruses (ODV), found within the protective occlusion bodies, that initiate infection of the midgut of the host upon oral ingestion of occlusion bodies (OB) and budded viruses (BV), that are responsible for cell-to-cell transmission and further systemic infection (Wang et al., 2016). ODVs (see figure 1-2) acquire their newly assembled envelopes in the nucleus in the late phase of infection, whereas BVs (see figure 1-1) obtain their envelope from the plasma membrane upon budding into the extracellular space or neighboring cells (Okano et al., 2006). The envelope consists amongst other proteins mainly of the gp64 glycoprotein, essential for virus-cell fusion and endocytosis by the host cell (Blissard et al., 1992).

When released from the occlusion body by the alkaline environment of the insect gut, the ODV initiates infection of the insect by attacking epithelial cells of the midgut (Monsma et al., 1996). Viruses are then produced from infected midgut epithelial cells in budded form, leading to systemic infection of the whole insect. In the late stage of infection ODVs are formed within the nuclei of infected cells and are released upon the death of the insect (Keddie et al., 1989).

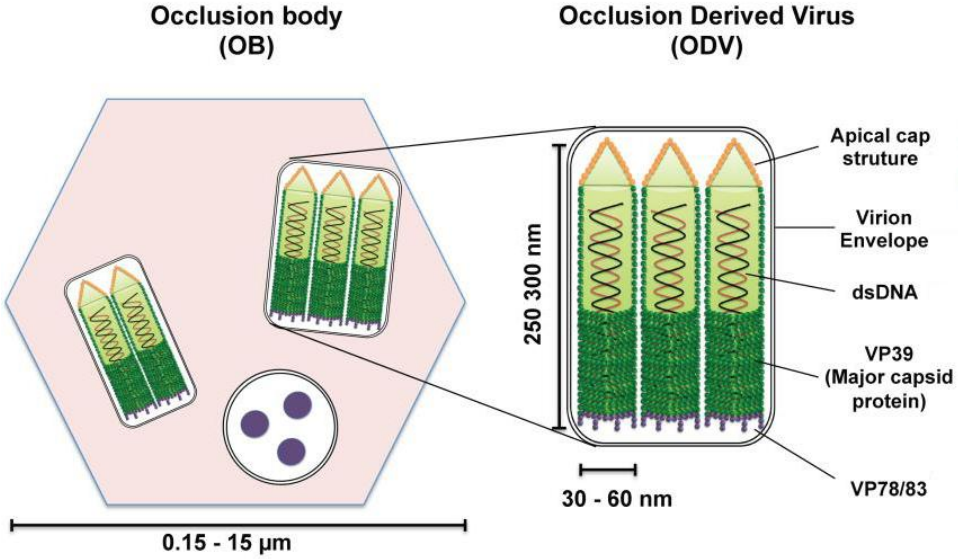


Figure 1-2: Schematic diagrams of the structure of baculovirus occlusion bodies (OB) and occlusion-derived virion (ODV). ODVs are embedded in a crystalline matrix of protein to form OBs. (Au et al., 2013)

1.2.4 Baculovirus as expression vector

The expression of polyhedrin and p10 is regulated by the strong polh and p10 promoters, respectively. These very late promoters can be employed for abundant transcription of very late genes. As a feature of baculoviruses both genes are necessary for the formation of ODV, but are dispensable for the life cycle of baculoviruses in insect cell culture, because BVs are the infectious morphotype needed for cell infection. Thus, both the polh (Smith et al., 1983) and p10 (Vlak et al., 1988) genes can be replaced by gene coding sequences of foreign genes, leading to recombinant baculoviruses. This replacement usually enables the production of large amounts of heterologous proteins (Jarvis, 2009), even more than 50% of the total protein in insect cells (Wickham et al., 1992).

1.2.5 Generation of recombinant baculoviruses

The MultiBac expression system (Fitzgerald et al., 2006, 2007) is particularly designed for the production of multiprotein complexes with many subunits. It consists of an array of small synthetic DNA plasmids, specially designed transfer vectors for assembly of multigene expression constructs, and an engineered baculovirus genome derived from the *Autographa californica* nuclear polyhedrosis virus (AcNPV) that is used to infect cells of the caterpillar *Spodoptera frugiperda* (Bieniossek et al., 2012). In the genome of AcNPV two baculoviral genes, v-cath and chiA, have been disrupted which leads to improved maintenance of cellular compartments during infection and protein production (Berger and Craig, 2011).
In this system gene insertions into the MultiBac genome take place in bacterial strains ($DH10^{MutliBac}$) that contain the MultiBac viral genome as an artificial chromosome together with a plasmid encoding the Tn7 transposon enzyme. The expression cassette, which is to be integrated and contains the gene of interest (GOI), is flanked by the Tn7L and Tn7R DNA sequences (Rohrmann, 2013; Bieniossek et al., 2012). The foreign DNA is inserted into the Tn7 attachment site (attTn7), located in the artificial chromosome, by Tn7 transposition.
Successful transposition into the attTn7 site additionally disrupts a LacZ encoding gene, which enables blue/white screening of colonies (Berger et al., 2013).
Subsequently, the bacmid DNA from positively screened colonies is purified and transfected into insect cells.

1.2.6 Protein display using baculoviruses

The MultiBac system was originally designed by X-ray crystallographers for studying multiprotein complexes (Berger et al., 2004) and has since been put to good use in structural biology. Many proteins and their complexes have been produced by the MultiBac system, often for the first time, for structure elucidation and providing important insight into their biological function (Bieniossek et al., 2012).

Another benefit of baculoviruses in general and the BEVS in particular is the relatively easy process to display proteins. Proteins of interest can efficiently be displayed on the surface of infected cells as well as on the baculovirus particles itself. There are various strategies to display proteins on baculovirus particles by fusing them to the major envelope protein gp64 or to other viral proteins like the capsid protein vp39 (Oker-Blom et al., 2003).

Proteins on the insect cell surface can be anchored by different sequences. Heterologous membrane proteins can be anchored by their native transmembrane domain or by insect cell specific sequences, like the gp64 membrane anchorage sequence (mars). Proteins are targeted to the cellular surface by different leader sequences, like the gp64 or melittin leader, and are anchored with insect cell specific proteins, by their native domains or by other sequences.

Recombinant clones are produced fast, thus, allowing the generation of libraries of adequate size and diversity. Especially the expression and display of complex eukaryotic proteins renders baculovirus surface display attractive. Eukaryotic surface display libraries, based on the BEVS, allow selecting for specific binding proteins, while providing sufficient post translational modifications.

The presentation of antigens, like cancer antigens, on insect cells is an important feature in the field of diagnostics in order to find binding proteins for therapy (Grabherr and Ernst, 2010).

1.2.6.1 Selected display model proteins

For cell display the model proteins Fcγ receptor I CD64, the human HIV anti-gp41 antibody 3D6 and the human epidermal growth factor receptor 2 HER2 (also known as ERBB2) were selected. CD64 and HER2 both are natural membrane bound receptors, whereas 3D6 is an artificial antibody.

- CD64 (cluster of differentiation 64) is an integral membrane glycoprotein with the unique functionality to be the only Fcγ receptor able to bind monomeric IgG with high affinity. Understanding the interaction between FcγRs and IgG is important for the application and engineering of antibodies (Hulett et al., 1998). Receptors for the Fc portion of IgG (FcγRs) play an important role for the induction of various IgG-dependent models of autoimmunity, inflammation, anaphylaxis, and cancer immunotherapy (Mancardi et al., 2013).
- The human HIV anti-gp41 IgG_1 antibody 3D6 is a monoclonal antibody, which has originally been isolated as IgG_1 isotype from seroconverted HIV-1 patients and bind to the immunodominant domain of gp41 (Reinhart et al., 2011). The 3D6 antibody is an artificial, therapeutic antibody which was displayed as whole antibody in contrast to the commonly conducted display of antibody fragments. The antibody can be used to generate recombinant antibody libraries for antibody affinity maturation, the identification of new ligands and finding of cellular interaction partners.
- The human epidermal growth factor receptor 2 (HER2) is a member of a family of receptors associated with tumor cell proliferation, apoptosis, adhesion, migration, and differentiation. HER2 is an important biomarker and key driver of tumorigenesis. Breast cancer patients show an overexpression of HER2, thus patients with HER2-positive tumors would benefit from treatment with anti-HER2 antibodies. (Bang et al., 2010).

1.3 Virus like particles

Virus like particles (VLPs) can be formed by the self-assembly of capsid proteins from many different viruses (Grgacic et al., 2006). The VLP, which may consists of one or more structural proteins, resembles the authentic virion but cannot replicate in cells (Liu et al., 2013). VLPs are replication as well as infection incompetent, due to the lack of any infectious genetic material (Buonaguro et al., 2011).

Natural VLPs fall in the size range of viruses (22-200 nm), but their exact size and morphology depend on the particular viral proteins incorporated. VLPs are promising candidates for the development of new vaccines against many diseases, because their repetitive, high density display of epitopes leads to strong immune responses, both humoral and cellular. This is further enhanced by their size, which seems to be optimal for uptake of nanoparticles by dendritic cells and subsequent antigen presentation (Grgacic et al., 2006).

Therefore, VLPs constitute a safe and effective approach for the induction of neutralizing antibodies to surface proteins (Vicente et al., 2011).

A wide range of virus like particles has been produced in order to conduct structural and functional studies and as promising vaccine candidates (Noad and Roy, 2003; Roy and Noad, 2008), like Cervarix ® (GlaxoSmithKline) and Gardasil ® (Merck), two Human Papillomavirus vaccines or Engerix ® (GlaxoSmithKline), a vaccine for Hepatitis B.

1.3.1 VLP generation using baculovirus expression systems

Many viral structural proteins have an intrinsic ability to spontaneously self-assemble into VLPs when expressed in insect cells with recombinant baculoviruses (Liu et al., 2013), using a baculovirus expression system. However, there is a need to express multiple proteins in order to construct VLPs. Two different viral expression strategies are available to generate complex VLPs. One possibility is to infect insect cells with multiple monocistronic baculoviruses (co-infection). The other possibility is to infect insect cells with a single polycistronic baculovirus (co-expression), encoding several proteins. Both approaches lead to the expression of more than one protein (see figure 1-3).

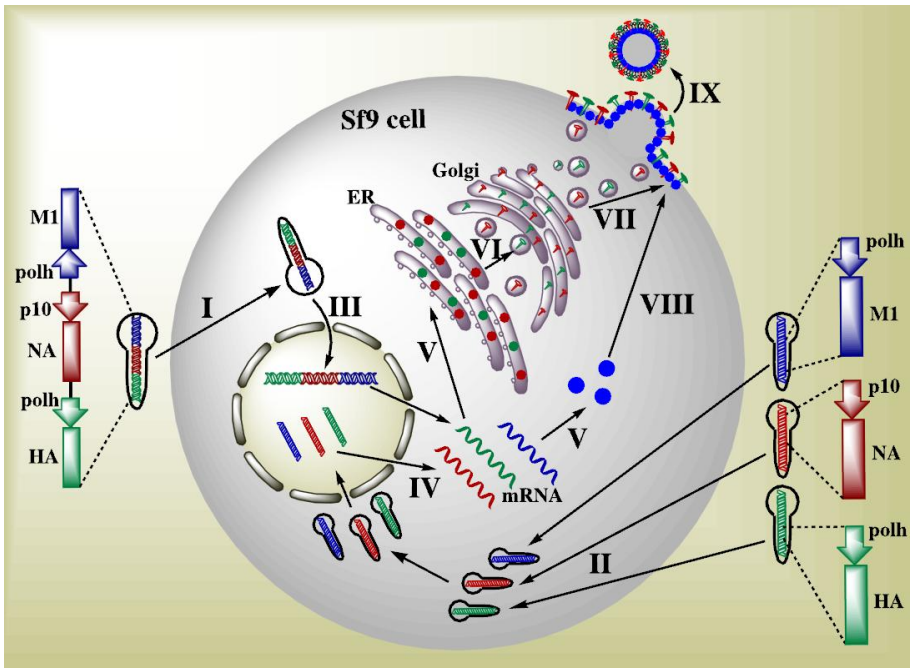


Figure 1-3: Main stages of co-expression (I) and co-infection (II) to generate influenza-like VLPs in insect cells III, baculoviral genome enters nucleus. IV, mRNAs are exported from the nucleus. V, the mRNAs are translated into structural proteins of influenza virus. VI and VII, hemagglutinin and neuraminidase are transported through the Golgi apparatus onto the plasma membrane. VIII, the M1 matrix protein is transported inside the plasma membrane. IX, a mature VLP buds from the surface of the cell. (Liu et al., 2013)

Influenza virus-like particles are also formed when the envelope protein hemagglutinin (HA) and neuraminidase (NA) are missing and only the matrix protein M1 is expressed. Therefore, the matrix (M1) protein is the only viral component which is essential for virus particle formation.

Consequently, recombinant baculoviruses containing the M1 matrix protein gene in combination with any other chosen protein, directed to the cellular surface, lead to the budding of VLPs consisting of these proteins. As seen in figure 1-4, the hemagglutinin (HA) is then substituted with another protein (e.g. model protein 3D6, CD64), that is targeted to the cell surface for display. In this case VLPs, designed for secretion, can then be harvested from the cell culture supernatant of suitable insect cell cultures and be further purified (Gómez-Puertas et al., 2000).

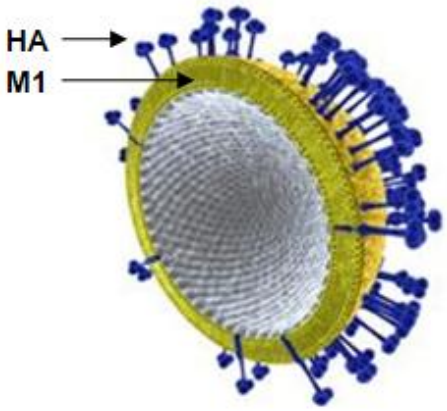


Figure 1-4: Schematic representation of Influenza VLP consisting of hemagglutinin (HA) and matrix protein (M1). (Krammer and Grabherr, 2010)

1.3.2 Applications of virus like particles

As already mentioned VLPs are promising vaccine candidates because they induce strong immune responses (Grgacic et al., 2006) and have potential in the fields of gene therapy and gene and drug delivery (Roldão et al., 2010). In addition, synthetic virus like particles displaying disease-specific biomarkers can be used in diagnostics. These biomarkers are proteins in their native folding that carry disease-specific amino acid sequences and/or post translational modifications. Subsequently VLPs, carrying biomarkers, act as probes for autoantibody detection assays and will help identify disease-specific monoclonal antibodies for diagnostics selected from antibody libraries with high diversity.

Cancer-specific antigens and other disease antigens can be presented on VLPs in order to find ligands by screening procedures. Moreover membrane proteins, which are difficult to study and to produce, due to their hydrophobic nature and complexity, can be secreted into the cell culture supernatant by virus particle budding (Krammer et al., 2010) and be harvested rather simply in comparison to intracellularly produced VLPs, where extraction prior to harvesting is essential.

1.3.3 VLP downstream processing

The first downstream processing (DSP) step depends on whether the VLPs are released to the extracellular medium, via secretion, or are produced intracellularly. If the VLPs are produced inside the cell or are not efficiently secreted, cell lysis or other extraction steps are required before further purification (Vicente et al., 2011).

The biophysical or biochemical separation of VLPs and baculoviruses, especially when VLPs are used for scientific or medical purposes, needs to be conducted. Baculoviruses have shown adjuvant activity in mice (Klausberger et al., 2014) and could induce unwanted synergistic effects on the immunologic response towards VLPs in humans (Liu et al., 2013). For scientific purposes, like structure elucidation or functionality studies, baculoviruses might interfere with the methods employed. Baculoviruses, which are co-produced as by-products in large amounts, and other undesired protein complexes do not differ significantly in size and molecular weight from VLPs, resulting in separation and purification difficulties (Roldao et al., 2011).

A method for purification is sucrose or cesium chloride gradient ultracentrifugation, a labor-intensive and time-consuming method, which at times fails to remove impurities, like recombinant baculoviruses (Liu et al., 2013). Newer, more sophisticated techniques, like tangential flow filtration, gel permeation, ion exchange, affinity and size exclusion chromatography and disposable membrane technologies, induce better purification and separation results (Roldao et al., 2011; Morenweiser, 2005, Vicente et al., 2008).

2 Objectives

The aim of this study was to display model proteins on the surface of insect cells and on the surface of virus like particles by multi-protein expression in insect cells using the baculovirus expression vector system. The chosen model proteins were a cellular anchored human monoclonal antibody (3D6), a Fc gamma receptor I (CD64) and the human epidermal growth factor receptor 2 (HER2).

1. Creating recombinant baculovirus working stocks for host cell infection in two different insect cell lines.

2. Display of model proteins and detection on the surface of insect cells.

3. Production of VLPs on the basis of the influenza A virus matrix protein M1 in two different insect cell lines.

4. Purification of VLPs by sucrose gradient centrifugation.

5. Characterization of VLPs by Western Blotting and transmission electron microscopy.

3 Material and Methods

3.1 Material

3.1.1 Equipment

Equipment used for experimental work can be found in table 3-1.

Table 3-1: Equipment

Device	Description	Supplier
Agarose gel electrophorese unit	Wide MiniSub® Cell GT	Biorad, USA
Balance	-	Sartorius, Germany
Cell counter TC20™	Automated Cell Counter	Biorad, USA
Centrifuge	Heraeus Megafuge 16R centrifuge	Thermo Scientific, USA
Centrifuge	Centrifuge 5415 R	Eppendorf, Germany
Centrifuge	PCV-2400	Grant Instruments, UK
Centrifuge	Centrifuge 5424	Eppendorf, Germany
Electroporator	ECM 630 Precision Pulse BTX®	Harvard apparatus, USA
FACS	Gallios™ Flow Cytometer	Beckman Coulter, USA
Fluorescence microscope	-	Leica, Germany
TEM	FEI Tecnai G² 200kV	FEI, USA
Gallios™ Flow Cytometer		Beckman Coulter, USA
Gel imaging system	Molecular Imager® Gel Doc™ XR+	Biorad, USA
Incubator	Thermo Scientific Incubator	Heraeus, Germany
Laminar flow	Safe 2020	Thermo Scientific, USA
Laminar flow	MSC-advantage	Thermo Scientific, USA
pH meter	inoLab pH 720	WTW, Germany
Pipette filler	S1	Thermo Scientific, USA
Pipettes	Pipetman® (2, 10, 20, 100, 200, 1000 µl)	Gilson, USA

Platform shaker	Unimax 1010	Heidolph, Germany
Power supply	EV202	Peqlab, Germany
Power supply	PowerPac™ Basic	Biorad, USA
Power supply	-	Invitrogen, USA
SDS-PAGE electrophoresis units	Mini-Protean® Tetra System	Biorad, USA
Semi dry blotter	-	Peqlab, USA
Shaking Incubator	MAXQ 8000	Thermo Scientific, USA
Shaking Incubator	3033	GFL, Germany
Spectrophotometer	NanoDrop 1000 Spectrophotometer	Peqlab, Germany
Sterile Syringe Filter	0,2 µm Cellulose Acetate	VWR, Austria
Stirrer	VMS-C7	VWR, Austria
Thermoblock	ThermoStat® plus	Eppendorf, Germany
Thermoblock	Thermomixer compact	Eppendorf, Germany
Thermocycler	C1000™ Thermal Cycler	Biorad, USA
UV-Microscope	DMIL LED	Leica, Germany
Vortex	Vortex Genie 2	Scientific Industries, USA

3.1.2 Laboratory consumables

Table 3-2 shows all consumables used during laboratory work.

Table 3-2: Laboratory consumables

item	**supplier**
1,5 ml reaction tubes	Eppendorf, Germany
15 ml SuperClear® Centrifuge Tubes	VWR, Austria
2 ml reaction tubes	Eppendorf, Germany
50 ml SuperClear® Centrifuge Tubes	VWR, Austria
5 ml Polystyrene Round-Bottom Tube	Corning, USA
6-well plates	Thermo Scientific, USA
Cell culture flasks (25, 75 and 175 cm^2)	Thermo Scientific, USA
Counting slides	Biorad, USA

Electroporation cuvettes (2 mm gap)	VWR, Austria
Falcon™ Round-Bottom Polystyrene Tubes	Thermo Scientific, USA
VivaSpin®6 , 10.000 MWCO	Sartorius, Germany

3.1.3 Reagents and kits

Chemicals and reagents used during this study are listed in table 3-3.

Table 3-3: Chemicals and reagents

item	**supplier**
10% (w/v) APS	Sigma-Aldrich, USA
2% (w/w) Bis-acrylamid solution	Biorad, USA
40% (w/w) Acrylamid solution	Biorad,USA
BCIP/NBT Color Development Substrate	Promega, USA
Bovine Serum Albumin	Sigma-Aldrich, USA
Coommassie Brilliant Blue	
CutSmart™ Buffer (10x)	New England BioLabs, USA
Ethanol	Sigma-Aldrich, USA
Ethidium bromide solution 10 mg/ml in H2O	Sigma-Aldrich, USA
FuGene HD Transfection Reagent	Promega, USA
Glycerol anhydrous	Sigma-Aldrich, USA
Heat inactivated FBS (fetal bovine serum)	Gibco, USA
Heparin	Sigma-Aldrich, USA
HEPES	Sigma-Aldrich, USA
Isopropanol	Sigma-Aldrich, USA
N,N,N′,N′-Tetramethylethan-1,2 diamin (TEMED)	Sigma-Aldrich, USA
One-Taq Standard Reaction Buffer (5x)	New England BioLabs, USA
peqGold Universal Agarose	Peqlab, Germany
Pluronic (10%)	Sigma-Aldrich, USA
Q5 Reaction Buffer (5x)	New England BioLabs, USA
Sodium chloride	Sigma-Aldrich, USA
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, USA
T4 DNA Ligase Reaction Buffer (10x)	New England BioLabs, USA

Trypan Blue Solution (0,4%)	Sigma-Aldrich, USA
Tween® 20	Sigma-Aldrich, USA

Plasmid purification and isolation kits used during this work are shown in table 3-4.

Table 3-4: Kits

item	**supplier**
NucleoBond® Xtra Midi Plus EF	Macherey-Nagel, Germany
NucleoSpin® Gel and PCR clean-up	Macherey-Nagel, Germany
NucleoSpin® Plasmid Quick Pure	Macherey-Nagel, Germany

DNA and protein markers used for gel electrophoresis are listed in table 3-5.

Table 3-5: DNA and protein markers

item	**Supplier**
2 -Log DNA ladder	New England BioLabs, USA
PageRuler™ Prestained Protein Ladder	Life Technologies, USA

3.1.4 Plasmids

3.1.4.1 pACEBac1

The acceptor vector pACEBac1 (EMBL-Grenoble) was used for generation of bacmids. The vector carries a ColE1 origin of replication allowing for maintenance of high plasmid copy number. Additionally, the vector carries a multiple cloning site (MCS) flanked by the viral polyhedrin promoter (polh) and a SV40 polyadenylation signal sequence (SV40 late polyA). Genes of interest can be cloned into this MCS. In order to select positive transformants in *E. coli* a gentamicin resistance marker (GentR) is incorporated. The locus of X-over P1 (LoxP) site allows CreLox recombination and thus combining acceptor and donor vectors.
The Tn7R and Tn7L sites enable the integration of the expression cassette in the baculovirus genome.

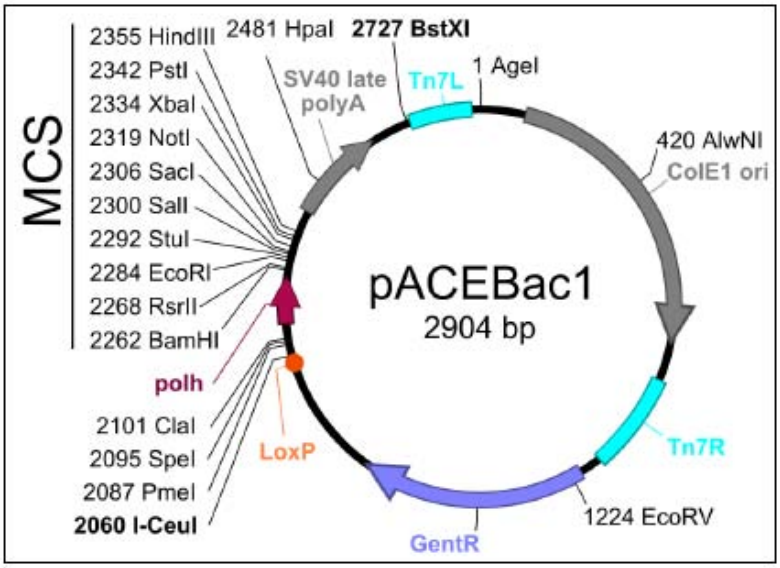


Figure 3-1: Map of pACEBac1 vector (EMBL-Grenoble)

3.1.4.2 pIDC

As a donor vector pIDC (EMBL-Grenoble) was used. This vector is similar to the acceptor vector but has a different resistance gene, namely the chloramphenicol resistance gene, and a conditional R6Kγ origin of replication which makes its propagation dependant on the expression of the *pir* gene in the *E. coli* PIRHC strain. Again genes of interest can be cloned into the multiple cloning site with its unique restriction sites.

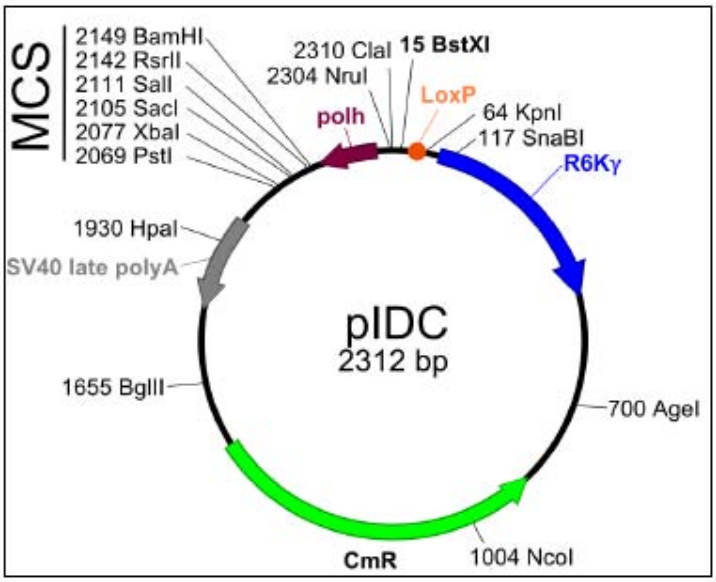


Figure 3-2: Map of pIDC vector (EMBL-Grenoble)

3.1.4.3 pSMART

The pSMART (Lucigen, USA) vector was used to amplify genes of interest or to assemble parts of a gene in one vector. The vector has an ampillicin resistance gene and a multiple cloning site with unique restriction sites for blunt end cloning.

3.1.5 Enzymes

For cloning experiments several enzymes, shown in table 3-6, were utilized.

Table 3-6: Enzymes

enzyme	**Function**	**supplier**
BamHI-HF	restriction endonuclease	New England BioLabs, USA
EcoRI-HF	restriction endonuclease	New England BioLabs, USA
EcoRV-HF	restriction endonuclease	New England BioLabs, USA
SalI-HF	restriction endonuclease	New England BioLabs, USA
SphI-HF	restriction endonuclease	New England BioLabs, USA
XbaI	restriction endonuclease	New England BioLabs, USA
XmaI	restriction endonuclease	New England BioLabs, USA
Alkaline phosphatase, calf intestinal (CIP)	dephosphorylation of 5' and 3' ends of DNA	New England BioLabs, USA
Cre Recombinase	CreLox recombination	New England BioLabs, USA
OneTaq® DNA Polymerase	DNA polymerase	New England BioLabs, USA
Q5® High-Fidelity DNA Polymerase	DNA polymerase	New England BioLabs, USA
Phusion® High-Fidelity DNA Polymerase	DNA polymerase	New England BioLabs, USA
T4 DNA Ligase	DNA Ligase	New England BioLabs, USA

3.1.6 Microorganisms and cell lines

3.1.6.1 Bacterial strains

- *E. coli* JM109 (New England BioLabs, USA)
- *E. coli* DH10MultiBacY cells (EMBL-Grenoble, France)
- *E.coli* DH10MultiBac (-Y) cells
- *E. coli* NEB 5-alpha (New England BioLabs, USA)
- *E.coli* PIRHC (EMBL-Grenoble, France)

3.1.6.2 Insect cell lines

- *Spodoptera frugiperda Sf9* cells (ATCC CRL-1711)
- *Trichopulsia ni* BTI-TN5B1-4 “High Five” cells (ATCC CRL-10859)

3.1.7 Growth media

3.1.7.1 Media for E. coli

- LB medium

All components listed in table 3-7 were dissolved in dH_2O and autoclaved at 120° C for 20 minutes. After autoclaving the medium was stored at 4° C. If required, antibiotics were added just before usage.

Table 3-7: Composition of LB medium

component	concentration
Peptone casein	10 g/l
Yeast extract	5 g/l
NaCl	10 g/

- LB agar

The components for LB agar listed in table 3-8 were dissolved in dH_2O, autoclaved and stored at 4° C. For the preparation of agar plates, the agar was melted in the microwave and the required antibiotics were added after some cooling. The plates were poured and stored at 4° C.

Table 3-8: Composition of LB agar

component	concentration
Peptone casein	10 g/l
Yeast extract	5 g/l
NaCl	10 g/l
Agar Agar	15 g/l

- SOC medium

SOC medium was prepared by dissolving all components, listed in table 3-9, and subsequent autoclaving. Sterile SOC medium was stored at 4° C.

Table 3-9: Composition of SOC medium

component	concentration
Peptone casein	20 g/l
Yeast extract	5 g/l
NaCl	10 mM
KCl	3 mM
$MgCl_2$ * 6 H_2O	10 mM
Glucose	20 mM
$MgSO_4$ * 7 H_2O	10 mM

3.1.7.2 Cell culture media

For insect cells HyClone™ SFM4 Insect media with glutamine (GE Healthcare, GB) was used. The medium was supplemented with 0.1% Pluronic, a non-ionic detergent that reduces hydrodynamic damage to the cells.

3.1.8 Antibiotics and media additives

Additions to media for preparing selective growth media or selective plates for *E. Coli* are shown in table 3-10.

Table 3-10: Media additives

additive	**working concentration**
Ampillicin	10 µg/ml
Chloramphenicol	25 µg/ml
Gentamycin	10 µg/ml
IPTG	50 µg/ml
Kanamycin	50 µg/ml
Tetracyclin	10 µg/ml
X-Gal	50 µg/ml

3.2 Methods

3.2.1 Molecular biology methods

3.2.1.1 Plasmid isolation from E. Coli cells (Mini-prep)

Plasmid isolation was carried out with the Macherey-Nagel Nucleo-Spin Plasmid Quick Pure Mini-prep kit (Macherey-Nagel, Düren, Germany) and was done according to the manufacturer's protocol.
12 mL overnight suspension culture were prepared in LB media, with a particular selective antibiotic, from a single colony picked from a LB-agar plate. 4 mL of this suspension were used for plasmid isolation carried out according to the manufacturer's protocol.

3.2.1.2 DNA Quantification

Amount of DNA was measured by using a spectrometer (Nanodrop 1000 Thermo Scientific, Wilmington, DE, USA).

3.2.1.3 Preparation of agarose gels

For agarose gel electrophoresis 1.0% agarose gels were prepared. The composition for 500 ml can be seen in Table 3-11.
Agarose in 1x TAE buffer was completely melted in the microwave. The solution was cooled down to about 50° C and ethidiumbromid was added.

Table 3-11: Composition of 1.0% agarose gel (500 ml)

amount	**component**
5 g	Agarose
10 ml	50x TAE buffer
15 µl	Ethidium bromid
	dH2O

3.2.1.4 Agarose gel electrophoresis

In agarose gel electrophoresis DNA fragments are separated based on their size. Smaller fragments travel through the sieve-like gel at faster speed than bigger ones thus separating them.

DNA samples were mixed with 6x BX-loading dye (0.25% bromphenol blue, 0.25% xylen cyanol, 30% glycerol) and loaded into the slots of the gel. A voltage of 130 V was applied to the electrophoresis chamber. Before the dye front reached the end of the gel the apparatus was turned off and the DNA bands on the gel were inspected with the Molecular Imager® Gel Doc™ XR System.

Table 3-12: Composition of 50x TAE buffer (1000 ml)

amount	**Component**
242 g	Tris(hydroxmethyl)aminomethane
57.1 ml	Glacial acetic acid
18.6 g	EDTA
	dH2O

Table 3-13: Composition of 1x TAE running buffer (10 l)

amount	**Component**
200 ml	50x TAE buffer
300 µl	Ethidium bromid
	dH2O

3.2.1.5 DNA size markers

DNA markers for assessment of sample size were purchased at New England Biolabs (Beverly, MA). The 2-log DNA ladder shown in figure 3-3 was used to compare the size of PCR products, plasmids and restriction digests with a standard. Consistently 6 µL of 2-log ladder was loaded into one or more slots of the gel.

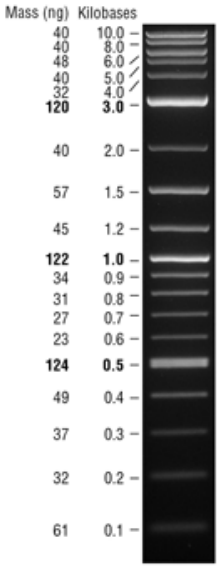


Figure 3-3: 2-log DNA ladder

3.2.1.6 Extraction of DNA from agarose gels

For preparative gel electrophoresis the DNA bands at the desired size were cut out with razorblade using an UV-transilluminator and purified by using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany). A voltage of 90 V was applied to the electrophoresis chamber for separation.

3.2.1.7 Polymerase chain reaction (PCR)

- **Amplification of DNA inserts**

For the amplification of DNA inserts Q5 High-Fidelity DNA polymerase was used. A master mix according to table 3-14 was prepared and transferred into 0.2 ml reaction tubes.

Table 3-14: PCR master mix for DNA amplification

Component	**50 µl reaction**
5x Q5 Reaction buffer	10 µl
10 mM dNTPs	1 µl
10 µM Primer-for	2.5 µl
10 µM Primer-back	2.5 µl
Q5 High-Fidelity DNA Polymerase	0.5 µl
Nuclease-free water	to 50 µl
Template DNA	

The primers used for amplification are listed below in table 3-15.

Table 3-15: Primers used for insert amplification

Primer	**Sequence 5`-> 3`**
HER2_ECD-G for	ACC CAA GTG TGC ACC GGC AC
HER2_ECD-G back	GTT GAT GGG GCA AGG CTG GC
HER2 gb1 SalI for	GAT GAT GTC GAC AAA ATG GAG CTG GCT GCT CTG TG
HER2 gb2 XbaI back	GAT GAT TCT AGA CTA CAC TGG AAC ATC GAG TCC
ECD-G XmaI for	GAT GAT CCC GGG TGC TGC TTC AAC CCA AGT GTG CAC

After preparing the master mix, the PCR reaction tubes were placed into the thermocycler and the cycling program was configurated. The standard cycling conditions are listed in table 3-16.

Temperature of annealing and time of the elongation step were adapted to the specific melting temperature (Tm) of the primers and to the length of the desired DNA fragment respectively.

Table 3-16: Thermocycler program

Step	temperature	time	
Initial denaturation	98° C	30 s	
Denaturation	98° C	10 s	30 x
Annealing	50 – 65° C	20 s	
Elongation	72° C	20 - 30 s/kb	
Final extension	72 ° C	2 min	

After completion, PCR products were analyzed by agarose gel electrophoresis, excised from the gel and purified as described in 3.2.1.1.

- **Colony PCR**

For screening positive *E. Coli* clones, after successful transformation, a PCR screening, using OneTaq DNA polymerase, was performed. Single colonies from the LB-agar plates with selective antibiotic were picked with a pipette tip and resuspended in 25 µl of the PCR master mix. With the same tip a small amount of suspension was streaked out on a fresh LB-agar plate (master plate). The plate was incubated at 37° C over night or until colonies were visible. For each colony PCR screening run 8 to 16 colonies were picked.

Table 3-17: PCR master mix for colony screening

Component	25 µl reaction
5x One Taq Standard Reaction buffer	5 µl
10 mM dNTPs	0.5 µl
10 µM Primer-back	0.5 µl
10 µM Primer-for	0.5 µl
One Taq DNA polymerase	0.2 µl
Nuclease-free water	to 25 µl

Primers used for colony screening were chosen from the available primers for amplification or specific sequencing primers were designed. (Table 3-15 and Table 3-19).

The appropriate cycling conditions are shown in table 3-18. The elongation time was adapted to the length of the desired fragment. The annealing temperature was selected according to the specific melting temperature of the primer recommended by New England Biolabs (UK).

Table 3-18: Thermocycler program colony screening

Step	temperature	time	
Initial denaturation	94° C	30 s	
Denaturation	94° C	10 s	
Annealing	45 – 68° C	30 s	30 x
Elongation	68° C	1 min/kb	
Final extension	68° C	5 min	

3.2.1.8 Digests

- **Control digest**

In order to check if the obtained plasmid contained the correct insert it was cut with different restriction enzymes and analysed with agarose gel electrophoresis. For one digest (30 µl total volume) 150 ng plasmid DNA, 1 µl restriction enzyme, 3 µl buffer and dH_2O were mixed. According to the optimal incubation temperature and time, given by the manufacturer, the mix was incubated. After gel electrophoresis, the bands on the gel were compared to the theoretical bands according to the plasmid map using the CLC main workbench (CLC bio, Aarhus, Denmark).

- **Restriction digest and dephosphorylation**

The pACEBac1 vector was linearized with *SalI*-HF and *XbaI*. Therefore a double digest was carried out.3 µg plasmid vector were mixed with 2 µl *SalI*-HF, 2 µl *XbaI*, 10 µl CutSmart buffer and 80 µl dH_2O. The preparation was incubated for 2 hours at 37° C. After heat inactivation (65° C, 20 minutes) 1 µl *CIP* (calf intestinal alkaline phosphatase) was added, to prevent relegation of the vector, and incubated for 30 minutes at 37° C. Afterwards DNA was purified by preparative gel electrophoresis and gel excision.

- **Restriction digest of DNA inserts**

The DNA insert was cut with the same enzymes as the vector resulting in overhangs which can be readily ligated.
For the digest a total volume of 50 µl was prepared consisting of 30 µl amplified PCR product (~500 ng), 5 µl CutSmart buffer, 1 µl *SalI*-HF, 1 µl *XbaI* and dH_2O. The mix was incubated for 1 hour at 37° C and heat inactivated at 65° C for 20 minutes.

3.2.1.9 Ligation

DNA ligation was carried out by using T4 DNA ligase. For the ligation reaction 100 ng linearized vector (pACEBAc1) and x ng insert DNA were used according to the equation below. Additionally 7 µl T4 ligase buffer, 4 µl T4 DNA ligase and 5.5 µl dH_2O were added. The mixture was incubated at room temperature for 2 hours.

Equation 1: Calculation of amount Insert for Ligation

$$\frac{100\ ng\ linearized\ vector}{2700\ bp\ linearized\ pACEBac1}\ x\ 5\ =\ \frac{x\ ng\ Insert\ DNA}{y\ bp\ Insert\ DNA}$$

3.2.1.10 Isopropanolprecipitation

For 20 µl of plasmid preparation 50 µl of Isopropanol and 2µl of NaAc (3M) were added. This approach was centrifuged at 4° C at maximum speed (16.000 rpm) for 15 minutes. The supernatant was discarded and 100 µl of Ethanol added. After washing with 96% ethanol, the preparation was centrifuged at room temperature at maximum speed for 5 minutes. The forming pellet was dried at room temperature for 5-10 minutes and resuspended in 10 µl of ddH_2O.

3.2.1.11 Preparation of electrocompetent E.Coli cells

Cells were made electro-competent by excessively washing to remove all remaining salts from culture media. The procedure ensures that during electroporation, charge is not conducted through medium, but rather through the cells themselves.

An *E.Coli* culture of JM109 was incubated overnight until OD600 of about 0.6 was reached. The cells were pelleted at 4°C at 4000 rpm for 8 minutes. The pellets were washed several times with chilled 1mM HEPES. With each washing step the volume of HEPES was reduced. The pellet was then resuspended in 10% glycerol, portioned at 50 µL in precooled tubes and shock frozen in liquid nitrogen. The cell aliquots were stored at -80° C.

3.2.1.12 Transformation into electrocompetent E.Coli cells

1 µl of plasmid DNA (about 1 ng total) was pipetted into 50 µl of thawed electrocompetent *E.Coli* cells (JM109). The mixture of plasmid DNA and cells was put into a prechilled electroporation cuvette (0.2 cm gap) and a pulse was applied (2.5 kV, 1000 Ohm, 25 µF). After pulsation, 200 µl of preheated (37° C) LB or SOC media was quickly pipetted into the cuvette. This mixture was then transferred into a tube of 950 µl preheated LB or SOC media. For cell recovery the suspension was incubated over night in LB-media or for 1-2 hours (SOC) on a thermoblock at 37° C and 750 rpm.
After recovery, dilutions (1:10, 1:100) were made and plated on selective LB-agar plates. The plates were then incubated at 37° C overnight.

3.2.1.13 Transformation into chemical-competent E.Coli cells

Chemically-competent NEB 5-alpha *E. coli* cells were thawed on ice for 10 minutes. Then 1 µl containing 1 pg-100 ng of plasmid DNA was added to the cell mixture. The tube was carefully flicked 4-5 times and placed on ice for 30 minutes. A heat shock was applied at exactly 42° C for exactly 30 seconds in a waterbath. The tube was placed on ice for 5 minutes and 950 µl of room temperature SOC was pipetted into the mixture. The cells were shaken at 250 rpm at 37° C for 1 hour. The solution was mixed by flicking the tube, then a 1:100 dilution was prepared and the cells were put onto selection plates and incubated overnight at 37° C.

3.2.1.14 Sequencing

Sequencing was conducted at Microsynth AG (Balgach, Switzerland). Plasmids were diluted with dH_2O to a concentration of 80 ng/µL in 12 µl total volume as recommended by the company. PCR products were diluted according to their size as recommended by Microsynth AG. Subsequently, 3 µl of sequencing primer were added and the whole mixture was sent for sequencing.

Primers used for sequencing are shown in table 3-19.

Table 3-19: Sequencing primer

Primer	**Sequence**
HER2 Insert Screening primer 1 for	TTC AAC CAC AGT GGC ATC TGT G
HER2 Insert Screening primer 2 for	ACT GTT TGC CGT GCC ACC CTG
HER2 Insert Screening primer 3 back	TGA CCA ACT CAC GGA AGC GC
HER2 Insert Screening primer 4 back	CAT CAT CTT CGA GAA GCG AGC G
HER2 Insert Screening primer 5 for	CAC TGA CTG CTG CCA TGA GCA G
pAB1 SV40 back	CCT CTA GTA CTT CTC GAC AAG
pAB1 -44 for	TTT ACT GTT TTC GTA ACA GTT TTG
pSMART Screening primer SL1	CAG TCC AGT TAC GCT GGA GTC
pSMART Screening primer SR2	GGT CAG GTA TGA TTT AAA TGG TCA GT
pSMART Screening primer 2 back	TAC GCC CGG TAG TGA TCT TAT TTC

For sequence alignment and confirmation of the sequence the CLC main workbench (CLC bio, Aarhus, Denmark) was used.

3.2.1.15 Cre-LoxP fusion reaction

Cre-LoxP fusion of acceptor and donor vectors for multigene expression and VLP generation in insect cells was carried out according to “ACEMBL Expression System Series MultiBac^{Turbo}, Multi-Protein Expression in Insect Cells, User Manuel, Version 3.0”

3.2.1.16 Bacmid preparation and blue/white screening

DNA was transformed (according to 3.2.1.12) into electro-competent DH10MultiBacY cells for the generation of viral bacmides. After cell recovery 1:10, 1:100 and 1:1000 dilutions were prepared and plated on selective agar plates containing gentamicin, kanamycin and tetracycline as selection markers, as well as IPTG and X-gal for subsequent blue/white screening. After incubation at 37° C for at least 48 hours clones that incorporated the DNA insert appeared white, whereas those who did not appeared blue. To verify this result, 7 white and 1 blue colony were picked and restreaked on a fresh selective agar plate (master plate). After verification a correct, white clone was picked and incubated overnight in 5 ml selective LB-medium containing gentamicin, kanamycin and tetracycline for isolating (mini-prep) the plasmid DNA. These plasmids were used for subsequent transfection of insect cells (see 3.2.2.2).

3.2.1.17 Cloning of expression constructs

The constructs influenza matrix protein M1, 3D6 antibody and Fcγ receptor I (CD64) were made available by other members of the working group. The theoretical steps of the cloning procedures are described below. In this work these constructs were used for further investigations. The cloning of the human epidermal growth factor receptor 2 (HER2) construct is shown in detail below.

3.2.1.17.1 Influenza matrix protein M1 (PR8)

As a control and for comparison naked VLPs consisting of M1 (PR8) only were generated. The sequence was ordered as GeneArt® String™ DNA Fragments (Invitrogen, USA) according to the Influenza Research Database A/Puerto Rico/8-1/1934 sequence nr. CY045765. The M1 (PR8) gene was PCR amplified and the obtained product was further digested with *EcoR*I/*Not*I and ligated into pACEBac1 vector that had been digested with the same enzymes, resulting in pACEBac1 + M1 (PR8) (or also referred to as M1 only construct).
For the purpose of Cre-LoxP fusion reaction a donor vector, pIDC, with the M1 (PR8) sequence was prepared. The sequence of M1 (PR8) was PCR amplified from the pACEBac1 + M1 (PR8) vector. The PCR product was digested with *SacI*/*XbaI* and cloned into pIDC donor vector, digested with the same enzymes, resulting in pIDC + M1 (PR8).

3.2.1.17.2 3D6 antibody

This construct was designed for baculovirus surface display of the human anti-gp41 antibody 3D6. Notably, it was aimed to express the whole 3D6 antibody and not antibody fragments.
The light chain (LC) of the 3D6 antibody was PCR amplified from pFBD-3D6 which has been constructed as previously described (Palmberger et al., 2011). The obtained product was digested with *Bam*HI/*Xba*I and ligated into pACEBac1 vector that had been digested with the same enzymes, resulting in pACEBac1 + LC 3D6. The heavy chain (HC) of 3D6 was generated as a gene fragment synthetized by Integrated DNA Technologies (IDT, Leuven, Belgium), containing the membrane anchorage sequence (mars) from the baculovirus gp64 major envelope protein on the C-terminus. The respective gene fragment was PCR amplified, digested with *Bam*HI/*Xba*I and ligated into pACEBac1 vector that had been digested with the same enzymes, resulting in pACEBac1 + HC 3D6 mars. Cloning of this synthetized gene fragment into pACEBac1 vector created a second undesired *Bst*XI restriction site disabling the construct to generate a multigene expression cassette via homing endonuclease/BstXI multiplication. The BstXI restriction site was removed by a PCR reaction with specifically designed primers. Subsequently, the multigene expression construct encoding 3D6 HC and LC was generated via homing endonuclease *I-Ceu*I and *Bst*XI multiplication leading to the pACEBac1 + 3D6 construct (HC + LC).

To obtain VLPs the pACEBac1 + 3D6 construct was further modified to additionally express the Influenza matrix protein 1 gene (M1 PR8) from strain A/Puerto Rico/8/34 Puerto Ricco (H1N1). As described before (see 4.2.1) the M1 (PR8) protein was PCR amplified from pACEBac1 + M1 (PR8), the obtained product was digested with *Sac*I/*Xba*I and cloned into pIDC donor vector that had been digested with the same enzymes, resulting in pIDC + M1 (PR8). Cre-LoxP fusion of acceptor vector pACEBac1 + 3D6 and donor vector pIDC + M1 (PR8), resulted in pACEBac1 + 3D6 + M1 (PR8).

3.2.1.17.3 Fcγ receptor I (CD64)

The CD64 gene coding for the protein in a soluble form was PCR amplified from plasmid pBacPAK8_CD64 containing the gene for CD64 in the soluble form. The obtained product was digested with *Bam*HI/*Xho*I. The gp64 mars sequence was PCR amplified from pACEBac1 + 3D6. The amplified product was further digested with *Xho*I/*Xba*I. The two

products were digested with *Xho*I, ligated and then cloned into pACEBac1 resulting in pACEBac1 + CD64 **sm** (soluble mars). The transmembrane domain and the cytoplasmic domain of the CD64 gene according to the UniProt protein sequence database, entry number P12314, were synthetized by Integrated DNA Technologies (IDT, Leuven, Belgium). To generate a construct encoding the whole CD64 gene the sequence of CD64 soluble was PCR amplified and digested with *Afl*II, the gene fragment encoding the rest of the whole CD64 gene was PCR amplified and digested with *Afl*II/*Xba*I. The two products were digested with *Afl*II, ligated and further cloned into pACEBac1 resulting in pACEBac1 + ∑CD64, encoding the whole CD64 gene with the native anchorage sequence.
In order to obtain VLPs both CD64 constructs were fused with the previously described pIDC + M1 (PR8) donor vector by CreLoxP fusion reaction leading to both constructs additionally containing the necessary influenza matrix protein M1 (PR8).

3.2.1.17.4 Human epidermal growth factor receptor 2 (HER2)

This construct was designed for surface display of the human epidermal growth factor receptor 2 (HER2) and for the generation of VLPs consisting of HER2 and M1 (PR8).
The DNA Sequence of a part of the ectodomain (ECD) of HER2 was provided by another group at BOKU. The sequence for targeting (signal peptide), the rest of the ectodomain, the transmembrane domain (TMD) and the cytoplasmic domain (CD) were ordered as gBlock at Integrated DNA technologies (IDT, Leuven, Belgium). The sequence assembly of these parts was carried out in the pSMART vector. After completion of the sequence, the whole HER2 sequence was cloned into pACEBac1 vector for bacmid preparation.

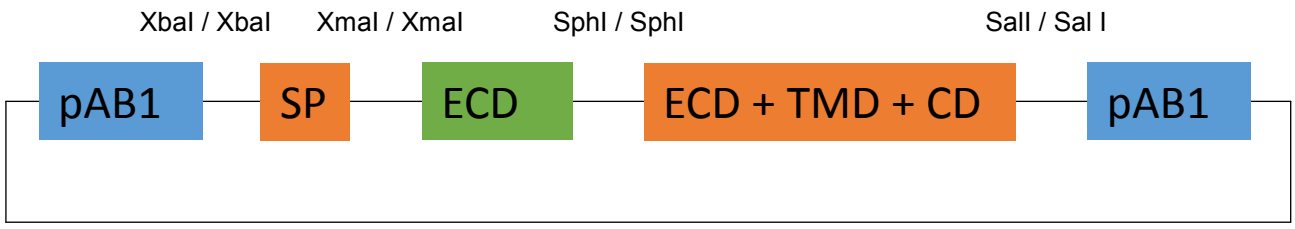


Figure 3-4: Schematic representation of DNA assembly strategy of HER2. The DNA fragments were digested with different restriction enzymes leading to complementary DNA overhangs, which allows for stepwise, seamless ligation and integration into ultimately the pACEBac1 acceptor vector.

- Preparation of insert DNA

The gBlock was PCR amplified using the primers HER2 gb1 *SalI* for and HER2 gb2 *XbaI* back. The DNA fragment was cut out of the gel and purified. The purified DNA was then phosphorylated and cloned into the pSMART vector according to the CloneSmart® Blunt Cloning Kit (Lucigen, USA).
The ECD was PCR amplified by using the primers ECD-G *XmaI* for and HER2_ECD-G back. The resulting DNA fragment was digested with *XmaI* and *SphI*-HF for cloning into the pSMART vector.

- Preparation of pACEBac1 vector

The pACEBac1 vector was linearized by double digestion using the enzymes *XbaI* and *SalI* (as described in 3.2.1.8). The estimated band at 2900 bp, obtained by preparative gel electrophoresis, was cut out and purified. Later, this vector was used for insertion of the whole HER2 sequence

- Ligation I – pSMART containing gBlock ligated with ECD

The pSMART vector containing the gBlock was cut with *XmaI* and *SphI*-HF. The ectodomain, cut with the same enzymes leading to complementary overhangs, was ligated into the vector resulting in the whole HER2 sequence. After purification this construct was transformed into competent JM109 *E.Coli* cells and striked out on LB agar plus ampillicin plates for selection. The pSMART vector contains an ampillicin resistance gene. For further screening a colony screening PCR was conducted by picking single colonies from the selection plates using HER2 Insert Screening primer 2 and HER2 Insert Screening primer 3 leading to a 1200 bp long band confirming the insertion of the sequence.

- PCR amplification of whole HER2 sequence

The Sequence of HER2 was PCR amplified using the primers HER2 gb1 *SalI* for and HER2 gb2 *XbaI* back, and pSMART + HER2 (whole sequence) as template. The band at 3800 bp was cut out of the gel and purified.

- Ligation II – HER2 ligated with pACEBAC1

The purified HER2 band was digested with *SalI*-HF and *XbaI*. The digest was used for ligation with the prepared pACEBac1 vector, cut with same enzymes, resulting in a 6704 bp band.

- CreLox fusion of acceptor and donor vector

The acceptor vector, pACEBac1 + HER2 (whole), and the donor vector, pIDC + M1 (PR8) (see 4.1.1), were used for CreLoxP fusion reaction, as described in the ACEMBL Expression System Series MutliBac^{Turbo} User Manual, resulting in the construct HER2 + M1 (PR8) with a length of 9693 bp. The M1 matrix protein from influenza virus is necessary to obtain VLPs. After the fusion reaction the construct was transformed into JM109 *E.Coli* cells. The cells were striked out on selective Agar plates with gentamicin (resistance gene on pACEBac1) and chloramphenicol (resistance gene on pIDC) as selection markers.

3.2.2 Cell culture methods

3.2.2.1 Cultivation of Sf9 and HighFive cells and determination of cell number

Sf9 (ATCC CRL-1711) and HighFive (ATCC CRL-10859) cells were cultivated in Hyclone medium supplemented with 0.1% pluronic at 27° C in shaking flasks at 100 rpm.
In order to maintain cell growth cells had to be passaged twice a week. The cell density and viability were measured with TC20^{TM} automated cell counter (Biorad, CA). 10 µl of cell suspension were mixed with 10 µl of 0.4% trypan blue and pipetted in counting slides. Cells were then diluted with Hyclone medium to a desired cell density of about 5 x 10^5 cells/ml and put back into the incubator at 27° C.

3.2.2.2 Transfection (FuGene HD)

In a 6 well plate 9 x 10^5 *Sf9* cells/well were seeded, filled up to 2 mL with Hyclone medium complemented with 3% FCS and let sit down.
In the meantime, in a Sarstedt tube 1-1.5 µg of Bacmid DNA filled up to 100 µl with Hyclone medium was prepared. In another Sarstedt tube 9 µl transfection reagent FuGene HD filled up to 100 µl with Hyclone medium was prepared. The content of both tubes was combined

and carefully mixed, incubated for 15 minutes at room temperature and applied to the cells in the 6 well plate.

After 4-5 days the cells were checked for YFP expression by conducting fluorescence microscopy to verify heterologous protein expression. The supernatant was harvested, constituting the seed stock. For baculovirus stock amplification and increasing the titer 100 µl of seed stock was transferred to 8 x 10^6 *Sf9* cells in T75 roux flasks filled up with Hyclone medium to a total volume of 12 ml. After 4 days the supernatant was harvested creating the intermediate stock. Again for purposes of virus amplification, 100 µl intermediate stock was transferred to 17.5 x 10^6 *Sf9* seeded cells in T175 roux flasks in a total volume of 25 ml. The harvested supernatant is the working stock (WS), which was used for infection experiments.

3.2.2.3 Infection for fluorescence activated cell sorting (FACS)

For FACS experiments 8 x 10^6 cells (*Sf9*, HighFive) were seeded in T75 flasks (12 ml volume). Subsequently cells were infected with virus working stocks at a MOI of about 5 – 10. For better infection 3% FCS (360 µl) was added. The flasks were incubated at 27° C for 48 or 72 hours. After 2 or 3 days post infection the cells were harvested and prepared for FACS analysis.

The various virus working stocks used in this work are listed in table 3-20.

Table 3-20: Virus working stocks for infection

Virus WS	**Description**
M1 (PR8) only	"Empty VLP", Influenza matrix protein 1 (PR8) only
CD64∑ + M1 (PR8)	Fc gamma receptor I, whole protein CD64, anchorage via original transmembrane domain
CD64 sm + M1 (PR8)	Fc gamma receptor I, CD64 **s**oluble + gp64 **m**ars (membrane anchorage sequence)
3D6 + M1 (PR8)	Human anti-gp41 whole antibody 3D6
HER2 + M1 (PR8)	Breast cancer marker protein
H1 Cal09	Hemagglutinin 1 from influenza virus (Cal09), no VLP – soluble protein

3.2.2.4 FACS preparation

The cells were inspected with fluorescence microscopy (Leica) to check for YFP (yellow fluorescent protein) expression and to confirm successful infection. The cells were carefully detached from the bottom of the flask and the cell density was measured, as previously described.

2-3 x 10^6 cells per flask were harvested and transferred into 50 ml falcon tubes. The tubes were centrifuged at 500 x g for 5 minutes. The supernatant was discarded and the cell pellet was resuspended in 0.5 ml PBS. Afterwards the resuspension was transferred into 1.5 ml Eppis and again centrifuged (500 x g, 5 min). Next, the cell pellet was resuspended in 100 µl PBS + 10% FCS and incubated for 30 minutes in order to block unspecific binding sites. Alternatively the cells were treated with a FcR blocking reagent (human, Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol.

After blocking, 100 µl PBS + 10% FCS + 2 µl specific first antibody (overall 1:100 diluted) were added and put at 4° C for at least 30 minutes. Next, 1 ml of PBS was added for washing purposes and centrifuged for 5 minutes at 500 x g. The supernatant was discarded and the pellet was resuspended in 200 µl PBS + 10% FCS + 2 µl secondary antibody. This mixture was incubated for at least 30 minutes at 4 °C in the dark.

Before measuring, the cells were washed two times with PBS and transferred in FACS tubes.

Table 3-21: Antibodies for FACS staining

Virus	**primary antibody**	**secondary antibody**
3D6	α human LC + Biotin (α kappa-Biotin) (Thermofischer, USA)	Strep PE Cy5 (eBioscience, USA)
CD64 ∑ / sm	α CD64 [3D3] (abcam, UK)	α mouse AF647 (Life Technologies, USA)

As negative control, cells infected with unrelated virus were treated the same way as the samples.

3.2.2.5 Infection for SDS-PAGE and subsequent WB

For SDS-PAGE 2.5 x 10^5 cells/ml were seeded in T25 flasks and filled up to 5 ml with Hyclone medium. After cell settling the cells were infected with different virus working stocks

at a MOI of about 10. The cells were incubated for 2 – 3 days at 27° C, harvested and used for SDS-PAGE analysis according to 3.2.3.2.

3.2.2.6 Infection for Ultracentrifugation

500 ml shaking flasks of 70 ml *Sf9* / HighFive cells at a concentration of 1 x 10^6 cells/ml were prepared. For infection 7 ml of virus working stock were added (which correlates to a MOI of about 5 to 10). The cells were incubated for 4 days at 27° C and 100 rpm.
The successful infection was controlled through yellow light exhibition (Yellow fluorescent protein) with fluorescence microscopy (Leica). The cells were harvested and centrifuged at 3000 x g for 10 min. The supernatant was centrifuged again at 18.000 x g for 10 min. Subsequently the supernatant was put into cell culture flasks and stored at 4° C.

3.2.3 Biochemical methods

3.2.3.1 Preparation of SDS-Gels

Gels for SDS-PAGE were prepared at RT according to the table 3-22.
First the separating gel was filled into the gel-cassette and overlayed with isopropanol to get a straight front. After polymerization of the separating gel isopropanol was discarded and the stacking gel was added to the cassette. Immediately after, the combs for the slots were inserted. Again, after polymerization the gels were either used directly for SDS-PAGE or stored at 4° C in wet torques.

Table 3-22: Composition of SDS-PAGE Gel (4 gels)

Separating gel		**Stacking gel**
7008 µL	40% acrylamide	712 µL
1475 µL	2% bis-acrylamide	325 µL
5625 µL	Separating / Stacking buffer	1250 µL
8104 µL	dH_2O	2700 µL
225 µL	10% SDS	50 µL
135 µL	10% Aps	40 µL
13.5 µL	TEMED	4 µL

Aps (Promega, USA) and TEMED (Promega, USA) were added right before filling into the cassette.

3.2.3.2 SDS-PAGE

SDS-PAGE was used for separating proteins of different molecular size. To identify different proteins a Western Blot was done subsequently.
SDS gels were transferred into a gel electrophoresis chamber filled with 1x Laemmli buffer. The 10x Laemmli buffer was prepared according to table 3-23.

Table 3-23: Composition of 10x Laemmli buffer

amount	**component**
30 g	TRIS base
144 g	Glycine
10 g	SDS
up to 1000 ml	dH_2O

Samples for SDS-PAGE were mixed with 2x SDS loading buffer (see table 3-24), heated to 95° C for 10 minutes and centrifuged for 5 minutes at 14.000 rpm to avoid loading of precipitates.
In order to determine the size, 5 µl of standard, the PageRuler™ Prestained Protein Ladder, was applied in one or more slots.
The conditions for electrophoresis were 200 V and 40 mA for about 1 hour and 30 minutes. After completion of electrophoresis the gels were further used for Western Blotting or directly stained with coomassie brilliant blue.

Table 3-24: Composition of 2x SDS-PAGE loading buffer

amount	**component**
1 g	SDS
2 ml	Glycerin
2 ml	0.1% Bromphenolblue
1.25 ml	1M Tris pH 6.8
up to 10 ml	dH_2O

3.2.3.3 Protein size marker

In order to determine the size of SDS-PAGE samples, PageRuler™ Prestained Protein Ladder (Thermo Scientific), shown in figure 3-4, was used.

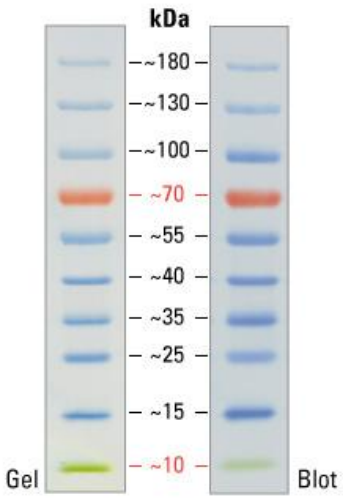


Figure 3-5: PageRuler™ Prestained Protein Ladder

3.2.3.4 Coomassie brilliant blue staining

The gels from electrophoresis or after Western Blotting were incubated in coomassie brilliant blue overnight on the shaker. On the following day, the gels were rinsed with dH_2O to get rid of excess staining solution and to visualize distinct protein bands.

3.2.3.5 Western Blot

In order to be able to detect specific proteins a Western Blot was performed after SDS-PAGE. Hereby proteins were blotted on a PVDF (GE Healthcare Life Sciences, UK) membrane and incubated with antibodies specific to target proteins.
The SDS-PAGE gel was equilibrated in TOWBIN buffer (see table 3-26) and put onto the PVDF membrane which had been activated with methanol and also equilibrated in TOWBIN buffer. Three filter papers, with the same size as the gel and membrane, were soaked in TOWBIN buffer and put in the blotting chamber. Then the gel on top of the membrane was transferred to the chamber, followed by another three filter papers. The blotting was carried out at 170 mA for 50 minutes.

Table 3-25: Composition of TOWBIN buffer

amount	component
3.03 g	TRIS base
14.4 g	Glycine
200 ml	Methanol
up to 1000 ml	dH2O

After blotting the membrane was incubated in a blocking solution (TPBS + 3% (w/v) BSA, see table 3-28) at 4° C over night.

Table 3-26: Composition of 10x PBS

amount	component
80 g	NaCl
2 g	KCl
2.4 g	KH_2PO_4
18.05 g	Na_2HPO_4*$2H_2O$
up to 1000 ml	dH_2O
	HCl for setting pH 7.4

Table 3-27: Composition of 1x TPBS (0.1% Tween 20)

amount	component
100 ml	10x PBS
1 ml	Tween 20
up to 1000 ml	dH_2O

Table 3-28: Composition of blocking solution

amount	component
1.5 g	BSA
50 ml	TPBS

The membrane was washed with dH_2O and equilibrated with TPBS. Then the membrane was incubated with one or more primary antibodies (see table 3-29) diluted in 12 ml TPBS +

1% (w/v) BSA for 1 hour on the shaker. The membrane was washed 3 times for at least 5 minutes with TPBS to remove excess antibody. Afterwards appropriate secondary antibody (see table 3-30) was added and incubated for another hour on the shaker. Again, the membrane was washed 3 times with TPBS and equilibrated in alkaline phosphatase (AP) buffer (see table 3-31). The blot was developed with 10 ml of AP buffer containing 66 µl NBT and 33 µl BCIP solution (Promega, USA) until bands were visible.

Table 3-29: Antibodies for Western Blot

primary antibody	**secondary antibody**
mouse α gp64 (1:2000)	α mouse IgG γ-chain AP (1:2000)
mouse α M1 (1:2000)	α mouse IgG γ-chain AP (1:2000)
mouse α CD64 [3D3] (1:2000)	α mouse IgG γ-chain AP (1:2000)
human Herceptin (1:1000)	α human γ-chain AP (1:2000)
α human heavy chain AP (1:2000)	-
α human light chain AP (1:2000)	-
α 3D6 antibody AP (binding to heavy and light chain) (1:2000)	-

Table 3-30: Composition of AP-buffer

concentration	**component**
100 mM	TRIS base
100 mM	NaCl
5 mM	$MgCl_2$
	pH 9.5

3.2.3.6 FACS

Subsequent preparation the samples were measured with Gallios™ Flow Cytometer (Beckman Coulter, Brier, CA) at a excitation wavelength of 633 nm (FL6). The acquired data was analysed using Kaluza® Flow Analysis Software.

3.2.3.7 Ultracentrifugation

- **Pelleting**

The culture supernatant was filled into 38 ml ultracentrifugation tubes and centrifuged for 1h 40 min at 30.000 rpm in a 38ti rotor (Beckmann) in vacuum. The forming pellet was resuspended in 0.5 ml PBS and stored at 4° C. A small sample was used for SDS-PAGE and WB.

- **Sucrose Gradient centrifugation**

Sucrose gradient preparation

For preparing the sucrose gradient volumes of 2 mL of 60 – 20% sucrose were filled into ultracentrifugation tubes. The gradient prepared was discontinuous consisting of 60%, 50%, 40%, 30% and 20% sucrose. After each addition the tube was put into the freezer for 2 hours to minimize mixing during preparation. Before usage the tube was thawed for about 2 hours at RT.

Gradient ultracentrifugation

The resuspended pellet (0.5 ml) was carefully filled onto the gradient in 13 ml ultracentrifugation tubes. PBS was added to fill the tube to the edge to prevent tube damage.
The ultracentrifugation was done at 38.000 rpm for 16 hours at 4° C in vacuum in a 42ti rotor (Beckmann)
After centrifugation, fractions of 1 ml each were removed from the top of the liquid and stored at 4° C for further Western Blot and SDS-PAGE analysis.

Pooling of fractions and pelleting

The desired fractions were pooled and diluted 1:1 with PBS to reduce sucrose concentration. The samples were centrifuged at 30.000 rpm for 1 h 40 min at 4° C in vacuum. The supernatant was discarded. The pellet was overlayed with 0.5 ml HEPES (20 mM) overnight to let the liquid soak in. On the following day the pellet was resuspended and stored at 4° C.

3.2.3.8 Transmission electron microscopy

30 µl of the resuspended pellet was used for TEM measurement (FEI). Samples were inspected at a magnification in the range between 10.000 and 100.000.

The samples were fixated on grids covered with pioloform and steamed with carbon. The coloration was done with uranyl acetate, leading to negatively stained samples.

4 Results and Discussion

4.1 General remarks

The aim of this study was to express different model proteins on the cell surface of insect cells and to create different budded virus like particles also displaying these proteins. Subsequently it was checked if the overexpressed proteins could be detected on the cell surface, virus like particles (VLPs) could be generated and if VLPs also displayed the model proteins.

Since successful production of VLPs is difficult to verify, several steps were conducted, each indicating additional information about VLP production. First, cloning of the baculovirus based display constructs was conducted, then expression of the model proteins was examined through SDS-PAGE and Western Blotting. Later the amount of model protein on cellular surfaces was analyzed through FACS experiments. Stepwise detection of positive results was performed before continuing with subsequent methods.
Ultimately, the separation of baculovirus and VLPs was pursued through gradient ultracentrifugation. The resulting samples were used for TEM measurements in order to visually detect VLPs.

4.2 Expression constructs

4.2.1 3D6 construct

The 3D6 construct was designed for baculovirus surface display of the human anti-gp41 monoclonal antibody 3D6. The recombinant baculovirus contained the 3D6 light chain and heavy chain, fused to the membrane anchorage sequence (mars) of gp64. In this manner the antibody was artificially anchored to the cellular surface and thus, was not secreted into the supernatant. The construct was made available by the working group and was used for further studies.

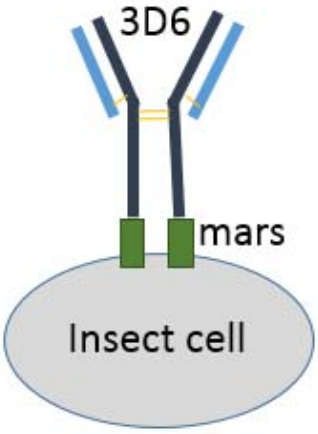


Figure 4-1: Schematic representation of the 3D6 antibody anchored to the cellular surface via the membrane anchorage sequence of gp64.

4.2.2 CD64 ∑ / sm construct

For the baculovirus surface display construct of CD64 two different version were designed. The anchorage of the Fcγ receptor I was made in two different ways. Once using the CD64 soluble gene sequence fused to gp64 mars sequence constituting a heterologous anchorage and once using the whole CD64 protein anchored via its original transmembrane domain. The construct was made available by the working group and was used for further studies.

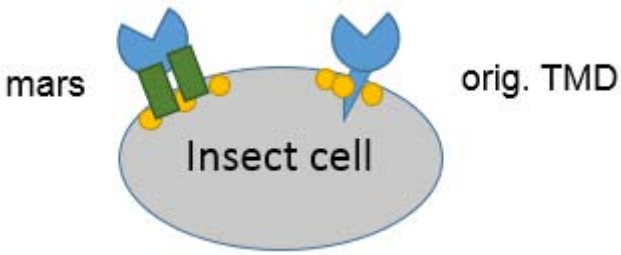


Figure 4-2: Schematic representation of the CD64 receptor anchored via the gp64 mars sequence and the original transmembrane domain.

4.2.3 HER2 construct

The HER2 construct was designed for surface display of the human epidermal growth factor receptor 2 (HER2) anchored via its native transmembrane domain. The HER2 construct was not available and was generated de novo.

- **Preparation of Insert DNA**

The HER2 gene was PCR amplified, according to 3.2.1.17.4., using specifically designed primers and applied to an agarose gel as shown in figure 4-3. In figure 4-3A the band for the PCR amplified gBlock can be seen at a size of about 2000 bp. In figure 4-3B the band at about 2000 bp is the PCR amplified ectodomain of HER2. In both cases a gel purification and subsequent excision of the correct band was required.

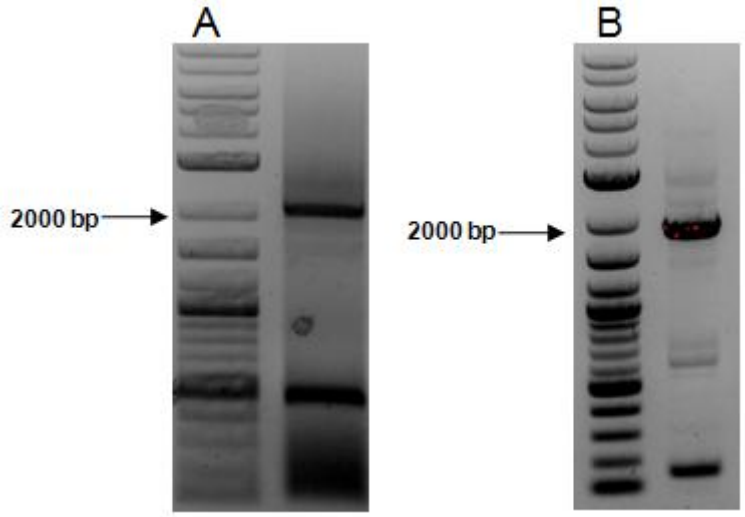


Figure 4-3: Gel pictures of PCR amplified insert DNA. **A:** PCR amplified gBlock. Quick-Load® Low Molecular Weight DNA Ladder was used as massruler. **B:** PCR amplified ectodomain of HER2. 2-log DNA ladder was used for size determination.

- **Ligation I – pSMART containing gBlock ligated with ECD**

The ECD was ligated into the pSMART vector containing the gBlock resulting in the assembly of the whole HER2 sequence. In figure 4-4A the band at about 4000 bp is the pSMART vector containg the gBlock digested with *XmaI/SphI*. In figure 4-2B several bands are visible. When successfully ligated the correct band size appears at 5631 bp. The bands visible above are pSMART vectors with multiple insertions of DNA. The band at about 2000 bp is the ECD alone, which wasn't ligated into the vector. The band at 4000 bp is the pSMART vector containing the gBlock but without the insertion of the ECD. Because of these unwanted bands a gel purification and excision of the correct band was required.

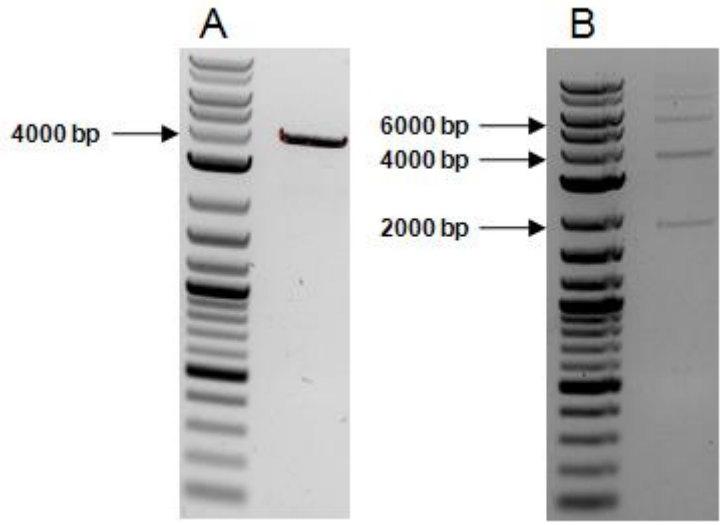


Figure 4-4: Gel pictures of ligation reaction outcomes. A: pSMART vector ligated with the gBlock and digested with XmaI/SphI with a size of 3831 bp. B: Digested pSMART vector containing the gBlock ligated with the ectodomain. 2-log DNA ladder was used as mass ruler.

- **Ligation II – HER2 ligated with pACEBac1**

The HER2 gene sequence was PCR amplified from the pSMART vector and ligated into the pACEBac1 acceptor vector. In figure 4-5 more than one band can be seen. The bands below the desired size at 2900 bp and 3800 bp are the vector pACEBac1 and the insert HER2 respectively. The bands visible above the desired size of 6704 bp are vectors with multiple insertions of insert DNA.

After ligation, isopropanolprecipitation and transformation of the obtained DNA into competent *NEB-5-alpha* cells, cells were striked out on selective LB agar plates containing gentamicin as selection marker. Due to the gentamicin resistance gene located on the pACEBac1 vector the selection of clones carrying the desired plasmid was possible. In order to check if the clones also contained the desired insert, a PCR screening was conducted using the primers HER2 Insert Screening primer 2 and HER2 Insert Screening primer3 leading to a 1200 bp long band.

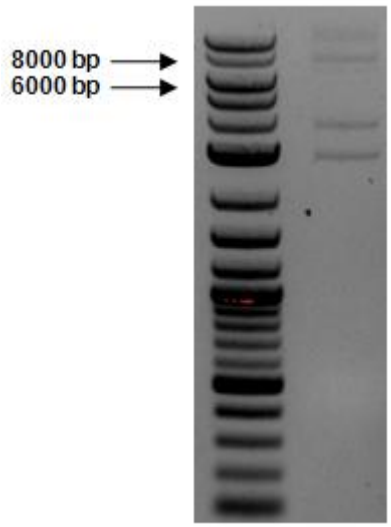


Figure 4-5: Agarosegel picture of the ligation of HER2 with the prepared vector pACEBac1. 2-log DNA ladder was used as mass ruler.

- **CreLox fusion of acceptor and donor vector**

The acceptor vector pACEBac1, containing the HER2 gene sequence, was recombined with the donor vector pIDC, containing the sequence for M1 (PR8).

Figure 4-6: Gel picture after the CreLoxP fusion reaction of donor and acceptor vector leading to a desired band of 9693 bp.

After plasmid preparation (Mini-prep) sample digests (figure 4-7) were done to confirm successful fusion of the two vectors and successful cloning of the construct. The bands on

the gel were compared to the theoretical bands according to the plasmid map using the CLC main workbench (CLC bio, Aarhus, Denmark).

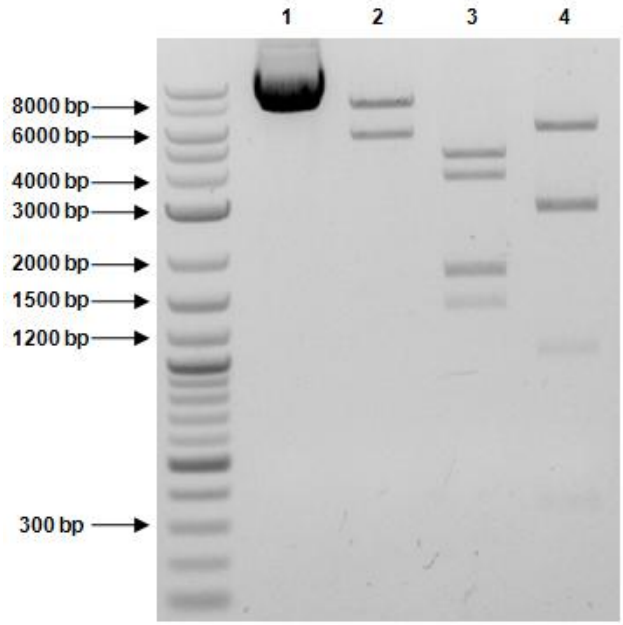


Figure 4-7: Gel picture of sample digest. In lane 1 the undigested plasmid can be seen with a length of about 9693 bp. In lane 2 the plasmid was digested with EcoRV leading to bands of 5300 bp and 4370 bp. In lane 3 the sample was cut with HIndIII leading to bands of 4370 bp, 3600 bp and 1700 bp in size. In lane 4 the sample was digested with BamHI leading to four bands with the sizes of 5660 bp, 2740 bp, 960 bp and 300 bp.

4.3 Comparison of protein expression

Generated virus working stocks (see 3.2.2.2) of the different expression constructs were used for infection of *Sf9* and HighFive cells. The reason for using two different insect cell lines was to examine differences in protein expression, secretion and for general comparison of the cell lines.

Expressed and assembled VLPs besides possible cell debris and baculovirus are expected in the supernatant. SDS-PAGE analysis followed by Western Blotting was conducted with all five different model proteins to check for protein expression. As negative control insect cells infected with non-related virus stocks (e.g. soluble hemagglutinin (H1 Cal09) from influenza) were used.

- Western Blot of **M1 only** construct:

In figure 4-8A the result of the Western Blotting of the M1 only construct expressed in HighFive (lane 1) and *Sf9* (lane2) cells can be seen. The upper band between 70 and 55 kDa represents the baculoviral gp64 major envelope protein (59 kDa) which is obligatory for propagation of the budded virus from cell to cell and thus for successful viral infection (Monsma et al., 1996). Detecting gp64 in the supernatant is an additional means of positive control, for e.g. successful infection, and concludes that there is baculovirus in the supernatant. The lower band at about 25 kDa represents the influenza matrix protein M1. For both cell lines M1 could be detected in the supernatant.
Gp64 represents the amount of baculoviruses in the supernatant and is comparable between the two cell lines, while there is slight difference in M1 expression found, maybe indicating that HighFive have a higher capacity for secreting proteins.

In comparison in figure 4-8B a whole H1M1 VLP construct (HA + M1) is shown. The bands for hemagglutinin (HA) and matrix protein M1 can be seen at a height of 70 kDa and 25 kDa respectively.

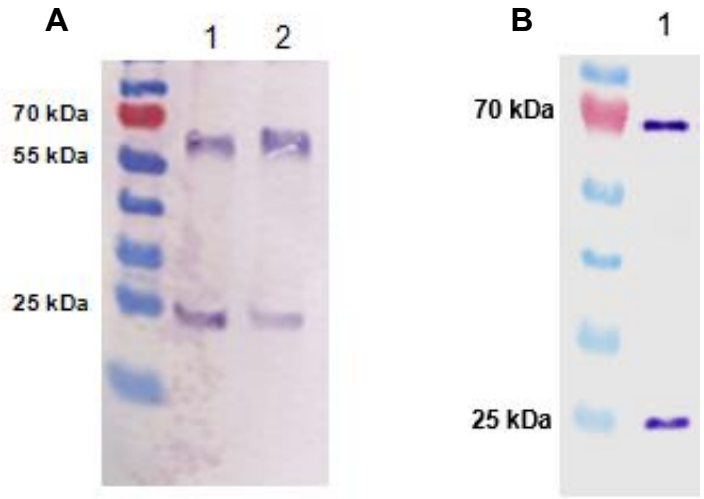


*Figure 4-8**A**: Western Blot showing the expression of gp64 and M1 (PR8) harvested three days post infection. The samples taken for this blot are from the supernatant fraction of an infection experiment. 1: gp64 and M1 expressed in HighFive cells. 2: gp64 and M1 expressed in Sf9 cells. PageRuler™ Prestained Protein Ladder was used as massruler. **B**: Western Blot showing the result of an infection experiment. The infection experiment and Western Blotting was done by another member of the working group. 1: The expression of HA and M1 in HighFive cells infected with a H1M1 expression construct. PageRuler™ Prestained Protein Ladder was used as massruler.*

- Western Blot of **CD64** (Σ/sm) constructs:

In figure 4-9A the Western Blot of the CD64 construct can be seen. Unfortunately, in the construct CD64 (Σ) with the native transmembrane domain specific CD64 could not be detected (lane 1 and 3) in this infection experiment. However, the construct CD64 (sm) with the gp64 mars sequence could be detected in both cell lines at a height of about 40 kDa (lanes 2 and 4). In figure 4-9B the Western Blot of another infection experiment can be seen. The bands visible represent specific CD64 (Σ) at a height of about 40 – 55 kDa which could be detected in both cell lines.

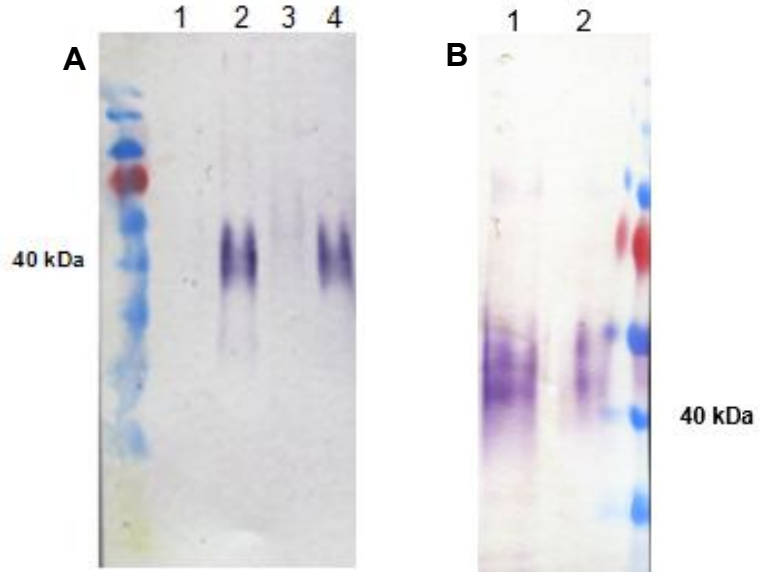


Figure 4-9 ***A:*** *Result of the Western Blot of the CD64 display constructs expressed in HighFive and Sf9 cells. The samples are taken from the supernatant fraction of an infection experiment. 1: CD64 (Σ) expressed in HighFive cells anchored with native domain. 2: CD64 (sm) expressed in HighFive cells anchored with baculoviral mars sequence.3: CD64 (Σ) expressed in Sf9 cells anchored with native domain 4: CD64 (sm) expressed in Sf9 cells anchored with baculoviral mars sequence. PageRuler™ Prestained Protein Ladder was used as massruler.*
B: *Result of the Western Blot of the CD64 ∑construct. The samples are taken from the supernatant fraction of an infection experiment 1: CD64 ∑ expressed in Hi5 cells. 2: CD64 ∑expressed in Sf9 cells. PageRuler™ Prestained Protein Ladder was used as massruler.*

- Western Blot of **3D6** construct:

Figure 4-10 shows the result of infection with the 3D6 + M1 display construct. In this experiment an antibody against the heavy and the light chain of 3D6 was used. The band for the heavy chain can be seen at the expected height of about 55 kDa. The light chain has a size of about 24 kDa and is also visible in the blot. Furthermore, the signal obtained is

stronger in the pellet fraction but also visible in the supernatant fraction at about the right height. The bands visible above 55 kDa are a result of unspecific binding. Overall, the signal for HighFive cells or *Sf9* cells is not significantly different and the heavy and light chain of 3D6 could successfully be detected in the pellet and supernatant fraction.

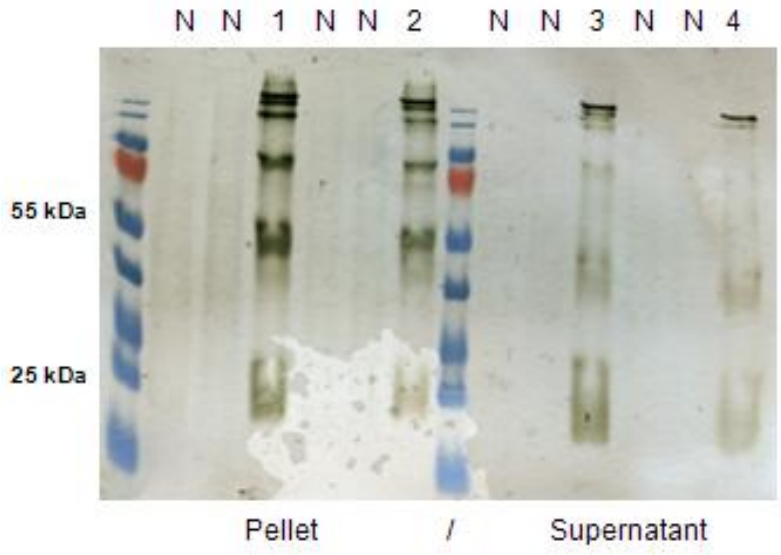


Figure 4-10: Western Blot of infection with 3D6 + M1 construct in HighFive and Sf9 cells. 1: Pellet fraction of HighFive cells infected with 3D6 + M1 virus. 2: Pellet fraction of Sf9 cells infected with 3D6 + M1 virus. 3: Supernatant fraction of HighFive cells. 4: Supernatant fraction of Sf9 cells. N: Negative control. HighFive/Sf9 cells infected with unrelated virus. PageRuler™ Prestained Protein Ladder was used as massruler.

- Western Blot of **HER2** construct:

Figure 4-11 shows the Western Blot of virus infection of HighFive and *Sf9* cells with the HER2 + M1 construct. The bands visible at about 25 kDa represent the matrix protein M1. A band for the human epidermal growth factor 2 (HER2) is not visible in any fraction. In lane 2, at the size of 130 kDa, a small band is visible which might be the desired band. Compared to the other bands the band is too light to be considered as positive result. Basically, M1 could be successfully expressed but not the rather large protein HER2.

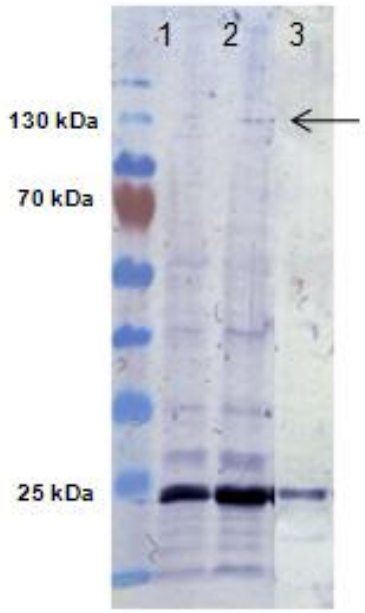


Figure 4-11: Result of Western Blot of virus infection with the HER2 + M1 construct. 1: Pellet fraction of HighFive cells. 2: Pellet fraction of Sf9 cells. 3: Supernatant fraction of Sf9 cells. PageRuler™ Prestained Protein Ladder was used as massruler.

4.3.1 Conclusion I – Verification of protein expression

All constructs showed results, meaning that the overexpressed proteins could be successfully detected in the supernatant (possible VLPs or soluble protein) shown by Western Blot and in the pellet fraction (data not shown). Therefore further experiments were conducted to obtain additional information.
Overall both cell lines showed comparable results at this stage and were used subsequently.
It needs to be highlighted, that there is the possibility that the positive signal at Western Blotting (samples from supernatant) is due to baculovirus itself, and not VLPs, carrying the expressed model proteins. Through virus budding from the cell surface, where the virus receives its envelope membrane from the host cell membrane, expressed membrane proteins can get incorporated into the virions from the baculovirus itself (Hötzel et al., 2011).

In figure 4-8B the expression of HA and M1 is shown as an example. The induction of expression and budding of particles consisting of HA and M1 is the most natural setup. Budding of particles, mediated by matrix protein M1, leads to the incorporation of many molecules of HA whereas it seems that in comparison fewer molecules of other model proteins, like CD64 or 3D6, are incorporated.

Moreover, viral envelope proteins, like gp64 and HA, are easier to express on the cellular surface and subsequently to direct to the surface of particles.

In case of the model protein HER2 there was no positive band visible at the desired size. The sequence of the ectodomain was not codon optimized for insect cells which might have been a problem. Another issue was, that a point mutation was incorporated into the sequence leading to a change in one amino acid. Also, HER2 is a very large protein (137 kDa) which might be hard to be targeted to the cell's surface which is necessary for detection since no cell disruption was done.
Due to these problems the model protein HER2 was not used further for subsequent analysis. A recloning of the construct would have been necessary.

4.4 FACS analysis

The amount of cells carrying display protein on its surface was analyzed through specific antibody binding and fluorescence detection by FACS analysis. Only cells displaying named proteins have the eventuality to produce VLPs through budding. In general, it was of interest to detect the percentage of cells displaying the model proteins.
As described in 3.2.2.3 insect cells were infected, harvested after 2-3 days, prepared and analyzed. As previously shown (Palmberger et al.,2011) it turned out to be ideal to incubate infected cells, with a MOI of about 10, for 3 days because at this point the virus is spread out completely. At incubation times longer than 3 days cell degradation and lysis, caused by infection, begins, resulting in many dead cells which is not desirable for this setup.
As negative control insect cells infected with an unrelated virus (baculovirus encoding influenza H1 from A/California/4/09) were used. The cells for the negative control (see 3.2.2.4) were treated in the same way as samples.

4.4.1 CD64Σ / sm

The CD64∑ construct is the native CD64 protein anchored via the original transmembrane domain whereas the CD64 sm construct was anchored via the heterologous membrane anchorage sequence (mars) of gp64. Both versions of the CD64 construct were expressed on the cellular surface of HighFive and *Sf9* cells.

In figure 4-12 native HighFive cells without viral infection are additionally shown in purple. The green peak represents the negative control. As stated before the negative control are cells infected with an unrelated baculovirus. The difference in the peaks of the cells without infection (purple) and the negative control cells (green) comes briefly from the fact that infected cells express many additional proteins and therefore are bigger, as observed in forward scatter versus side scatter plots (data not shown). Additionally, the granularity is higher which also leads to different light scatter patterns and autofluorescence. Nonetheless, the main reason for the difference is unspecific antibody binding of primary and secondary antibodies to the surface of insect cells which can not be completely avoided.
The blue peak represents the actual sample, cells infected with the CD64 **Σ** construct. In both cases a peak shift is observable, meaning that the desired proteins were successfully displayed. 38% HighFive and 32% of the *Sf9* cells displayed protein CD64 on their surface.

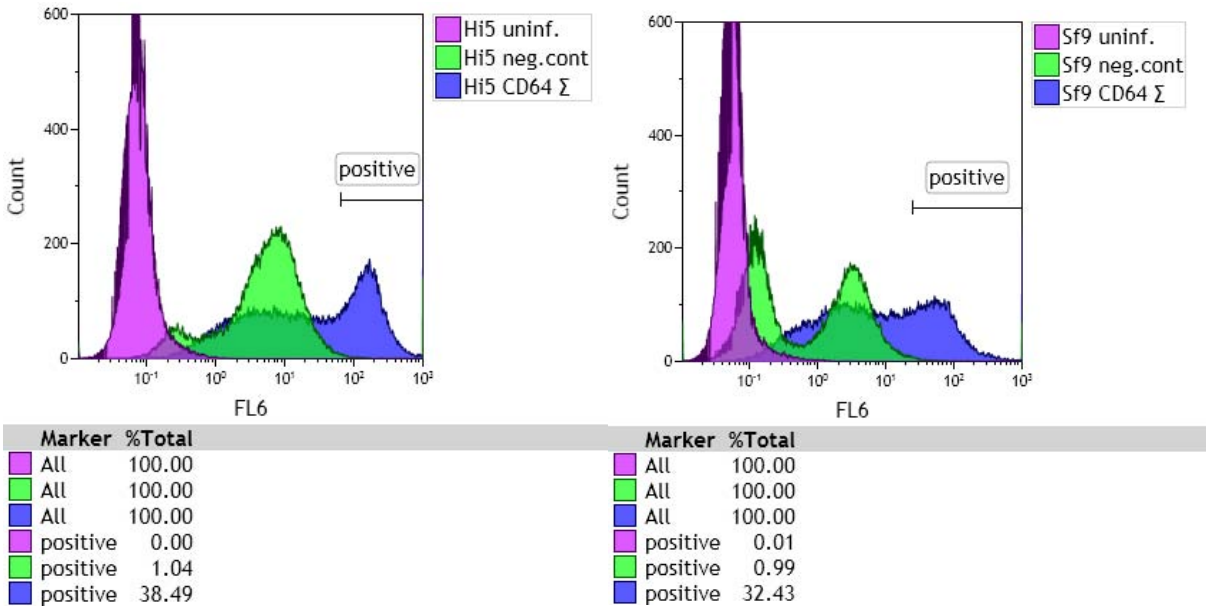


*Figure 4-12: Results of FACS analysis of CD64 **Σ** construct expressed in HighFive (left) and Sf9 (right) cells. The antibodies applied for detection were α CD64 [3D3] (primary antibody) and α mouse AF647 (secondary antibody).*

In figure 4-13 the result of FACS analysis of the CD64 sm construct is shown. In the left histogram (HighFive cells) the peaks are overlapping resulting in a lower number of positive cells (27%). Overall the number of positive cells are pretty similar to the CD64 Σ construct, both showing a percentage of about 30% cells displaying the construct.

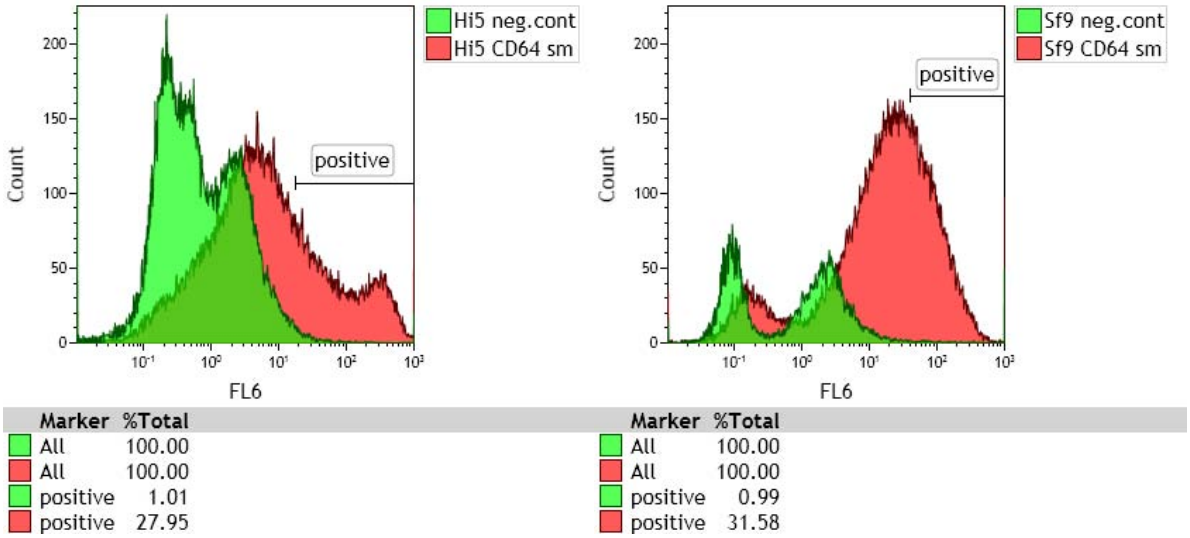
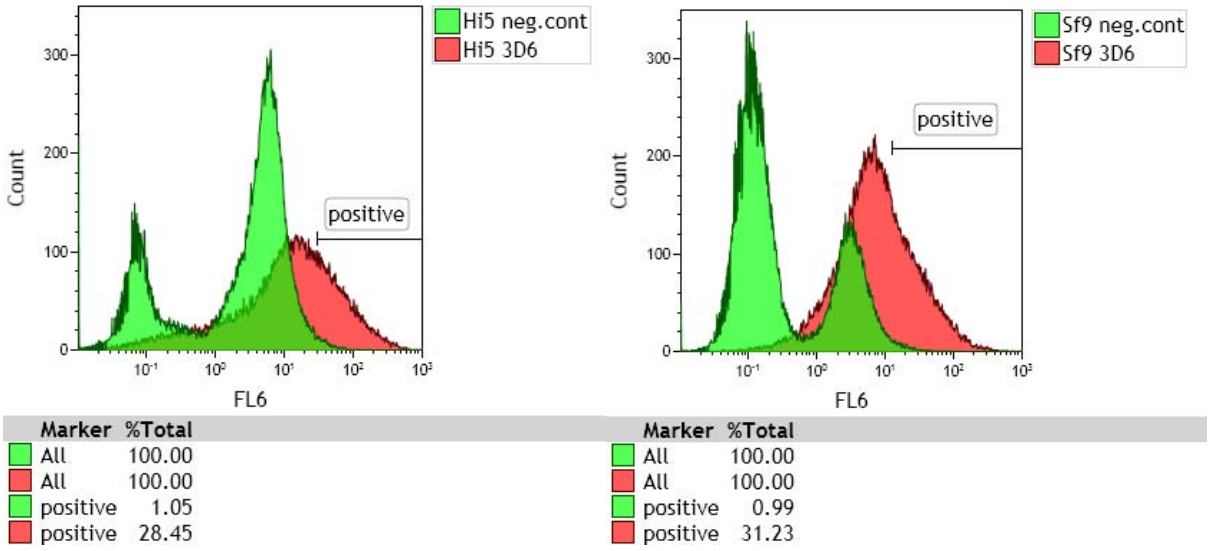


Figure 4-13: Results of FACS analysis of CD64 ***sm*** *construct expressed in HighFive (left) and Sf9 (right) cells. The antibodies applied for detection were α CD64 [3D3] (primary antibody) and α mouse AF647 (secondary antibody).*

4.4.2 3D6

The human monoclonal antibody 3D6 was anchored via the gp64 membrance anchorage sequence (mars) and expressed on the cellular surface of HighFive and *Sf9* cells.

Again, the histograms for the 3D6 display construct, as seen in figure 4-14, are quite similar to those of the CD64 constructs. No distinct peak shifts could be observed, meaning a rather low number of 3D6 positive cells (28% for HighFive, 31% for *Sf9* cells). For detection an antibody against the light chain of the 3D6 antibody display construct was applied.



Marker	%Total
All	100.00
All	100.00
positive	1.05
positive	28.45

Marker	%Total
All	100.00
All	100.00
positive	0.99
positive	31.23

Figure 4-14: Results of FACS analysis of 3D6 construct expressed in HighFive (left) and Sf9 (right) cells. The antibodies applied for detection were α human LC + Biotin (α kappa-Biotin) (primary antibody) and Strep PE Cy5 (secondary antibody).

In figure 4-15 the forward versus side scatter plot of HighFive cells, infected with the 3D6 display construct, is shown as an example. In the plot two different populations (1, 2) of cells can be seen. Both of these populations showed expression of yellow fluorescent protein and the desired display protein, meaning that both populations of cells are infected, maybe to a different extent. For calculations both populations were included without applying a gate.

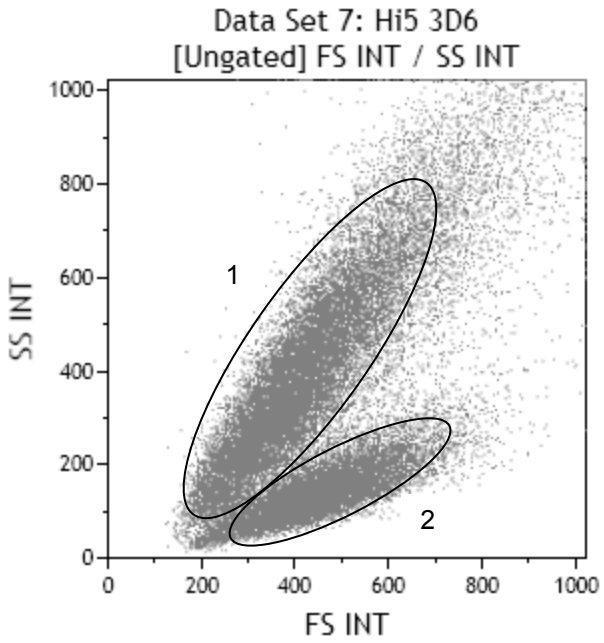


Figure 4-15: Forward scatter (FS) vs side scatter (SS) plot of 3D6 construct expressed in HighFive cells.

4.4.3 Conclusion II - FACS

Table 4-1: Results from FACS analysis for different constructs in HighFive and Sf9 cells.

Construct	**HighFive**	***Sf9***
CD64 Σ - positive	38%	32%
CD64 sm - positive	28%	32%
3D6 - positive	28%	31%

FACS analysis showed an overall number of around 30% cells which express the specific proteins on its surface (see table 4-1). Both cell lines and all constructs showed about the same amount of positive cells.

Prior to FACS analysis YFP expression was checked with fluorescence microscopy in order to verify successful virus infection. The reasons why the number of cells, which display model proteins, is not higher might be that the metabolic stress for the insect cells, caused by viral infection leading to the expression of various viral proteins as well as the desired model proteins and matrix protein M1, is higher. Subsequently, the specific expression and targeting of the model protein might be shut down, explaining the detectable amounts of proteins on the cellular surface. In contrast, YFP is produced internally and has no need to be secreted as it is detectable through the cell membrane.

Another problem was finding the optimal protocol for this setup. The expression of YFP, a good means to draw early conclusions, interferes, due to its excitation (550 nm) and emission wavelength, with the most common secondary staining antibodies. To avoid overlap, weaker fluorophores, which have higher emission and excitation (660 nm) spectra, had to be used. In addition, it couldn't be ruled out completely that YFP distorts the results in the higher regions as well. A solution for this problem would be to clone constructs without the YFP sequence, e.g. DH10MBY strains without YFP (-Y).

Due to unspecific antibody binding it was tried to introduce as many washing steps as possible, without damaging the cells and to block the cell's unspecific binding sites with FCS. Too many washing steps lead to cell disintegration as well as to loss of cells due to washing out. Another attempt was made by using a Fc receptor blocking solution (in case of CD64). Unfortunately, this problem could not be eliminated. In general it was hard to get reproducible data because of variable cell growth and variable degrees of virus infection while using exactly the same protocols for cell seeding and virus infection.

In all the histograms of *Sf9* derived samples the negative control shows double peaks. The occurance of these double peaks is independent of the display construct and the antibodies applied. This means that two different populations of cells, as also seen in figure 4-13, are present. One population might be infected, partly damaged cells and the other uninfected or less infected, intact cells, leading to two different peaks. Otherwise, the grade of infection was checked prior to FACS analysis by fluorescence microscopy and it is assumed that 2-3 days post infection all cells are infected at usual conditions. In comparison to HighFive cells *Sf9* cells tended to be better infected by baculoviruses, indicated by lower cell viability, also checked prior measurement, and therefore lower number of cells available for analysis.
Inspection of the forward versus side scatter plots of all samples and their respective negative controls showed that two different populations occured in all of them. In order to not distort the results all cells (both populations) were included for calculation without applying a gate, because it could not be determined which population is more relevant. In all cases the population 2 (see figure 4-15) expressed more YFP but less display protein and population 1 more display protein but less YFP. This could be caused by a competition for the expression machinery to produce YFP and display proteins inside the cell.

Later it could be shown that better results are obtained by infecting cells with a single monocistronic virus rather than using a polycistronic virus (co-expression). The amount of cells displaying the model protein is about half when additionally expressing the matrix protein M1, due to a higher metabolic burden.
For the purpose of protein surface display, infection with a single monocistronic virus is naturally beneficial because the aim is to detect a single protein on the surface and not a multicomplex protein or several proteins, and the expression of multiple proteins is generally more demanding for cells. In this work co-expression was the method of choice because subsequently the generation of VLPs was intended, requiring two different proteins for assembly.
Another strategy for multiprotein expression in insect cells is co-infection. By using a co-infection strategy viral DNA replication and mRNA transcription occur much faster than in co-expression systems, resulting in higher final DNA and mRNA concentrations, but also result in a quicker onset of cell death. Nevertheless the usage of co-expression has become more prominent recently, especially for the purpose of multigene expression. The limitations of co-infection, namely the uneven distribution of virus taken up by the cells, the need to copy genetic material of several different viruses and the possibility of incomplete co-infection, could be overcome by co-expression (Sokolenko et al., 2012). However, comparing results

from co-expression and co-infection experiments showed the same outcome (data not shown).

Nonetheless, the detection of the expressed model proteins on the cell surface could be successfully demonstrated.

4.5 Ultracentrifugation

4.5.1 Pelleting

The pelleting step was performed in order to achieve a better sample purity and to reduce the volume of sample to obtain a higher concentration of particles.
As described in 3.2.3.7 the harvested culture supernatant was ultracentrifuged. A SDS-PAGE and Western Blot were done to ensure that the desired proteins were expressed and secreted, optimally in form of correctly assembled VLPs consisting of M1 (PR8) and the model protein, into the supernatant.

- Western Blot of M1 and gp64:

First it was checked if the influenza matrix protein M1 (band at 25 kDa) was expressed. Additionally, an antibody against baculoviral gp64 (59 kDa) was applied in order to check if gp64 is present. Gp64 could be detected on the surface of virus like particles and/or on baculovirus budded virions. As seen in figure 4-16 both proteins were expressed in all fractions and both cell lines. Furthermore, it can be noted that in this case *Sf9* cells (annotation 2 figure 4-16) express more protein than HighFive cells (annotation 1 figure 4-16). The samples from *Sf9* cells induce smears on the Western Blot meaning that more particles are present and that in comparison to HighFive cells the samples are more pure.
Altogether the concentration of particles was sufficient for subsequent gradient ultracentrifugation.

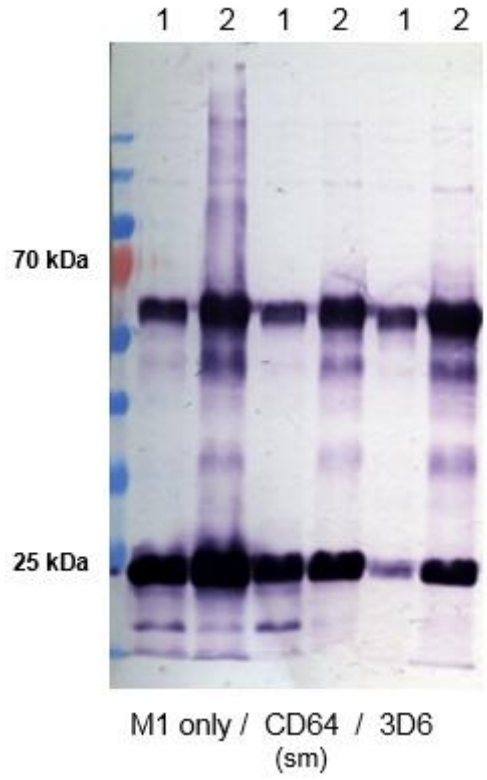


Figure 4-16: Western Blot showing the bands against matrix protein M1 and baculoviral gp64. 1: Infection of HighFive cells. 2: Infection of Sf9 cells. PageRuler™ Prestained Protein Ladder was used as mass ruler.

- Western Blot of specific proteins (CD64, 3D6):

In addition, it was tested if the specific display proteins (CD64, 3D6) have been successfully expressed. In lane 1 and 2 (see figure 4-17) the heavy (about 55 kDa) and light chain (about 25 kDa) of 3D6 could be detected. As seen before (figure 4-16) the expression of protein was better in *Sf9* cells. In lane 2, 3 and 4 of figure 4-17 a lot of unspecific bands and smears are visible because of cross reactivity of the antibodies applied, general unspecific and weak binding or other reasons.

The band for CD64 (sm) at the right size is detectable (black arrow), but, because of the many unspecific bands, no real conclusion could be drawn. Nevertheless, since M1 was specifically detectable (see figure 4-16), the samples were used further for sucrose gradient ultracentrifugation.

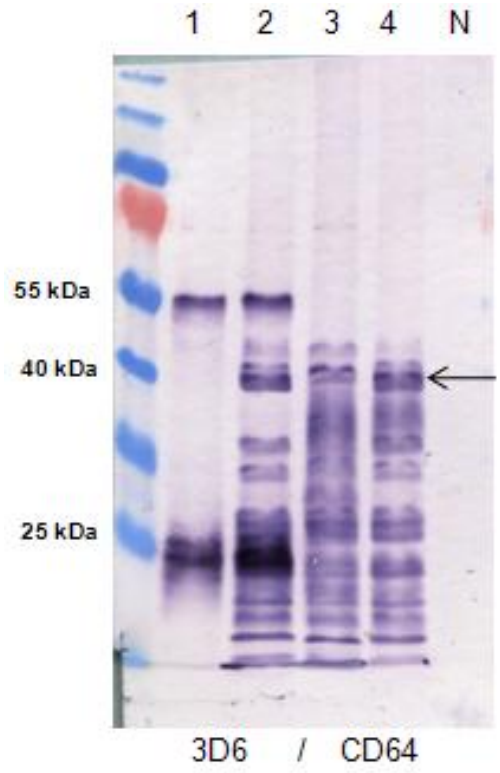


Figure 4-17: Western Blot showing the expression of the display constructs 3D6 and CD64 (sm). 1: 3D6 expressed in HighFive cells. 2: 3D6 expressed in Sf9 cells. 3: CD64 (sm) expressed in HighFive cells. 4: CD64 (sm) expressed in Sf9 cells. N: Unrelated baculovirus H1 Cal09 used as negative control. PageRuler™ Prestained Protein Ladder was used as mass ruler.

4.5.2 Conclusion III - Pelleting

The results from the Western Blots (figure 4-16 and 4-17) showed that the bands for *Sf9* cells are stronger than those of HighFive cells. Hence it can be assumed that in the resuspended pellets, derived from *Sf9* cells, more particles and baculovirus can be found. It has previously been shown (Wilde et al., 2014; Krammer et al., 2010) that HighFive cells have better secretion properties and are easier infected, whereas *Sf9* cells produce more baculoviral particles and can be better amplified. In this case the data obtained comes to the same conclusion. Nevertheless, it has to be stated that, especially in HighFive cells, the growth and protein expression were very variable. Even without viral infection, HighFive cells sometimes showed affected growth resulting in cell clumps and low viability. One possible explanation might be an overall too high number of passages of the cells in culture, negatively affecting cell growth properties. Because of these reasons full reproducibility is not given.

Also, the nature of CD64 as a Fcγ receptor I might lead to unspecific antibody binding (IgG), as seen in figure 4-17. CD64 is a Fc receptor that binds monomeric IgG-type antibodies with

high affinity (Hulett et al., 1998). Thus, primary or secondary IgG-type antibodies used for WB might get bound unspecifically by CD64. Additionally, CD64 may be differently processed by the insect cells leading to varying glycosylation patterns. Thus, the model protein CD64 shows different heights of bands at Western Blot according to the level of glycosylation.

4.5.3 Gradient ultracentrifugation

Following the pelleting step, a gradient ultracentrifugation was to done in order to separate baculovirus from VLPs. After centrifugation, the gradient was split up into 12 fractions and the pellet fraction and examined on a Western Blot. As shown in the previous Western Blot (see figure 4-16) a lot of gp64 could be detected suggesting that baculovirus is present.

4.5.3.1 VLP production in Sf9 cells

In figure 4-18 the results of the Western Blots after sucrose gradient ultracentrifugation are shown. The distinct bands at about 25 kDa represent the matrix protein M1 and can be seen in all four instances (A, B, C, D). In figures 4-18 A, B, D the bands for the specific display proteins (CD64, 3D6) can be seen. To avoid overlap of bands the antibody against gp64 was left out.

Figure 4-18C shows the construct M1 only with antibodies against baculoviral gp64 and matrix protein M1. It could be previously shown (Valley-Omar et al., 2011) that VLPs that bud from insect cells become enveloped with the insect host cell membrane, which contains significant amounts of baculovirus gp64 envelope protein. Therefore, antibodies binding to other baculoviral structural proteins (e.g. baculovirus major capsid protein VP39), which are not incorporated into VLPs, are needed to distinguish baculoviruses from VLPs.

However, the fractions with strong bands were pooled followed by preparations for transmission electron microscopy.

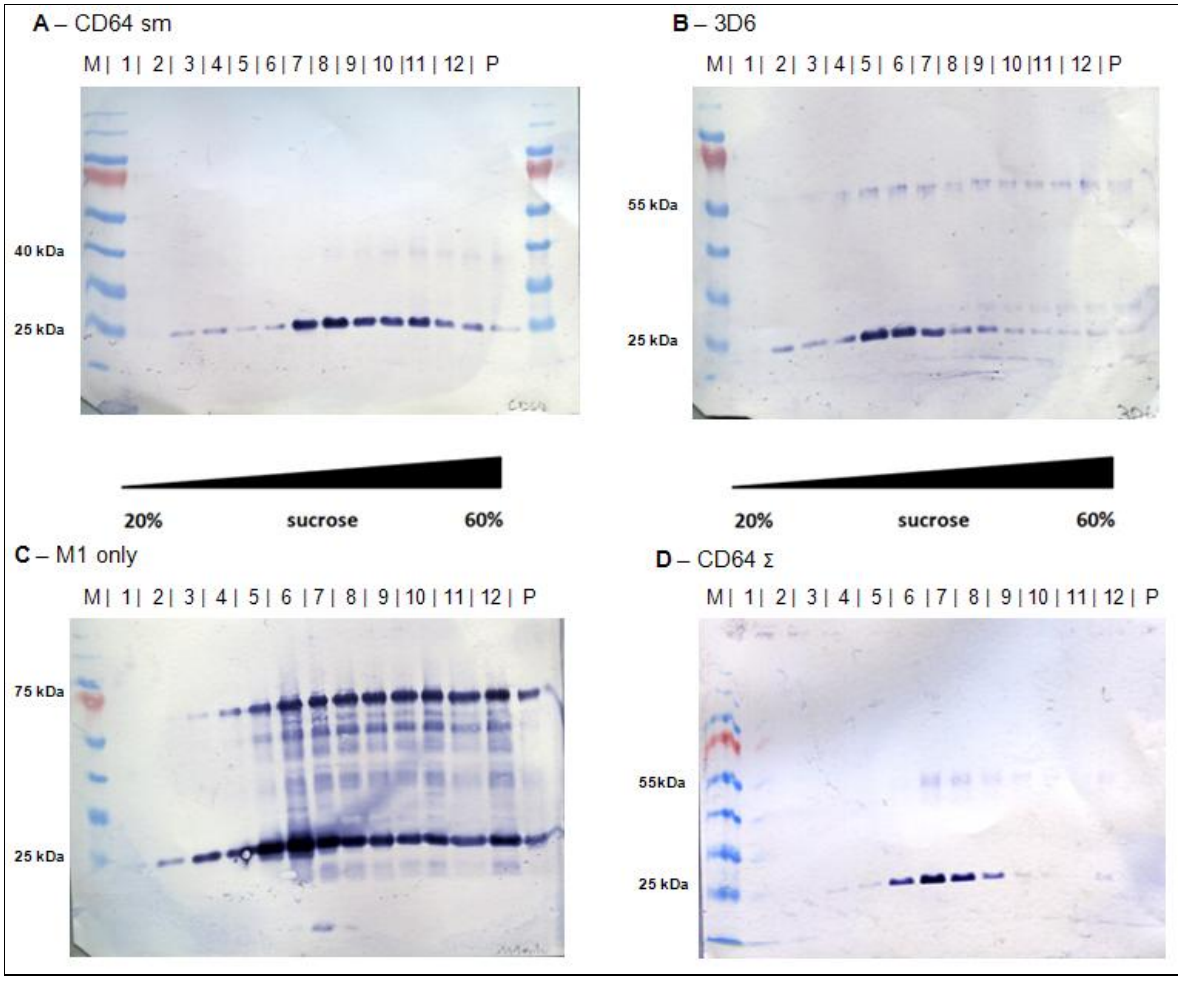


*Figure 4-18: Western Blots after the gradient ultracentrifugation of the four different display constructs in **Sf9** cells. **A:** Gradient of CD64 (sm) with mars sequence. **B:** Gradient of infection with 3D6 + M1 virus working stock. **C:** Gradient of infection with M1 only construct. **D:** Gradient of infection with CD64 (construct with native transmembrane domain. M: PageRuler™ Prestained Protein Ladder was used as massruler. 1-12: Fractions of the sucrose gradient. P: Pellet of sucrose gradient.*

4.5.3.2 VLP production in HighFive cells

In figure 4-19 the Western Blots after gradient ultracentrifugation of culture supernatant of HighFive cells can be seen. As expected the signal is weaker than in *Sf9* cells because of previous findings (see figure 4-16). In addition, the samples, obtained from the pelleting step, are strongly diluted with sucrose leading to weak or no bands. In figure 4-19A weak bands for M1 can be seen in fraction 4,5,6,7,8,12 and in the pellet fraction but no bands at all for CD64 sm. In figure 4-19B very weak bands for the heavy chain of 3D6 can be seen in fraction 5, 6, 7 and 8. The blot of the M1 only construct (figure 4-19C) shows distinct bands whereas the blot of CD64 ∑ shows no bands at all.

Accordingly to the procedure with *Sf9* derived samples, the fractions with the strongest bands or bands at all got pooled and prepared for transmission electron microscopy. In case of CD64 ∑ (D) the fractions 4-11 got pooled.

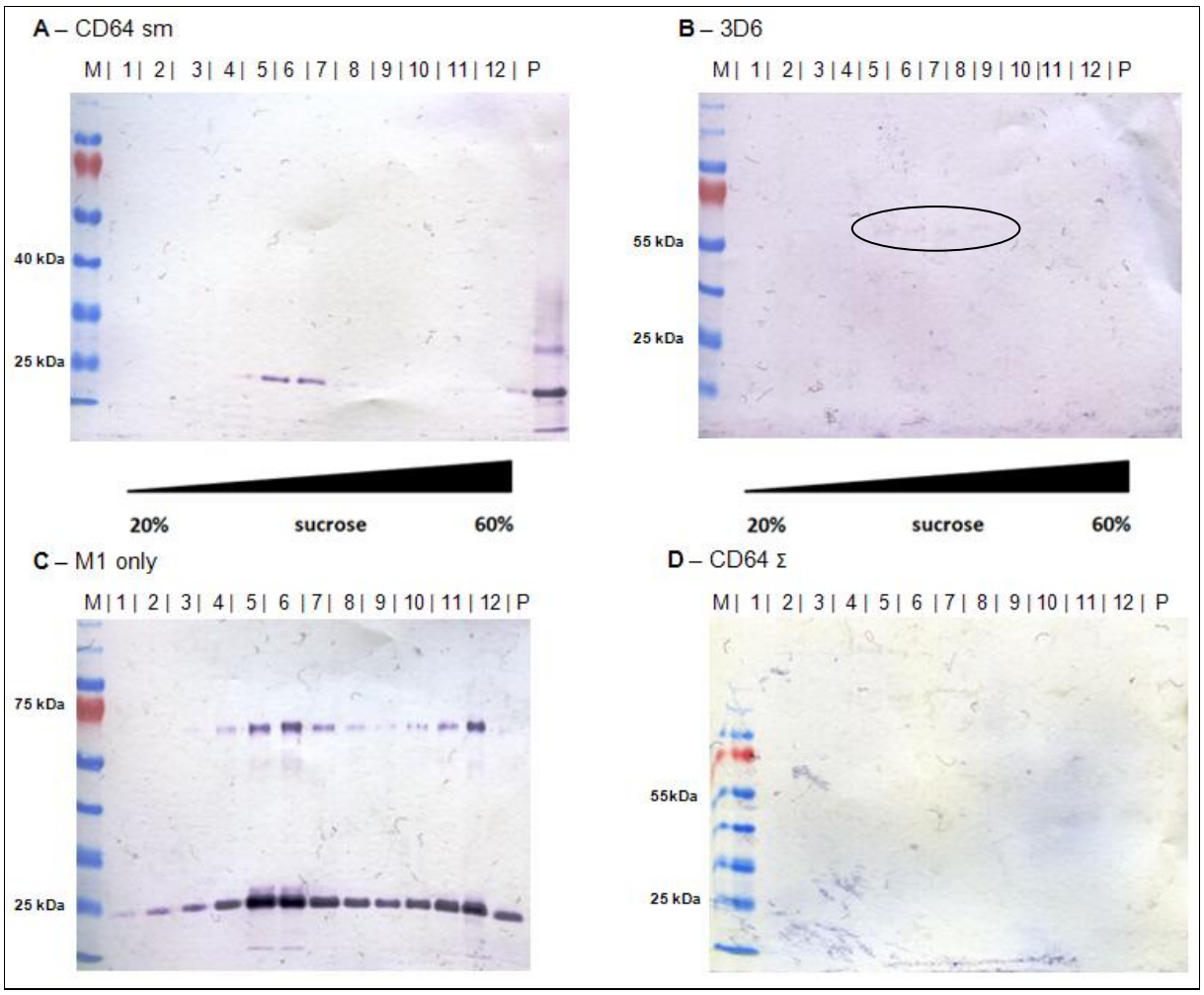


*Figure 4-19: Western Blots after the gradient ultracentrifugation of the four different display constructs in **HighFive** cells. **A:** Gradient of CD64 (sm) with mars sequence. **B:** Gradient of infection with 3D6 + M1 virus working stock. **C:** Gradient of infection with M1 only construct. **D:** Gradient of infection with CD64 (construct with native transmembrane domain. M: PageRuler™ Prestained Protein Ladder was used as massruler. 1-12: Fractions of the sucrose gradient. P: Pellet fraction of sucrose gradient.*

4.5.4 Conclusion IV – Gradient ultracentrifugation

The obtained results from Western Blot showed that separation might have been achieved, but it was hard to differentiate between baculoviruses and VLPs with the antibodies available, because of gp64 incorporation into VLPs. In this case the gradient ultracentrifugation can be seen like a cleaning or clarification step, cutting off larger particles or contaminations.

Overall, even after the strong dilution with sucrose, the model proteins as well as the matrix protein M1 could be detected in Western Blot meaning that there have to be VLPs and or baculovirus carrying model proteins. The construct M1 only showed the best result, meaning that in many fractions a strong signal was detectable. The metabolic burden for the insect cells is the lowest because besides viral proteins only one foreign protein, the matrix protein M1, has to be expressed. All other constructs elicit the expression of two proteins, requiring more cell capability and additional processing pathways.

As stated before HighFive cells showed affected growth and expression compared to *Sf9* cells due to cell clumping in culture and the high passage number. In addition, it has been shown (Krammer et al., 2010) that the baculovirus background is lower using HighFive cells compared to *Sf9* cells. Hence, the signal at Western Blot is weaker at the HighFive derived samples (figure 4-19), because fewer baculovirus particles, consisting of baculoviral proteins as well as model proteins are present.

As next step transmission electron microscopy was performed.

4.6 Transmission electron microscopy

Subsequently, transmission electron microscopy (FEI) from the pooled samples was conducted.

In figure 4-20 the rod shaped baculovirus are visible. At both ends of the rods cloud like structures can be seen, consisting of the major envelope protein gp64 (Wang et al., 2016).

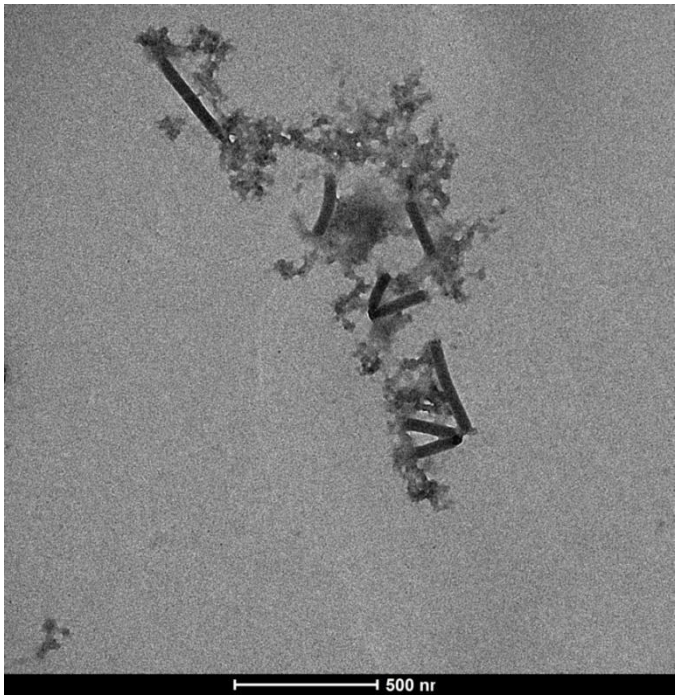


Figure 4-20: Image of TEM measurement of (Sf9 CD64 ∑ + M1) at 19000 x magnification.

Figure 4-21 shows baculoviruses at a higher magnification. Again gp64 can be observed at the end of the rods (darker regions).

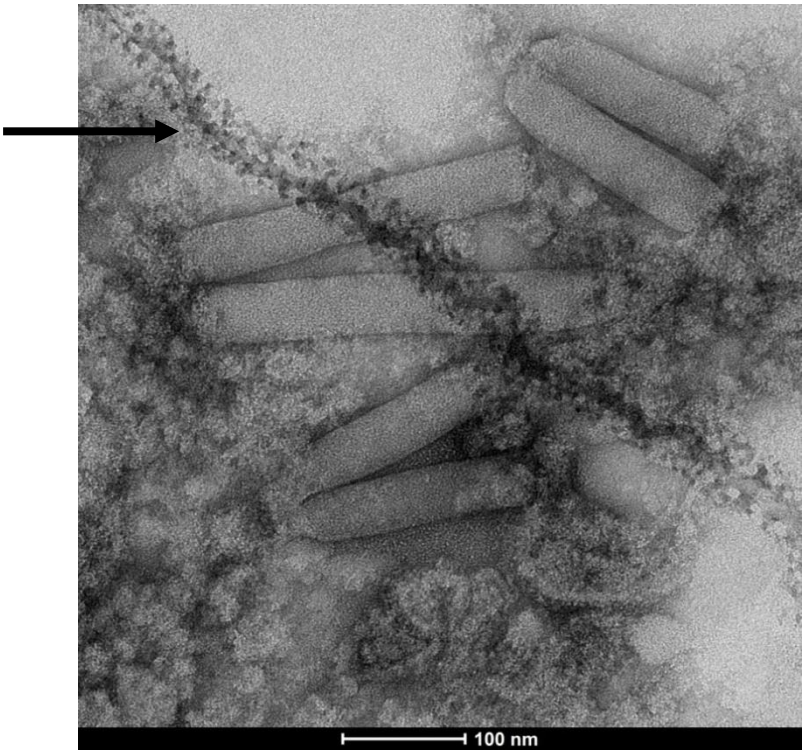


Figure 4-21: Image of TEM measurement of sample (Sf9 M1 only) at 80000 x magnification. The black arrow indicates the grid on which the samples were fixated.

Figure 4-22 shows two examples of spherical structures found in the samples which could be VLPs. The darker regions visible at the edge around the sphere could be the influenza matrix protein M1. In figure 4-22A a somehow damaged or incomplete VLP can be seen. The size of the particles is larger than those of wild-type influenza VLPs, which are in a range of 80-120 nm (Krammer et al., 2010). Generally, the size of VLPs depends on the size and structure of the proteins incorporated.

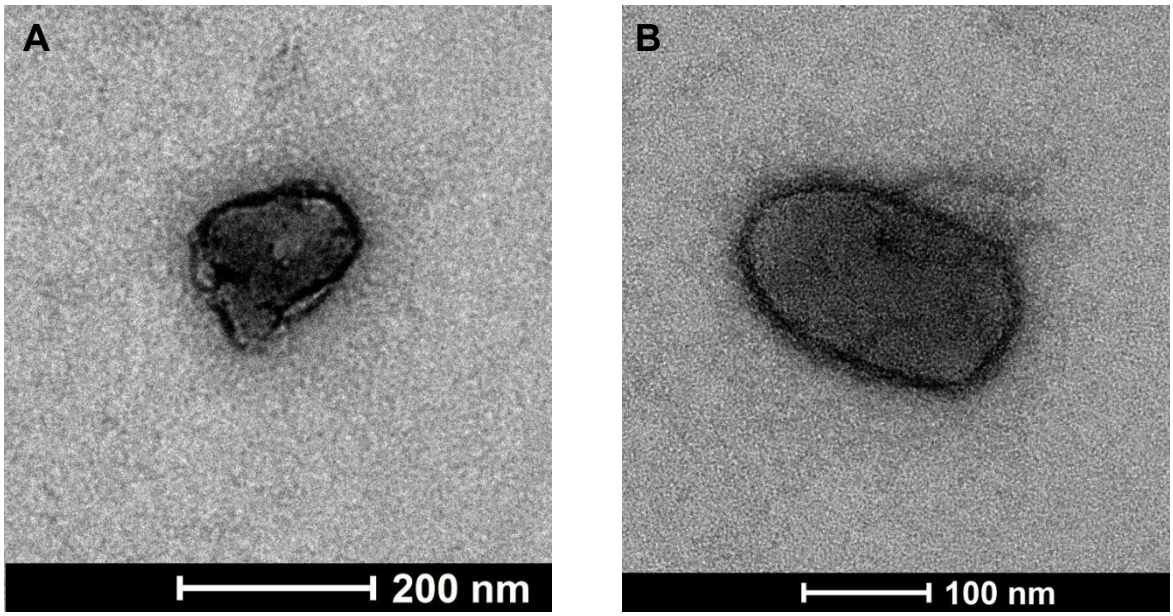


Figure 4-22: Image of TEM measurement of negatively stained samples (HighFive CD64 ∑).

4.6.1 Conclusion V - TEM

The spherical structures observed in figure 4-22 could be assumed to represent virus like particles. However, in order to be sure that VLPs, displaying the model proteins, were assembled, antibodies, which bind to the display constructs and are conjugated with gold particles (immunogold labeling), would be needed to be applied (Wu et al., 2010).
Recently it has been shown (Wang et al., 2016) that a better way to visualize the structure of baculovirus and probably VLPs is to conduct cyro-EM. To sum it up, the overall approach has to be changed, including additional means of staining (labeling) and other electron microscope techniques, to be able to detect and identify VLPs properly.
Generally, the sample preparation and staining for TEM is complex and the analysis itself bears difficulties. High purity of the samples is a precondition for TEM analysis (Thompson et al., 2013), which could be achieved through multiple runs of ultracentrifugation.
In a recent study it has been shown (unpublished data) that the matrix protein M1 from influenza is not very efficient for VLP generation because M1 shows bad budding properties as compared to other matrix proteins, like the structural protein gag from HIV.

5 Conclusion

The aim of this thesis was to recombinantly overexpress selected proteins in insect cells and to direct them to the cellular surface. By mutli-protein expression, using the baculovirus expression vector system, it was also tried to assemble virus like particles, formed by the influenza matrix protein M1, acting as capsid protein, and the selected 3D6 antibody or Fcγ receptor I CD64, located at the particle surface.

Initially it was checked if the overexpressed proteins could be found in the pellet fraction and supernatant of the insect cell slurry, revealing that all proteins could be successfully detected. Next the amount of cells displaying the model proteins was analysed through FACS. A possibility to improve the expression levels, bacmids without the YFP sequence could be generated in order to reduce the metabolic stress of the insect cells to express three heterologous proteins and to render FACS analysis easier in terms of fluorophore utilization and measurement itself. For the purpose of protein display, the usage of a single monocistronic virus would lead to more cells displaying desired proteins.

Since protein display and expression of the chosen model protein was to a greater extent successful, the concentration of particles and separation of VLPs from baculoviruses was attempted by ultracentrifugation. The obtained results showed that separation might have been achieved, but it was hard to differentiate between baculoviruses and VLPs on Western Blot with the antibodies available, because of gp64 incorporation into VLPs. An antibody against the capsid protein VP39 would tackle this problem.

Nevertheless the ultracentrifugation proved beneficial as a means of purification for the following transmission electron microscopy. The images from TEM measurement illustrated the morphology of baculoviruses nicely and spherical structures could be found that can be assumed to represent virus like particles.

To sum it up, the baculovirus expression vector system (BEVS) in combination with HighFive and *Sf9* cells is definitely suitable for expression of particle structures and protein display. In order to facilitate the identification of VLPs, the conditions and procedures may be changed. For increased yield of particles, other matrix proteins, like the viral structural protein gag from HIV, could be tested instead of matrix protein M1. In addition, the co-expression of chaperones, assisting in correct folding, could be beneficial. Moreover, sucrose gradient ultracentrifugation is a good means of sample purification and preparation for TEM analysis or other applications where highly pure samples are required. Correctly assembled VLPs can be further used as biomarkers in diagnostics, to identify binding partners, to display antigens for screening procedures and to test the functionality of expressed proteins.

6 Abbreviations

3D6	Human HIV anti-gp41 IgG_1 antibody
ABTS	2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt
AcMNPV	*Autographa californica* multinucleocapsid NPV
AP	Alkaline phosphatase
APS	Ammonium persulfate
attTn7	Tn7 attachement site
BCIP	5-bromo-4-chloro-3'-indolyphosphate
BEVS	Baculovirus expression vector system
BSA	Bovine serum albumin
CD64	Fc gamma receptor I
EDTA	Ethylendiaminetetraacetic acid
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
YFP	Yellow fluorescent protein
GOI	Gene of interest
GV	Granulovirus
HER2	Human epidermal growth factor receptor 2
HighFive	*Trichopulsia ni* BTI-Tn5B1-4 “HighFive™” cells
LB	Luria-Bertani
M1	Influenza matrix protein
mars	Membrane anchorage sequence
MCS	Multiple cloning site
MOI	Multiplicity of infection
NBT	nitro-blue tetrazolium
NPV	Nucleopolyhedrovirus
OB	Occlusion body
ODV	Occlusion derived virus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
polh	Polyhedrin
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sf9	*Spodoptera frugiperda* cells

VLP	Virus like particle
TEM	Transmission electron microscopy
TEMED	N,N,N´,N´-Tetramethylethan-1,2 diamin
TPBS	Phosphate buffered saline + tween
WS	Working stock

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