University of Natural Resources and Life Sciences

Department of Biotechnology

HEMAGGLUTININ SURFACE DISPLAY ON VIRUS LIKE PARTICLES GENERATED IN TWO DIFFERENT INSECT CELL LINES

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ABSTRACT

Recurring antigenic drift and antigenic shifts of the influenza virus necessitate regular vaccine updates and the availability of new vaccines within a short time. Rapid response is especially necessary in the case of a pandemic setting and can be achieved through VLP based vaccines produced with the baculovirus expression system in insect cells. The aim of this work was to produce H1 A/California/4/2009 (H1N1) and H3 A/Hiroshima/52/2005 (H3N2) displaying HIV-1 Gag VLPs in the insect cell lines *Sf*9 and *Tnms*42 and compare these two systems in terms of product yield. A HIV-1 p24 ELISA was used to determine the amount of produced VLPs. The widely used *Sf*9 cells yielded more empty VLPs (Gag-only VLPs) than *Tnms*42. However, regarding VLPs displaying a hemagglutinin on their surface the *Tnms*42 cell line generated three times more HA-Gag VLPs per infected cell than the *Sf*9 cells. This results show the potential of using *Tnms*42 cell for producing VLP-based vaccines. A H1-ELISA with a self-produced standard was developed in order to quantify the HA on the VLPs in the expression supernatant. We could increase HA expression 3-fold by having a second HA expression cassette on the baculovirus. The produced VLPs were purified by sucrose gradient centrifugation and subsequently analyzed with NanoSight, Zetasizer and TEM. In general, all hemagglutinin constructs showed comparable results regarding purity and VLP integrity.

Keywords: insect cells, baculovirus, recombinant protein expression, VLP, hemagglutinin, influenza

ZUSAMMENFASSUNG

Immer wiederkehrende Antigendrifts und Antigenshifts des Influenza Virus erfordern regelmäßige Impfstoffaufrüstungen und die Verfügbarkeit von neuen Impfstoffen in kürzester Zeit. Schnelles Handeln ist besonders notwendig bei Pandemien und kann mithilfe von Virus-like particle basierenden Impfstoffen, die durch das Baculovirus Expressions-Vektor-System in Insektenzellen produziert werden, erreicht werden. Das Ziel dieser Arbeit war die Produktion von H1 A/California/4/2009 (H1N1) und H3 A/Hiroshima/52/2005 (H3N2) HIV-1 Gag VLPs in den Insektenzelllininen *Sf*9 und *Tnms*42 und der Vergleich dieser zwei System bezüglich Produktausbeute. Ein HIV-1 p24 ELISA wurde dazu verwendet, um die Menge an produzierten VLPs zu bestimmen und dadurch die zwei Zelllinien zu vergleichen. Die weit verbreitete *Sf*9 Zelllinie produzierte mehr leere VLPs (HIV-1 Gag_only VLPs) als *Tnms*42. Aber, in Bezug auf VLPs mit Hämagglutinin an der Oberfläche stellte die *Tnms*42 Zelllinie dreimal mehr HA-Gag VLPs pro infizierter Zelle her als die *Sf*9 Zellen. Diese Resultate zeigen das Potential der Verwendung von *Tnms*42 Zellen zur Herstellung von VLP-basierenden Impfstoffen. Ein H1 ELISA mit selbst hergestelltem Standard wurde entwickelt, um die Menge an H1 Trimeren an der Oberfläche eines VLPs zu bestimmen. Ungefähr 400 H1, um das Hämagglutinin auf den VLPs im Expressionsüberstand zu quantifizieren. Wir konnten die Hämagglutinin-Expression durch eine zweite HA Expressionskassette im Baculovirus dreifach erhöhen. Trimere konnten auf der Oberfläche der produzierten HIV-1 Gag VLPs gefunden werden. Die produzierten VLPs wurden über einen Sucrosegradienten gereinigt und anschließend mit dem NanoSight, Zetasizer und TEM analysiert. Im Allgemeinen zeigten die unterschiedlichen Hämagglutininkonstrukte vergleichbare Resultate bezüglich Reinheit und VLP Qualität.

ABBREVIATIONS

*Ac*MNPV	*Autographa californica* multiple nucleopolyhedrosisvirus
APS	Alkaline phosphatase buffer
BCIP	5-bromo-4chloro-3'-indolyphosphate
BEVS	Baculovirus expression vector system
bp	Basepairs
BS	Burstsequence
BSA	Bovine serum albumin
BV	Budded virion
ddH2O	Double destilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose containing nucleoside triphosphate
DPBS	Dulbecco's phosphate-buffered saline
EDTA	Ethylendiaminetetraacetic acid
GP64	Glycoprotein 64
GV	Granulosisvirus
HQ water	High Quality water
MNPV	multiple nucleopolyhedrosisvirus
MOI	Multiplicity of infection
ODV	occlusion derived virion
Polh	polyhedrin
RNA	ribonucleic acid
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNPV	single nucleopolyhedrosisvirus
TCID50	50% Tissue Culture infective dose
TEMED	N,N,N',N'-Tetrametylethan-1,2diamin
TPBS	Phosphate buffered saline + Tween 20
TRIS	tris(hydroxymethyl)aminomethane
WS	Working Stock
YFP	Yellow fluorescence protein

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1. INTRODUCTION

1.1. RECOMBINANT PROTEIN PRODUCTION IN INSECT CELLS

During the last years recombinant protein production in insect cells using baculoviruses infection has increasingly gained importance and has been widely accepted for biotechnological applications in molecular biology, agriculture and animal health (Granados *et al*., 2008). Advantages of the baculovirus expression vector system (BEVS) include high-yield recombinant protein expression and the expression of biologically active and correctly folded proteins with posttranslational modifications (glycosylation, phosphorylation, fatty acid acylation and disulphide bond formation) similar to mammalian cells (Murhammer *et al*., 2016) Furthermore, the system is amenable for the expression of multi-protein complexes, which is important for VLP production. Another advantage of insect cells is that they can be cultivated easily without serum, which lowers costs and raises biosafety and the scale up is simple (Altmann *et al.,* 1999). The most widely used insect cell lines for academic research and commercial applications (Granados *et al.,* 2008) are *Sf*9 and *Sf21* cells, derived from the fall army worm *Spodoptera frugiperda* (Vaughn *et al.,* 1977) and BTI-TN5B1-4 “High Five” cells that have been isolated from the American cabbage looper *Trichoplusia ni* (Durocher *et al.,* 2009). *Sf*9 cells are robust, can be grown in suspension and grow fast so that high cell densities can be reached. Furthermore, they can be used with serum free media and for scale up. (Granados *et al.,* 2008) On the other side, large scale insect cell cultures are more sensitive to shear stress and have higher oxygen consumption as mammalian cells (Altmann *et al.,* 1999).

TABLE 1-1: COMPARISON OF DIFFERENT EXPRESSION SYSTEMS

	E.coli	**Yeast**	**Insect cells**	**Mammalian cells**
Cell growth	rapid	rapid	slow	slow
Cultivation expense	low	low	medium	high
Scale up	easy	easy	easy	difficult
Protein folding	bad	bad	good	good
Posttranslational modifications relative to human	none	bad	good	very good
Application	prokaryotic proteins, simple eukaryotic proteins	intracellular/secreted proteins, disulfide-bonded proteins, glycosylated proteins	membrane proteins, large-size proteins, viral-vaccines, signaling proteins	complex eukaryotic proteins, proteins with accurate posttranslational modifications

The *Autographa californica* multiple nuclear polyhedrosis virus *(Ac*MNPV), is the most frequently used baculovirus vector for recombinant protein production in these cells (Davies *et al.,* 1994; Luckov *et al*., 1995). It can be used for vaccine production (Cox et al., 2012) but also is being utilized as gene therapy vector (Airenne *et al*., 2013; Hu *et al*., 2011; Rivera-Gonzales *et al.,* 2011; Rychlowska *et al.,* 2011). In order to achieve high protein expression foreign proteins are expressed with the viral polyhedrin promoter. (Altmann *et al.,* 1999) To make purification easier purification tags such as polyhistidine can be added. (Janknecht *et al.,* 1991; Schmidt *et al.,*1998; Zhu *et al.,* 1996)

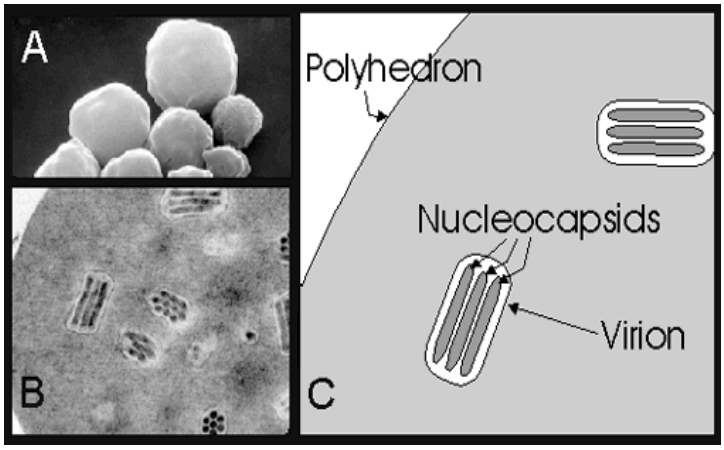


FIGURE 1-1: A - POLYHEDRA (BACULOVIRUS PARTICLES); B - CROSS-SECTION OF A POLYHEDRON; C - SCHEMA OF POLYHEDRON CROSS-SECTION. ELECRON MICROGRAPHS (TAKEN FROM [1])

As baculovirus infection is a lytic infection and the polyhedrin promoter drives recombinant protein synthesis shortly before cell lysis, this may lead to improper processing of plasma membrane and secretory proteins because of lack of compounds of the posttranslational pathway (Altmann *et al.*, 1999). The use of baculovirus promoters active earlier in the infection cycle can improve protein processing but may decrease protein expression levels (McCarroll *et al.,* 1997). As a result of continuous development and improvement of the baculovirus expression vector system (BEVS) the platform is nowadays well established and there are several commercial kits available (Baculogold (BD Biosciences), Bac-to-Bac (Invitrogen), MultiBac and SweetBac (Geneva Botech)).

1.2. BACULOVIRUSES

Autographa californica multiple polyhedroviruses are the most frequently used baculoviruses in Biotechnology and are part of the *Baculoviridiae* family (Krammer *et al*. 2011). They are enveloped, double-stranded DNA viruses containing a circular genome of 88 to 200 kbp encoding for 90 to 180 genes. (Burgess 1977 summers and Anderson 1972)

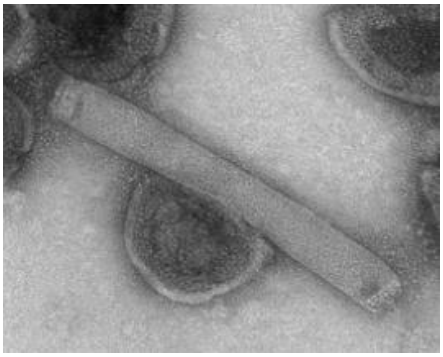


FIGURE 1-2: BACULOVIRUS TEM IMAGE (BUDDED VIRUS)

The viral genome is packed into rod-shaped nucleocapsids of a size of 200-400 nm in length and 30-70 nm in diameter (Jehle et al., 2006) and forms together with the Vp39, the major capsid protein, a spiral structure.

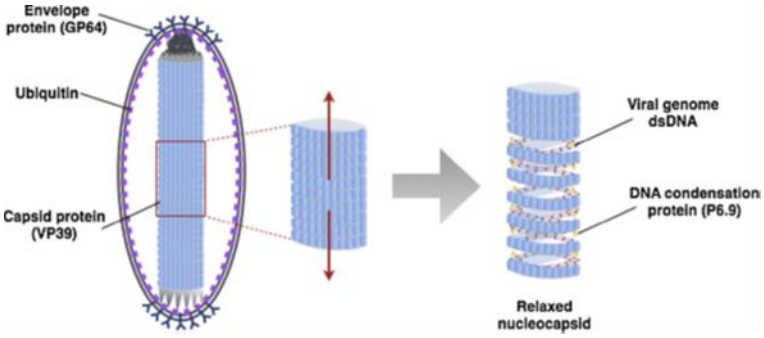


FIGURE 1-3: SCHEMATIC REPRESENTATION OF THE BACULOVIRUS BUDDED VIRUS (BV) REPRESENTED BY ACMNPV. FIGURE TAKEN FROM WANG *ET AL*., 2013

Baculoviridae can be separated in *four genera including Alpha-baculovirus, Betabaculovirus, Gammabaculovirus* and *Deltabaculovirus* based on genome phylogeny (Herniou at al., 2012). *Alphabaculoviruses* and *Betabaculoviruses* comprise lepidopteran (moths and butterflies) *nucleopolyhedroviruses* (NPVs) and *granuloviruses* (GVs), whereas*Gammabaculoviruses* and *Deltabaculoviruses* contain diptera-specific (mosquitoes and flies) and hymenoptera-specific (wasps and bees) NPVs (Yin *et al*., 2013). The Alphabaculoviruses can be further divided into two subgroups based on the type of envelope fusion glycoprotein (gp64 or F protein) present on the budded virion envelope (Jehle *et al*., 2006; Zanotto *et al.,* 1993).

1.3. BACULOVIRUS LIFE CYCLE

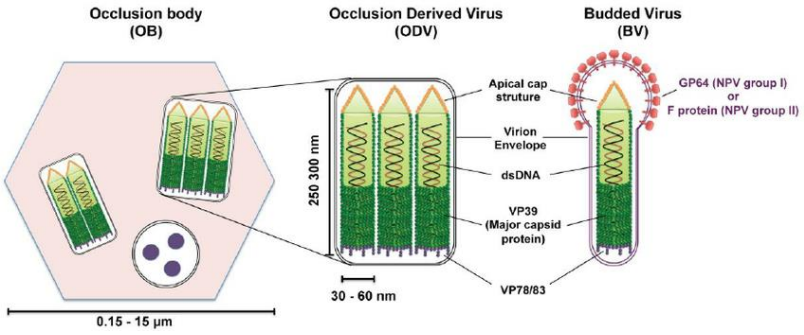


FIGURE 1-4: SCHEMA OF THE TWO DIFFERENT TYPES OF INFECTIOUS PARTICLES. TAKEN FROM AU *ET AL*., 2013

Throughout the baculovirus life cycle two different kind of virions are formed – budded virions (BV) and occlusion derived virions (ODVs). The virions are similar in their nucleocapsid structure and carry the same genetic material but differ in the composition of their envelopes and their function as well as the time they are produced within the baculovirus life cycle. The occlusion-derived virus is occluded in a crystalline protein matrix (Jehle *et al.*, 2006) of polyhedrin, produced in the very late phase of baculovirus replication and is responsible for establishing primary infection of the host. The budded virus consists of a plasma membrane-derived envelope which is generated by budding of nucleocapsids through the surface of infected cells.

The viral envelope fusion protein GP64 is incorporated into the envelope. GP64 is important for virus attachment, membrane fusion and virion budding and therefore essential for viral propagation (Lung *et al.,* 2002). The budded virus is produced during the initial phase of replication and initiates secondary/systemic infection of the host. (Rohrmann *et al*., 2013; Herniou *et al*., 2012)

1.4. BACULOVIRUS INFECTION CYCLE

Baculoviral occlusion bodies are taken up by insects during feeding. After transport to the midgut the alkaline pH causes the occlusion bodies to dissolve, nucleocapsids are released and the infection cycle is started. Budded viruses are produced in the nucleus by the assembly of nucleocapsids that afterwards bud from the surface of infected cells while receiving a plasma membrane-derived envelope. They are able to infect neighbouring cells and initiate an infection cascade. (Okano *et al*., 2006)

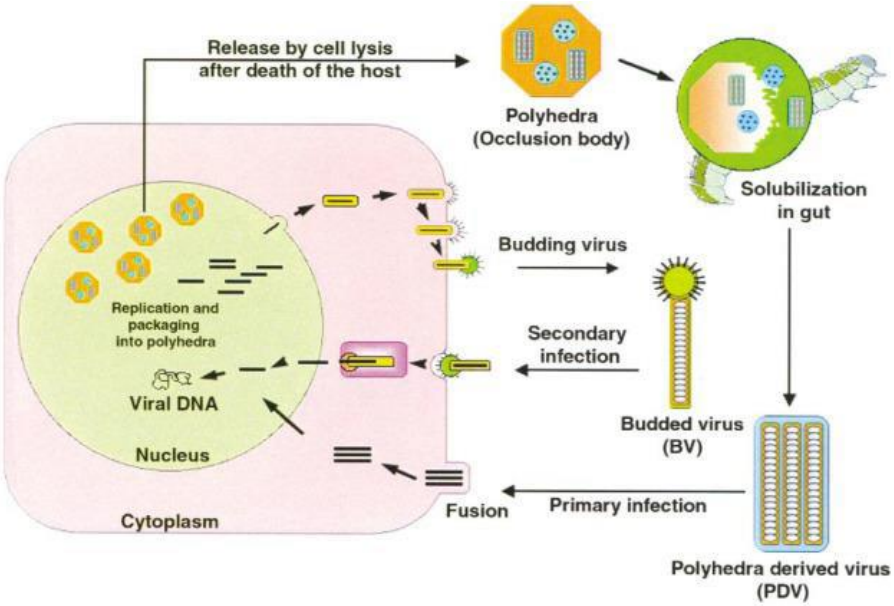


FIGURE 1-5: BACULOVIRUS LIFE CYCLE. OCCLUSION BODIES ARE SOLUBILIZED IN THE INSECT GUT AND RELEASED. OCCLUSION DERIVED VIRUSES ARE NOW ABLE TO INFECT INSECT CELLS. THE VIRAL DNA IS REPLICATED AND PACKAGED IN THE NUCLEUS USING THE HOST CELL MACHINERY. ONE PART LEAVES THE CELL BY BUDDING THROUGH THE CELL MEMBRANE (BUDDED VIRUS), THE OTHER PART (OCCLUSION BODIES) IS RELEASED DURING CELL LYSIS. FIGURE TAKEN FROM GHOSH *ET AL*., 2002.

The BV enters insect cells through endocytosis. After viral entry the endosome is acidified and the viral envelope fuses with the host endosomal membrane. This results in the release of the nucleocapsids that are transported to the nucleus. The viral DNA is released and replication of the viral genome and transcription of viral early genes using the host cell machinery starts.

Progeny viruses are assembled leave the nucleus and bud through the cell membrane receiving an envelope. New BVs are generated that can infect neighboring cells. (Au *et al.,* 2013) Occlusion derived viruses remain in the nucleus and are released during lysis of the host cell.

1.5. MAKING RECOMBINANT BVS BY THE MULTIBAC SYSTEM

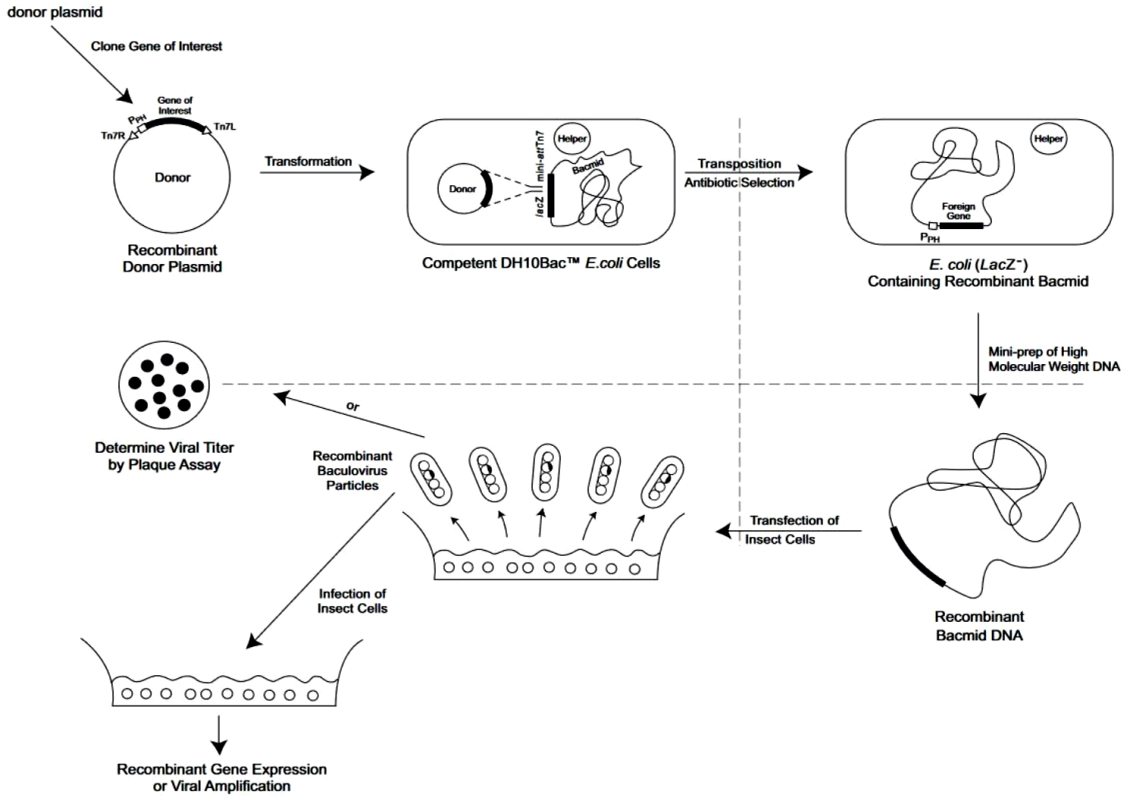


FIGURE 1-6: THE MULTIBAC SYSTEM. THE DONOR PLASMID CARRYING THE GENE OF INTEREST (GOI) IS INSERTED INTO THE TN7 ATTACHMENT SITE OF THE BACMID. POSITIVE CLONES ARE SELECTED THROUGH BLUE/WHITE SCREENING AND BACMIDS ARE ISOLATED AND TRANSFECTED INTO INSECT CELLS. THE VIRUS IS HARVESTED FROM THE SUPERNATANT AND AMPLIFIED FOR INFECTING INSECT CELLS FOR RECOMBINANT PROTEIN PRODUCTION. FIGURE TAKEN FROM THE MULTIBAC MANUAL VERSION 3.0, 2011.

The most widely used BEVS platforms include the Baculogold (BD Biosciences), Bac-to-Bac (Invitrogen) and MultiBac (Geneva Biotech) systems. In the presentwork the MultiBac system was utilized for recombinant baculovirus generation. There deletion of the viral genes for the protease v-cath and the chitinase chiA from the bacmid and maintenance of cellular compartments during the course of infection the baculovirus protein expression could be improved (Berger and Craig, 2011).

The system offers several transfer vectors (acceptor and donor vectors) with the viral promoters polh and p10 and different selection markers for molecular cloning of the gene of interest.

In order to generate multigene complexes acceptor and donor vectors are fused by Cre-loxP site-specific recombination. Insertion of these vectors into the MultiBac genome occurs in bacterial strains (DH10^{MultiBac}) that apart from the bacterial genome containing a bacterial artificial chromosome harboring the baculovirus genome and a helper plasmid encoding the enzyme Tn7 transposase. Integration of the resulting transfer vector into the baculoviral genome occurs through Tn7 transposition, whereby the integration of the foreign genes leads to the disruption of the LacZ encoding gene located at the bacmid. Afterwards cells carrying the genes of interest are selected by blue/white screening using selective agar plates containing X-Gal (Berger *et al*., 2013). Insect cells are transfected with the purified recombinant baculoviral DNA isolated from white colonies and virus stocks are made by sequential amplification of the virus seed stock harvested after transfection.

1.6. INFLUENZA VIRUS

Influenza viruses are single-stranded negative-sense RNA viruses (Lamb *et al*., 2001) and belong to the *Orthomyxoviridae* family (Harris *et al.,* 2006). They are divided into three classes A, B and C, where influenza A and B viruses are only infecting humans while influenza viruses C have a more diverse host range (Sultan *et al*., 2010). They contain a segmented genome which means that multiple ribonucleoprotein (RNP) complexes (Inglis *et al*., 1976) are enclosed by a continuous envelope of matrix protein. Moreover, trimeric hemagglutinin (HA) and tetrameric neuraminidase (NA) (Laver *et al*., 2002), the major glycoproteins and influenza antigens, are integrated in the envelope. Influenza A viruses can be classified into 16 different hemagglutinin subtypes (H1-H16) and 9 different neuraminidase subtypes (N1-N9) (Chen *et al.,* 2009). Due to genetic shifts and drifts that lead to minor or major antigentic changes of the hemagglutinin or neuraminidase proteins, influenza viruses are able to evade the host immune system (Mandell *et al*., 2005). Genetic drifts are little changes (point mutations) of the HA and/or the NA proteins that yield proteins with new antigenic properties, that are not well recognized by antibodies induced by previous seasonal vaccinations. In contrast, genetic shifts, promoted through the segmented genome, lead to the generation of a novel influenza virus subtype (re-assortment) against which no preexisting immunity may exist. Former pandemics occurred because of antigenic shifts, with the “Spanish flu” 1918 being the most devastating in documented history (Mayo *et al*., 2010).

1.7. TRADITIONAL INFLUENZA VACCINES

TABLE 1-2: COMPARISON FO DIFFERENT VACCINE TYPES (TAKEN FROM [2])

Vaccine Type	Description	Challenges	Examples
Live Attenuated	Weakened version of living microbe that can't cause disease	Mutation; storage	Measles, mumps, rubella, yellow fever
Inactivated or "killed"	Microbes killed with chemicals, heat or radiation	Weaker immune response; need boosters	Cholera, flu, hepatitis A, rabies
Subunit	Include antigens (or epitopes) that best stimulate immune system	Identifying specific antigen takes time	Hepatitis B, pertussis
Toxoid	Formalin inactivated toxins used as vaccine	Used when main cause of illness is a bacterial toxin	Diphtheria, Tetanus
Conjugate	Specialized subunit vaccine where antigens are linked to polysaccharides	Most effective for immature immune system of infants	H. Influenza subtype b

Every year influenza viruses A and B cause severe illnesses and mortality worldwide. Especially infants, elderly and immunocompromised people are affected. The most effective way to protect individuals from influenza is vaccination. The major influenza vaccine antigens are the viral hemagglutinin (HA) and neuraminidase (NA). The hemagglutinin is an essential protein in the initial phase of infection because it is responsible for attachment and intrusion of viral particles into the cell. Neuraminidase mediates the release of virions through the cell membrane. Due to antigenic drifts in the HA and NA proteins available vaccines are not always effective. Every year new vaccines against the most prominent strain of each circulating influenza virus subtype have to be produced, which takes about 6 to 9 months. The World Health Organization (WHO) meets with academies, regulatory and national laboratories twice a year (Northern and Southern Hemisphere) to make recommendations on the composition of the new influenza vaccine. In the end each country decides on their own which influenza strain will be included in their influenza vaccine. Since the 1940s influenza vaccines have been produced in embryonated hen's eggs. As the composition of the next influenza vaccine is determined, the virus is propagated in chicken eggs for some days. The produced life virus is then isolated, purified and has to be inactivated. However, in the case of pandemic, where large amounts of a vaccine are needed within short time, there is the risk of lack in available eggs and lack in time and infrastructure (biosafety levels 2 and 3 facilities) for the production of such vaccines. Moreover, egg-derived proteins can cause allergic reactions in humans.

The most frequently used vaccines for viral diseases are attenuated or inactivated live viruses although there are high risks associated with them. Through incomplete inactivation or attenuation individuals may develop diseases. Furthermore, epitopes can be modified by the inactivation/attenuation treatment, leading to diminished vaccine efficacy (Okano *et al*., 2006). Recombinant subunit vaccines can be a good alternative to live virus vaccines. However, several immunizations with high doses of the antigen and the addition of adjuvant may be necessary to achieve the same immunity as with live vaccines (Okano *et al*., 2006). A special type of subunit-vaccines are virus-like particle-based vaccines, which are explained under 1.8. The advantage is that large amounts of an effective vaccine can be produced in a short time. However, these vaccines also have disadvantages because they contain live baculoviruses that can cause excessive immunological damage by integrating the bacuoviral genome (Hu *et al*., 2008). (Klausberger *et al*., 2014; Krammer *et al*., 2010; Roldão *et al*., 2010) Influenza virus-like particles are engineered as a non-egg and non-animal cell culture-based vaccine to protect from infection. They consist of a structure matrix protein which self-assembles to form the VLP and hemagglutinin.

1.8. VIRUS-LIKE PARTICLES

VLPs are a special type of subunit vaccine but are generally more immunogenic than other subunit vaccines because of their particulate structure and the display of viral antigens in their native conformation (Noad *et al.*, 2003). VLPs can be produced with different expression systems including mammalian cell lines with viral expression vectors, insect cell lines with baculovirus expression system, yeast, *Escherichia coli* and other bacteria. Virus-like particles consist of one or more viral proteins that self-assemble to form structures that resemble natural virions (Liu *et al.,* 2013). As they do not contain genetic material, VLPs cannot propagate and infect other cells (Buonaguro *et al.,* 2011).

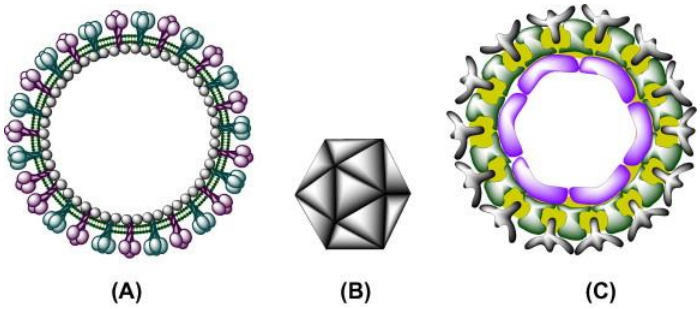


FIGURE 1-7:SCHEMATIC REPRESENTATION OF A HIV-1 GAG VLP DISPLAYING HEMAGGLUTININ TRIMERS: THE GAG PROTEIN BUILDS THE INNER STRUCTUR OF THE VLP, COVERD BY A CELL MEMBRANE DERIVED FROM THE BUDDING PROCESS. OUTSIDE ARE THE HEMAGGLUTININ TRIMERS THAT ARE ANCHOORED THROUGH THE TRANSMEMBRANE DOMAIN. TAKEN FROM LIU ET

VLPs may range in size between 22 and 200 nm depending on the incorporated viral proteins (Liu *et al*., 2013). They offer a new possibility of developing vaccines for infectious diseases, cancer and are a promising alternative where soluble protein vaccines were non-successful (Buonaguro *et al.*, 2011; Grgacic *et al.*, 2006). VLPs were shown to stimulate both the humoral and cellular arm of the immune system. By activating B-cells through antigen presentation to antigen-presenting cells, strong immune responses may be reached. Reasons for that are the high density display of epitopes and cross-presentation with cytotoxic T cells and T-helper cells. Membrane-bound proteins can be displayed in their natural conformation, resembling the native virus structure. VLP based vaccines commercially available are the two Human Papilloma vaccines Cervarix ® (GlaxoSmithKline) and Gardasil ® (Merck), and Engerix ® (GlaxoSmithKline), a vaccine against Hepatitis B. The first Malaria vaccine Mosquirix™RTS, S (GalaxoSmithKline) has been recently approved by the European regulators.

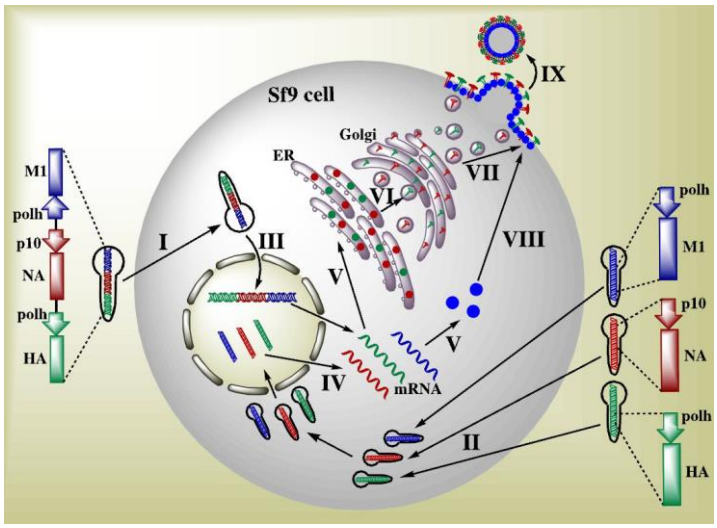


FIGURE 1-8: IMAGE OF CO-EXPRESSION (I) AND CO-INFECTION (II) IN/OF INSECT CELLS GENERATING INFLUENZA VIRUS-LIKE PARTICLES. THE BACULOVIRAL GENOME ENTERS THE NUCLEUS (III), WHERE IT IS TRANSCRIBED TO MRNA. AFTERWARDS THE MRNA IS TRANSPORTED OUTSIDE THE NUCLEUS (IV) WHERE TRANSLATION TAKES PLACE AND STRUCTURAL INFLUENZA PROTEINS ARE MADE. HEMAGGLUTININ AND NEURAMINIDASE GO THROUGH THE GOLGI APPARATUS TO THE PLASMA MEMBRANE (VII). THE MATRIX PROTEIN IS ALSO TRANSPORTED TO THE PLASMA MEMBRANE (VIII) WHERE THE BUDDING PROCESS (IX) TAKES PLACE. FIGURE TAKEN FROM LUI *ET AL*., 2013

There are two different strategies to produce VLPs displaying multiple proteins. One way is to develop multiple monocistronic baculoviruses and to co-infect insect cells with these viruses. The other possibility is to insert genes for multiple proteins into a single polycistronic baculovirus infecting insect cells subsequently.

1.8.1. HIV 1 GAG

Human immunodeficiency virus (HIV) is an enveloped single-stranded RNA virus that encodes the Gag polyprotein. The Pr55Gag has a molecular weight of 55 kDa and can be used for the generation of virus-like particles because of its ability to self-assemble. HIV-1 Gag VLPs bud through the cellular membrane of infected cells using the same mechanism as the native HIV virus. In the same course the produced VLPs are receiving an envelope that consists of the host cell membrane.

Moreover, additional cellular vesicles like microvesicles and exosomes are secreted (Akers *et al.*, 2013). (González-Domíngoez *et al*., 2016) The only difficulty is the separation of the baculovirus from the produced VLPs because they are similar in size. This protein was used in this work for producing VLPs and displaying different hemagglutinins on their surface. It was the structure protein in order to form VLPs.

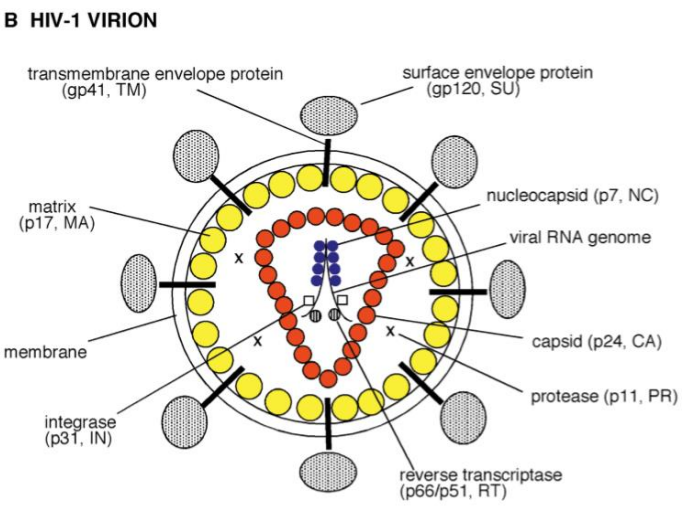


FIGURE 1-9: SCHEMATIC REPRESENTATION OF THE HIV-1 GENOME AND VIRION ORGANIZATION

1.8.2. HEMAGGLUTININ H1 OF INFLUENZA VIRUS, A/CALIFORNIA/4/2009 (H1N1)

In 2009 the World Health Organization (WHO) classified a new strain of influenza A virus as a Phase 6 pandemic virus (maximum threat) (WHO. Influenza A (H1N1): pandemic alert phase 6 declared, of moderate severity. [3]) It was the first pandemic virus since 40 years and spread all over the world in few weeks. Influenza A H1N1 2009 is a genetic combination of segments of previous avian, human and swine influenza viruses and is capable of human-to-human transmission. (Qaboos 2010; Sullivan *et al*., 2010)

1.8.3. HEMAGGLUTININ H3 OF INFLUENZA VIRUS, A/HIROSHIMA/52/2005 (H3N2)

In 1968 the influenza A H3N2 virus emerged from H2N2 through an antigenic shift. It led to the "Hong Kong flu" pandemic and killed around 700000 humans. (Rajagopal and Treanor 2007). Afterwards H3N2 has been used as vaccination for seasonal influenza [4].

2. OBJECTIVES

The aim of this study was to display different hemagglutinins on the surface of virus like particles by multi-protein expression in insect cells using the baculovirus expression vector system. Furthermore, the comparison of the expression in the cell lines *Sf*9 and *Tnms*42 was part of this work. Hemagglutinin H1 of influenza virus, A/California/4/2009 (H1N1) and hemagglutinin H3 of influenza virus, A/Hiroshima/52/2005 (H3N2) were displayed on HIV1-Gag VLPs.

1. Creating recombinant baculovirus working stocks for host cell infection in *Sf*9cells.
2. Production of different hemagglutinin VLPs in the insect cell lines *Sf*9 and *Tnms*42.
3. Determination of the amount of produced VLPs by Gag-ELISA.
4. Development of a H1-ELISA and subsequent analysis of the H1-VLPs.
5. Purification of VLPs through sucrose gradient centrifugation.
6. Characterization of VLPs by Western Blotting, NanoSight, Zetasizer and transmission electron microscopy.

3. MATERIAL AND METHODS

3.1. MATERIALS

3.1.1. CELLS

E.COLI

- JM109, New England BioLabs, USA
- DH10MultiBacY cells (EMBL-Grenoble, France)
- pirHC/LC

INSECT CELL LINES

- *Spodoptera frugiperda Sf*9 cells (ATCC CRL-1711)
- *Tnms*42 subcloned from BTI-TN5B1-4 cells (Chen *et al*. 2013), abbr. *Tnms*42

3.1.2. PLASMIDS

- pACEBac1 (EMBL-Grenoble, France)

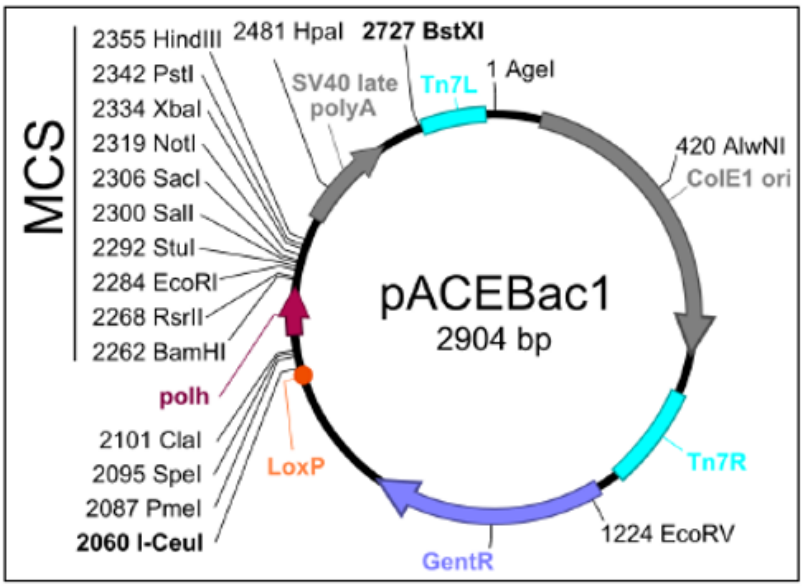


FIGURE 3-1: MAP OF PACEBAC1 VECTOR (EMBL-GRENOBLE)

The acceptor vector pACEBac1 (EMBL-Grenoble) was used for molecular cloning the influenza hemagglutinin. 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) and a gentamicin resistance marker (GentR) for selecting positive transformants in *E.coli*. The Tn7R and Tn7L sites enable the integration of the expression cassette into the baculovirus genome.

- pIDC (EMBL-Grenoble, France)

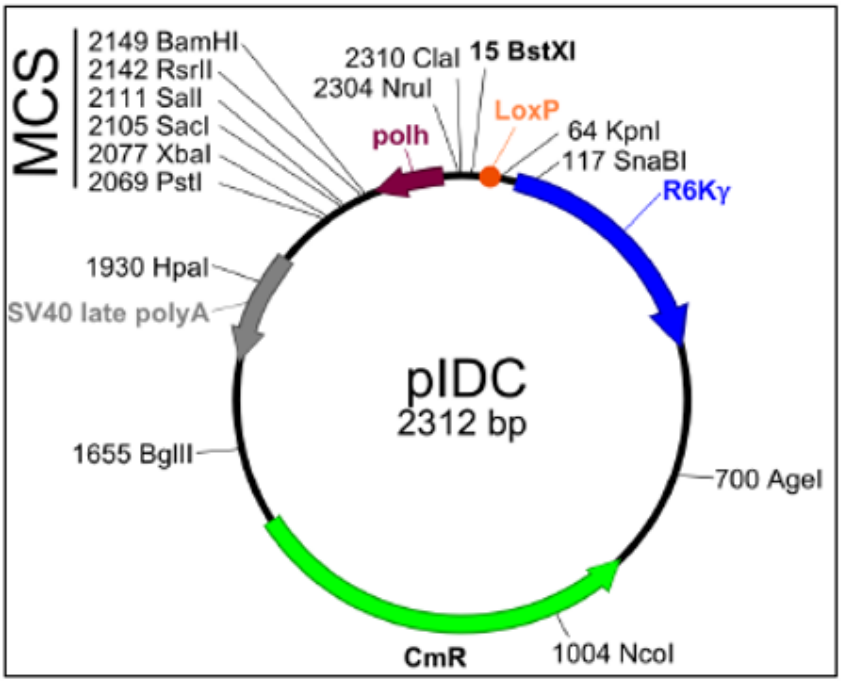


FIGURE 3-2: MAP OF PIDC VECTOR (EMBL GRENOBLE)

As a donor vector pIDC (EMBL-Grenoble) was used for cloning of the Gag gene. This vector carries the same promoter and terminator signals as the pBAC1 vector but has a different resistance gene (chloramphenicol) and a conditional R6Kγ origin of replication which makes its propagation dependent on the expression of the pir gene in the E. coli PIRHC strain. Both vectors carry a P1 (LoxP) site that allows CreLox recombination and thus combining acceptor and donor vectors to form a multiexpression vector.

Proteins

- H1 HA: Hemagglutinin H1 of influenza virus, A/California/4/2009 (H1N1)
- H3 HA: hemagglutinin H3 of influenza virus, A/Hiroshima/52/2005 (H3N2)
- HIV-1 Gag (HXB2) (GenBank accession no. K03455.1)

3.1.3. MEDIA

LYSOGENY BROTH –MEDIA (LB) PH 7.5 ADJUSTED WITH NAOH

The components listed in Table 3-1 were dissolved in ddH2O and autoclaved at 120°C for 20 minutes. After autoclaving the medium was stored at 4° C. If required, antibiotics and additives were added just before usage.

TABLE 3-1: COMPOSITION OF LB MEDIUM

Component	Concentration
Peptone casein	10 g/L
Yeast extract	5 g/L
NaCl	10 g/L

LB AGAR

The components for LB agar listed in Table 3-8 were dissolved in dH2O, 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 cooling the medium to 50°C. The plates were poured and stored at 4°C.

TABLE 3-2: 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

SUPER OPTIMAL BROTH WITH (SOC)

SOC medium was prepared by dissolving all components, listed in Table 3-9, except for the glucose to prevent Maillard reaction, and subsequent autoclaving. The glucose was added after autoclaving and the sterile SOC medium was stored at 4° C.

TABLE 3-3: COMPOSITION OF SOC MEDIUM

Component	Concentration
Peptone casein	20 gL
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

CELL CULTURE MEDIA

Insect cells were cultivated in HyClone™ SFM4 Insect media with glutamine (GE Healthcare, GB). The medium was supplemented with 0.1% (v/v) Pluronic-68, a non-ionic detergent that reduces hydrodynamic damage to the cells.

ANTIBIOTICS AND MEDIA ADDITIVES FOR SELECTIVE GROWTH MEDIA OR PLATES

Antibiotics and media additives were added to liquid and solid media to ensure selective *E.coli* growth and were added at the working concentration given in Table 3-4.

TABLE 3-4: CONCENTRATIONS OF ANTIBIOTICS

Additive	Working concentration
Anti-Clumping Agent	0.1% (v/v)
Ampicillin	10 µg/mL
Chloramphenicol	25 µg/mL
Gentamycin	15 µg/mL
IPTG	40 µg/mL
Kanamycin	50 µg/mL
Tetracyclin	10 µg/mL
X-Gal	100 µg/mL

3.2. METHODS

3.2.1. MOLECULAR CLONING EMPLOYED IN THIS WORK

This is a short description of the steps done to receive the desired virus constructs. All protein sequences were cloned into the multiple cloning site of the plasmids. Sequences of inserts and primers can be found in the appendix. The description for each method can be found in chapter.

3.2.2. 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).From a LB-agar plate a single colony was picked and cultivated overnight in 5 mL LB-medium including antibiotics. The cells were pelleted for 5 minutes at 3000 x g and plasmid isolation carried out according to the manufacturer's protocol.

3.2.3. DNA QUANTIFICATION

The DNA concentration was determined by measuring the absorbance at 260 nm using a spectrometer (Nanodrop 1000 Thermo Scientific, Wilmington, DE, USA).

3.2.4. PREPARATION OF AGAROSE GELS

For agarose gel electrophoresis 1.5% (x/v) agarose gels were prepared. The composition per liter can be seen in Table 3-5. The agarose in 1x TAE buffer was completely melted in the microwave. Afterwards the solution was cooled, and ethidium bromide added.

TABLE 3-5: COMPOSITION OF 1.5% AGAROSE GEL

Component	Amount
Agarose	15 g/L
50x TAE buffer	20 mL/L
Ethidium bromid	30 µL/L
dH_2O	

3.2.5. AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a method for separating DNA fragments based on their size. Smaller fragments travel through the sieve-like gel at higher speed than bigger ones.

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

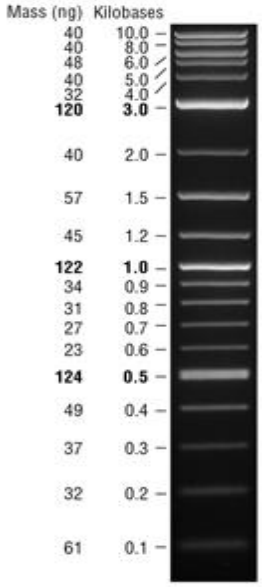
TABLE 3-6: COMPOSITION OF 50X TAE BUFFER

Component	Amount
Tris(hydroxmethyl)aminomethane	242 g/L
Glacial acetic acid	57.1 mL/L
EDTA	18.6 g/L
dH_2O	

TABLE 3-7: COMPOSITION OF 1X TAE RUNNING BUFFER (1 L)

Component	Amount
50x TAE buffer	20 mL/L
Ethidium bromid	30 µL/L
dH_2O	

3.2.6. DNA SIZE MARKERS



The 2-log DNA ladder (New England Biolabs (Ipswich, MA) shown in Figure 3-3 was used to compare the size of PCR products, plasmids and restriction digests with a standard. 6 µL of 2-log ladder were loaded onto analytical gels, whereas 15 µL were used for preparative gel electrophoresis.

FIGURE 3-3: 2-LOG DNA LADDER. FIGURE TAKEN FROM [5].

3.2.7. EXTRACTION OF DNA FROM AGAROSE GELS

For preparative gel electrophoresis the DNA bands at the desired size were cut out with a razorblade using an UV-transilluminator and purified by using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany).

3.2.8. AMPLIFICATION OF DNA INSERTS

For the amplification of DNA inserts the Q5 High-Fidelity DNA polymerase (New England Biolabs, Ipswich, MA) was used. A master mix according to Table 3-8 was prepared and transferred into PCR reaction tubes.

TABLE 3-8: 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	1 ng – 1 µg

The primers used for amplification are listed in Table 3-9.

TABLE 3-9: PRIMERS USED FOR INSERT AMPLIFICATION

Primer	Sequence 5`-> 3`
A/H1 Cal09 NotI-for	5`-GAT GAT GCG GCC GCT CA TTAA ATA CAT ATT CTA CAC TGT AGA-3`
A/H1 Cal09 EcoRI-back	5`-GAT GAT GAA TTC ATG AAG GCA ATA CTA GTA GTT CT-3’
A/H3 Hiroshima NotI-for	5’ GAT GAT GCG GCC GCT CAT TCA AAT GCA AAT GTT GCA 3’
A/H3 Hiroshima SalI-back	5’ GAT GAT GTC GAC ATG AAG ACT ATC ATT GCT 3’

After aliquoting the master mix, the template DNA was added, and the PCR reaction tubes were placed into the thermocycler. The standard cycling conditions are listed in Table 3-10. 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. They were calculated with the Tm-Calculator by New England Biolabs (UK). The annealing temperature for H1 is 56°C, for H3 it is 55°C and the elongation time is 50 s for both sequences.

TABLE 3-10: THERMOCYCLER PROGRAM

Step	Temperature	Time	Cycles
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.

3.2.9. COLONY PCR

For screening *E. coli* transformants, for the take-up of the desired plasmids, PCR screening, using OneTaq DNA polymerase (New England Biolabs), was performed. Single colonies from the LB-agar plates with selective antibiotics 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 stroken 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 colonies were picked.

TABLE 3-11: PCR MASTER MIX FOR COLONY PCR

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 3-13). The appropriate cycling conditions are shown in Table 3-12. The elongation time was adapted to the length of the desired fragment, resulting in 102 s for both the H1 and the H3 sequence. The annealing temperature was selected according to the specific melting temperature of the used primers recommended by New England Biolabs (UK). 47°C were used as annealing temperature for the H1 sequence and 52°C for the H3 sequence.

TABLE 3-12: THERMOCYCLER PROGRM FOR COLONY SCREENING

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

3.2.10. CONTROL DIGEST

In order to check if the obtained plasmid contained the correct insert it was cut with different restriction enzymes and analyzed by agarose gel electrophoresis. For one digest (20 µL total volume) 1 µg plasmid DNA, 1 µLof each restriction enzyme, 2 µL10x buffer and dH2O were mixed. The restriction digest was incubated as per the manufacturers recommendations and digested, undigested samples were analyzed by gel electrophoresis. The migration pattern of the digested plasmid was determined with the Software CLC main workbench 6 (CLC bio, Aarhus, Denmark).

3.2.11. RESTRICTION DIGEST AND DEPHOSPHORYLATION (VECTOR PREPARATION)

The pACEBac1 vector was linearized with two restriction enzymes (EcoRI and NotI for H1 and SalI and NotI for H3). Therefore, a double digest was carried out. 1 µg plasmid vector was mixed with 1 µL of each enzyme, 5 µl of the appropriate buffer and filled up to 50 µL with dH2O. The mixture was incubated for 1.5 hours at 37° C.

For dephosphorylation 1 µL CIP (calf intestinal alkaline phosphatase) was added in order to prevent religation of incompletely digested vector and incubated for 20 minutes at 37° C. Afterwards the DNA was purified by preparative gel electrophoresis and gel excision.

3.2.12. Restriction digest of DNA inserts

The DNA insert was cut with the same enzymes as the vector resulting in overhangs that can be readily ligated. For the digest a total volume of 50 µL was prepared consisting of 1 µg amplified PCR product, 5 µL apropriate buffer, 1 µL of each restriction enzyme (EcoRI and NotI for H1 and SalI and NotI for H3) and filled up with dH2O. The mixture was incubated for 1.5 hours at 37° C and purified by preparative gel electrophoresis and gel excision.

3.2.13. DNA Ligation

DNA ligation was carried out with the T4 DNA ligase (New England). For the ligation reaction 100 ng linearized vector (pACEBAc1) and a 5-fold molar surplus of insert DNA added as calculated according to the equation below. Additionally, 2 µl T4 ligase buffer, 1 µl T4 DNA ligase and dH2O were added. The mixture was incubated at 16°C overnight.

EQUATION 3-1: CALCUALTION OF AMOUNT INSERT NEEDED FOR LIGATION

$$\frac{100\ ng\ linearized\ vector}{1200\ bp\ linearized\ pACEBac1} * 5 = \frac{x\ ng\ insert\ DNA}{y\ bp\ insert\ DNA}$$

3.2.14. DNA precipitation

For 20 µL of plasmid sample 50 µL of Isopropanol and 2µL of NaAc (3M) were added. Afterwards the samples were centrifuged at 4° C at maximum speed (16.000 rpm) for 15 minutes. The supernatant was discarded and 50 µL of 70% Ethanol were added. After centrifugation at room temperature at maximum speed the supernatant was removed, and the pellet dried for 5 minutes at room temperature. The pellet was resuspended in 8 µL of HQ-H_2O, vortexed and spinned down.

3.2.15. Transformation into electrocompetent *E.Coli* cells

50 µL of thawn electrocompetent *E.Coli* (JM109) were added to 8 µL precipitated plasmid. This mixture of plasmid DNA and cells was transferred to a prechilled electroporation cuvette (0.2 cm gap) and a pulse was applied (2500 V, 200 Ohm, 25 µF). After pulsation, 100 µL of prewarmed (37° C) SOC media was quickly pipetted into the cuvette. This mixture was then transferred to the remaining 850 µL of prewarmed SOC media.

For cell recovery the suspension was incubated at 37°C and 550 rpm for 45 minutes. After recovery, 100 µL, 300 µL and 600 µL were plated on selective LB-agar plates. The plates were then incubated at 37° C overnight or at room temperature over the weekend.

3.2.16. SEQUENCING

Sequencing was conducted at Microsynth AG (Balgach, Switzerland). Plasmids were diluted with dH_2O to a concentration of 60-120 ng/µL in 12 µL total volume as recommended by the company. 3 µL of sequencing primer were added and the whole mixture was sent for sequencing.

Primers used for sequencing are shown in Table 3-13.

TABLE 3-13: SEQUENCING PRIMERS

Primer	Sequence
A/H1 Cal09 EcoRI-back:	5'-GAT GAT GAA TTC ATG AAG GCA ATA CTA GTA GTT CT-3'
A/H1 Cal09 NotI-for	5'-GAT GAT GCG GCC GCT CA TTAA ATA CAT ATT CTA CAC TGT AGA-3'
A/H3 Hiroshima SalI-back	5' GAT GAT GTC GAC ATG AAG ACT ATC ATT GCT-3'
A/H3 Hiroshima NotI-for	5' GAT GAT GCG GCC GCT CAT TCA AAT GCA AAT GTT GCA-3'
H1/H3_Screening Primer_1for	5'-GAT GAT ATT TTA CTG TTT TCG TAA CA-3'
H1/H3_Screening Primer_2rev	5'-GAT GAT GGG GAG GTG TGG GAG GTT TT-3'
H1_Insert Screening Primer_3for:	5'-GAT GAT TCT GGT ATT ATC ATT TCA GA-3'
H3_Insert Screening Primer_4for	5'-GAT GAT TCA ATA ATG AGA TCA GAT GC-3'
pAB1 SV40 back	5'-CCT CTA GTA CTT CTC GAC AAG-3'
pAB1 -44 for	5'-TTT ACT GTT TTC GTA ACA GTT TTG-3'

For sequence verification and alignments, the CLC main workbench (CLC bio, Aarhus, Denmark) Software was used.

3.2.17. CRE-LOXP FUSION REACTION

Cre-LoxP fusion of acceptor and donor vectors for multigene expression in insect cells was carried out according to "ACEMBL Expression System Series MultiBacTurbo, Multi-Protein Expression in Insect Cells, User Manuel, Version 3.0". Therefore, an acceptor vector carrying the hemagglutinin sequence and a donor vector with the sequence of the Gag structure protein were generated, both carrying a LoxP site. Through Cre-Lox recombination these two vectors were fused together resulting in a vector carrying the sequence for one of the hemagglutinins and the sequence for the Gag structure protein.

3.2.18. BACMID PREPARATION AND BLUE/WHITE SCREENING

DNA was transformed into electro-competent DH10MultiBacY cells in order to generate recombinant bacmids. After cell recovery a dilution series (undiluted, 1:10 and 1:100 dilutions) were plated on selective agar plates containing gentamicin, kanamycin and tetracycline as selection markers, as well as IPTG and X-gal for blue/white screening. After incubation at 37° C for 24 - 48 hours clones that incorporated the DNA insert have a white phenotype, whereas those who did not appeared blue. To verify this result, 7 white and 1 blue colony were picked and restroken on a fresh selective agar plate (master plate). After verification a 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. Isolated bacmids were used for subsequent transfection of insect cells.

3.2.19. CLONING OF EXPRESSION CONSTRUCTS

- Gag_only
- Gag + H1
- Gag+H1/H1
- Gag + H3

The template constructs of the Influenza A Hemagglutinin H1, H3, pACEBac1 and pIDC_Gag were made available by other members of the working group. The cloning procedure of Gag+H1 is described in detail below. In this work these constructs were used for further investigations, the naked VLPs consisting of the Gag structure protein only were used as a control and for comparison with hemagglutinin displaying constructs.

GAG + H1 CONSTRUCT

This construct was designed for baculovirus surface display of the Influenza A Hemagglutinin (A/California/04/09) (1701 bp) Swine flu H1. First of all, the H1sequence was amplified using PCR. The obtained product and the pACEBac1 vector were double digested with the restriction enzymes EcoRI and NotI, followed by ligation to form pACEBac1_H1 (Acceptor vector). In order to obtain VLPs the pACEBac_H1 construct was further modified to additionally express the retrovirus structural protein Gag. Therefore Cre-LoxP fusion of Acceptor vector pACEBac1_H1 and Donor vector pIDC_Gag was performed, resulting in pIDC_Gag+H1/H1 because the pACEBac1_H1 vector had been integrated twice. To get a construct with only one H1 sequence the vector was digested with PmeI.

Thereby one pACEBac1_H1 sequence was cut out and the resulting pIDC_Gag+H1 sequence was ligated to form a vector. After ligation the construct was transformed into JM109 E.Coli cells. The cells were stroken out on selective Agar plates with gentamicin (resistance gene on pACEBac1) and chloramphenicol (resistance gene on pIDC) as selection markers.

3.3. Cell culture methods

3.3.1. Cultivation of *Sf*9 and *Tnms*42 cells and determination of cell number

*Sf*9 (ATCC CRL-1711) and *Tnms*42 *(Tnms*42 subcloned from BTI-TN5B1-4 cells (Chen *et al*. 2013)) cells were cultivated in HyClone medium supplemented with 0.1% (v/v) Pluronic-68 at 27° C in shaking flasks at 100 rpm. To avoid aggregation of the *Tnms*42 cells 0.1% (v/v) of Heparin was added. In order to maintain cell growth cells had to be passaged twice a week. The cell density and viability were measured with a TC20™ automated cell counter (Biorad, CA). 10 µL of cell suspension were mixed with 10 µL of 0.4% (v/v) trypan blue and pipetted in counting slides. Cells were diluted with HyClone medium to a desired cell density of about 0.5 x 10^6 cells/mL and further incubated at 27°C.

3.3.2. Transfection (FuGene HD)

For transfection *Sf*9 cells were seeded in 6 well plates at a density of 1 x 10^5cells/cm^2area (1 well: ~ 10 x 10^5 cm^2) and wells were filled up to 2 mL with Hyclone medium supplemented with 3% (v/v) FCS. Cells were let adhere in the incubator for at least 10 minutes at 27°C. In the meantime, a DNA solution with 2 µg of Bacmid DNA in 100 µL HyClone medium was prepared in Sarsted tubes. Another Sarstedt tube containing 8 µL FuGENE®HD Transfection Reagent (Promega) and 92 µL with HyClone medium was prepared. The contents of both tubes were combined and carefully mixed. After incubation for 10-15 minutes at room temperature the mixtures were added to the cells in the 6 well plate. After 3-4 days the cells were checked for YFP expression by fluorescence microscopy to verify successful virus rescue. The harvested supernatant constituted the seed stock and was stored at 4°C, constituting the seed stock. For baculovirus amplification 150 µL of seed stock were transferred to 7,5 x 10^6 *Sf*9 cells in T75 roux flasks filled up with HyClone medium to a total volume of 12 mL supplemented with 3% (v/v) FCS. After 3 days the supernatant was harvested creating the intermediate stock.

Again, for purposes of virus amplification, 200 µL intermediate stock were transferred to 17.5 x 10^6 *Sf*9 seeded cells in T175 roux flasks in a total volume of 25 mL with 3% (v/v) FCS. The harvested supernatant constitutes the working stock (WS), which was used for recombinant protein expression experiments.

3.3.3. 50% TISSUE CULTURE INFECTIVE DOSE (TCID50)

For determining the infectios titer of the generated virus working stocks the TCID50 method was applied. This assay was preferred for constructs containing yellow fluorescence protein (YFP) on the viral backbone as of easy monitoring of infection by the fluorescent phenotype; otherwise plaque assay was the method of choice. The information about the virus titer was necessary to be able to compare different constructs with each other by infecting cells with the same multiplicity of infection, MOI. All dilutions were made with HyClone media substituted with 0.1% (v/v) Pluronic F68. One 96-well-plate was used per virus sample. *Sf*9 cells were diluted to a concentration of 0.2 x 10^6 cells/mL. 100 µL of this dilution were pipetted into each well of the 96 well plate and incubated for approximately 1 hour at 27°C in order to let cells adhere. Meanwhile the virus dilutions were prepared in a 96-well-plate. 240 µL media was pipetted into each well of one column. One row of virus dilutions is needed for infecting one plate *Sf*9 cells. The virus stocks were first diluted 1:2000 (first 1:100 then 1:20). Then 60 µL of each virus dilution were added to the first well of each column (1A-12A) on the virus dilution plate. Further dilutions could be done using the multichannel pipette. 60 µL of the first well were transferred to the second well (1B-12B). After proper mixing through pipetting up and down for several times, the pipette tips were changed and 60 µL were again pipetted into the next (third) well until G1-12 was reached. 60 µL of each well in the last row were discarded, row H had served as negative control. One column of virus dilution served to infect a whole 96 well plate. By adding 15 µL virus from the respective dilutions to 12 wells of the 96 well plate containing the seeded cells. Finally, the plates were incubated at 27°C for 5 days in a plastic bag where a wet paper had been added to ensure that the plates do not dry out. After 5 days the plates were inspected under the fluorescence microscope and the titer was calculated.

PROPORTIONATE DISTANCE (PD)

$$= \frac{\%\ \text{of wells infected at dilution rate above } 50\% - 50\%}{\%\ \text{of wells infected at dilution above } 50\% - \%\ \text{of wells infected at dilution below } 50\%}$$

LOG10(TCID50) = $\log \text{total dilution above } 50\% - (\text{PD x } \log \text{ h})$

TCID50 = $10^{\text{Log10(TCID50)}}$

TCID50/mL = $\frac{\frac{1}{TCID_{50}}}{inoculation\ volume}$

PFU/mL = 0.69 x TCID50/mL

3.3.4. HA-GAG VLP EXPRESSION IN *SF*9 AND *TNMS*42 INSECT CELLS

20 mL *Sf*9/*Tnms*42 cells at a concentration of 1 x 10^6 cells/mL were infected with H1-Gag, H3-Gag or Gag-only expressing baculovirus working stocks at an MOI of 5 in 100 mL shaking flasks in triplicates. Cells were incubated for 3 days at 27°C and 100 rpm. Infection progress was monitored by evaluating YFP expression using the fluorescence microscope, the cell concentration and viability were determined with the TC20^{TM} automated cell counter (Biorad, CA).

3.3.5. FLOW CYTOMETRY

1-2 mL of the infected cell suspension was transferred into 2 mL Eppendorf tubes and centrifuged 5 min at 500 rpm. The supernatant was discarded, the pellet washed with PBS and centrifuged for 5 min 500 at rpm. The supernatant was again discarded, the cells were taken up in 1 mL PBS and analyzed on a Gallios Flow Cytometer (Beckman Coulter, Vienna, Austria) for YFP expression.

3.3.6. VLP EXPRESSION AND PURIFICATION

100 mL *Sf*9 or *Tnms*42 cell suspension at a concentration of 1 x 10^6 cells/mL were infected with 10 mL of virus working stock and incubated in a 500 mL flask at 27°C and 100 rpm. After 4 days of incubation the VLPs were harvested by low-speed centrifugation (10 min at 3000 rpm) using conical 150 mL tubes and a Heraeus Megafuge 16R centrifuge (Thermo Fisher Scientific). The supernatant was transferred into fresh 50 mL tubes and a centrifugation step for 10 min at 18000 rpm performed. Finally, each supernatant was split into three 38 mL ultracentrifugation tubes and the samples were ultracentrifuged for 1h and 40min at 30000 rpm in a 32 Ti rotor (company) in vacuum. The resulting pellet was resuspended in 300 µL PBS, covered with Parafilm and stored at 4°C overnight in order to dissolve the pellet. Subsequently sucrose gradient purification was performed, see 3.4.7.

3.3.7. DIFFERENT VLP EXPRESSION METHODS (*TNMS*42)

1. The **Gag+H1/H1** sample was treated as under 3.3.6., which means that the *Tnms*42 cells were grown to a cell density of 3x10^6 cells/mL, diluted to a concentration of 1x10^6 cell/mL and infected with a MOI of 5.

2. For the **Gag+H1/H1 + media change** samples *Tnms*42 cells were also grown to a density of 3x10^6 cell/mL. However, before dilution a centrifugation step was added in order to remove the old media and replace it by new, unspent media so that all substances required for cell growth had been available. Afterwards the cells were diluted to a concentration of 1x10^6 cells/mL and infected with a MOI of 5 as before.

3. **The Gag+H1/H1 + ACA** sample differentiates to the original procedure only in the addition of anti-clumping agent (ACA) before infection. Anti-clumping agent reduces building of aggregates which *Tnms*42 tend to do and therefore makes the cells more accessible for viral infection.

4. The **Gag+H1/H1 + media change + ACA** sample is a combination of condition 2 and 3. After cell growth to a density of 3x10^6 cells /mL the cells were centrifuged down and the old media was removed. They were taken up in new media and diluted to concentration of 1x10^6 cell/mL. Moreover, ACA was added to reduce clumping and the cells were infected with a MOI of 5.

3.3.8. Soluble H1 HA Expression

300 mL of *Tnms*42 cells with a density of 1 x 10^6 cells/mL were infected with 15mL H1soluble virus working stock (MOI=5) and incubated for 2 days.

3.3.9. His-tag purification

After incubation the suspension was centrifuged 10 min at 3000 rpm. The supernatant was split into two fractions of 150 mL whereby one fraction was supplemented with 16.6 mL 10x PBS for pH adjustment to pH 7.5. For each supernatant fraction, three mL nickel-slurry was washed in 45 mL PBS and were pelleted at4000 rpm for 10 minutes. The supernatant was discarded and the beads were added to the two fractions. This was followed by an incubation of 2 hours at 100 rpm. The two supernatant fractions were transferred into two columns and were washed 4 times with 15 mL wash buffer. Afterwards the protein was eluted with 2 mL of elution buffer which was incubated for 5 min. This elution step was repeated 2 times. From each step a sample for Western Blot analysis was taken. Vivaspin® 20 tubes (Sartorius) with a cut-off of 30kDa were used to concentrate the protein sample and for buffer exchange. Membrane filters were equilibrated with 15 mL PBS. Afterwards the three eluates were pooled and pipette onto the membrane filter reservoir. The proteins were concentrated to 500 µL and 15 mL PBS buffer was added for buffer exchange.

The tubes were centrifuged 15 min at 3500 rpm and 4°C, the concentrated samples again were filled up with PBS two times followed by a centrifugation step until the protein sample was concentrated to ~ 200 µL. The concentrated sample was aliquoted and stored at -80°C.

TABLE 3-3-144: COMPONENTS FOR WASH BUFFER (1 LITER)

Component	Amount for 1 liter
50 mM NaH_2PO_4	6.90 g NaH_2PO_4*H_2O (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
20 mM imidazole	1.36 g imidazole (MW 68.08 g/mol)
pH adjustment to 8.0 using NaOH	

TABLE 3-15: COMPONENTS FOR ELUTION BUFFER (1 LITER)

Component	Amount for 1 liter
50 mM NaH_2PO_4	6.90 g NaH_2PO_4*H_2O (MW 137.99 g/mol)
300 mM NaCl	17.54 g NaCl (MW 58.44 g/mol)
250 mM imidazole	17.00 g imidazole (MW 68.08 g/mol)
pH adjustment to 8.0 using NaOH	

3.4. BIOCHEMICAL METHODS

3.4.1. PREPARATION OF SDS-GELS

Sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE, is used to separate proteins based on their size, independent of secondary structures. The mobility of the molecules depends therefore on their length. Gels for SDS-PAGE were prepared at RT according to Table 3-16. First the separating gel was filled into the gel-cassette and overlayed with isopropanol to get a straight migration. After polymerization of the separating gel isopropanol was discarded and the stacking gel was added to the cassette. Subsequently, the combs for the slots were inserted. After polymerization the gels were either used directly for SDS-PAGE or stored at 4° C in wet torques in a plastic bag.

TABLE 3-16: COMPOSITION OF SDS-PAGE GEL (12% ACRYLAMIDECONCENTRATION; 1.5 GELS))

Component	Separating gel	Stacking gel
30% acrylamide	6.25 mL	0.833 mL
Separating / Stacking buffer	5.625 mL	0.625 mL
dH2O	2.843 mL	3.462 mL
10% SDS	150 µL	50 µL
10% Aps	120 µL	25 µL
TEMED	12 µL	5 µL

Aps (Promega, USA) and TEMED (Promega, USA) were added just before pipetting the solution into the cassette.

3.4.2. SDS-PAGE

SDS-PAGE was used for separating proteins of different molecular size. SDS gels were transferred into a gel electrophoresis chamber filled with 1x Laemmli buffer. The 10x Laemmli buffer was prepared according to Table 3-17.

TABLE 3-17: COMPOSITION OF 10X LAEMMLI BUFFER

Component	Amount
TRIS base	30 g/L
Glycine	144 g/L
SDS	10 g/L
dH2O	

The samples for SDS-PAGE were mixed with 2x SDS loading buffer (see Table 3-24), heated to 70° C for 10 minutes. In order to determine the size, 5 µL of standard, the PageRuler™ Prestained Protein Ladder (Figure 3-4), was used. 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.

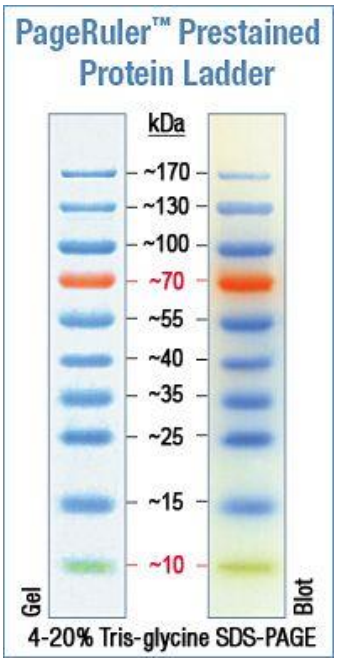


FIGURE 3-4: PAGERULER PRESTAINED PROTEIN LADDER. FIGURE TAKEN FROM [6].

TABLE 3-18: COMPOSITION OF 2X SDS-PAGE LOADING BUFFER

Component	Amount
SDS	1 g
Glycerin	2 mL
0.1% Bromphenolblue	2 mL
1M Tris pH 6.8	1.25 mL
dH2O	up to 10 mL

3.4.3. WESTERN BLOT

Western blotting was performed in order to verify and identify a protein. The proteins in the SDS-PAGE gel were transferred onto a membrane with the help of an electric field. Subsequently they were detected with two antibodies.

First the membrane was incubated with an antibody which was specific to the target protein. After a washing step the second antibody which was specific to the first antibody and conjugated to

alkaline phosphatase was added. By adding the alkaline phosphatase substrate a colorizing reaction occurred and the target protein bands were made visible.

The SDS-PAGE gel was put onto the PVDF membrane (GE Healthcare Life Sciences, UK) which had been activated with methanol and equilibrated in TOWBIN buffer (see Table 3-26). 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 3-19: COMPOSITION OF TOWBIN BUFFER

Component	Amount
TRIS base	3.03 g
Glycine	14.4 g
Methanol	200 mL
dH2O	up to 1000 mL

After blotting the membrane was incubated in a blocking solution (TPBS + 3% (w/v) BSA, see Table 3-22 at 4°C overnight.

TABLE 3-20: COMPOSITION OF 10X PBS

Component	Amount
NaCl	80 g
KCl	2 g
KH_2PO_4	2.4 g
$Na_2HPO_4*2H_2O$	18.05 g
dH_2O	up to 1000 mL
HCl for setting pH 7.4	

TABLE 3-21: COMPOSITION OF 1X PBST (0.1% TWEEN 20)

Component	Amount
10 x PBS	100 mL
Tween 20	1 mL
dH_2O	up to 1000 mL

TABLE 3-22: COMPOSITION OF BLOCKING SOLUTION

Component	Amount
BSA	1.5 g
PBST	50 mL

The membrane was washed with dH2O and equilibrated with TPBS. Then the membrane was incubated with a primary antibody (see Table 3-23) diluted in 10 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 an appropriate secondary antibody (see Table 3-23) 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 3-24). The blot was developed with 5 mL of AP buffer containing 33 µL NBT and 16.5 µL BCIP solution (Promega, USA) until bands were visible.

TABLE 3-23: ANTIBODIES USED FOR WESTERN BLOT

Primary antibody	Secondary antibody
H1 serum, mouse received from Dieter Palmberger)	a-mouse whole chain A5153 (1:2000)
H3N2, mouse monoclonal antibody (MBS832169 mybiosource)	a-mouse whole chain A5153 (1:2000)
H1 (A/California/06/2009) (H1N1) a-mouse (IT-26D11)	a-mouse whole chain A5153 (1:2000)

TABLE 3-24: COMPOSITION OF AP-BUFFER

Component	Concentration
TRIS base	100 mM
NaCl	100 mM
MgCl2	5 mM
pH 9.5	

3.4.4. Flow Cytometry

Infected cells were analyzed on a Gallios™ Flow Cytometer (Beckman Coulter, Brier, CA) for determination of the proportion of infected cells in the expression cultures. Cells were washed and resuspended in PBS and analyzed using an excitation wavelength of 488 nm (FL6). The acquired data was analyzed using Kaluza® Flow Analysis Software.

3.4.5. Ultracentrifugation (Pelleting)

The culture supernatant was filled into 38 mL ultracentrifugation tubes and centrifuged for 1h 40 min at 30.000 rpm in a 32Ti rotor (Beckmann) in vacuum. The forming pellet was resuspended in 0.5 mL PBS and stored at 4° C.

3.4.6. Sucrose Gradient centrifugation

Preparation

For preparing the 60-20% sucrose gradient, volumes of 2 mL 60%, 50%, 40%, 30% and 20% sucrose were filled into ultracentrifugation tubes and stored in the freezer for 2 hours to minimize mixing during preparation after each added layer.

Gradient ultracentrifugation

The resuspended pellet (0.5 mL) was carefully loaded onto the gradient in 13 mL ultracentrifugation tubes. PBS was added to fill the tube to the minimum filling level to prevent tube damage.

The ultracentrifugation was performed 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 gradient and stored at 4° C for further Western Blot and SDS-PAGE analysis.

3.4.7. Pooling of fractions and pelleting

The desired fractions were pooled and diluted 1:2 with PBS to reduce sucrose concentration. Subsequently 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 dissolve the pellet. The dissolved pellet was filled up to 1 mL with 20 mM HEPES and the concentrated VLPs were stored at 4° C until further analyses.

3.4.8. Transmission electron microscopy

30 µL of the purified VLP suspension were used for TEM measurement (FEI Tecnai G2 200 kV, FEI, Hillsboro, Oregon, USA). The samples were absorbed on grids that were previously covered with pioloform and steamed with carbon, were fixed using 2.5% glutaraldehyde and were examined at various magnifications. The coloration was performed with uranyl acetate, leading to negatively stained samples.

3.4.9. Nanoparticle Tracking Analysis (NTA)

NTA measurements were performed to determine the VLP concentration using a NanoSight LM 10 (Malvern Instruments Ltd., Worcestershire, UK) equipped with a blue laser (405 nm).

Sucrose gradient purified VLP samples were diluted 1:1000, 1:2000 and 1:4000 in particle free water to obtain a suitable concentration (60-100 particles per video frame) for analysis and 1 mL each was injected into the device. Videos of three dilution steps for each sample were captured for 60 s and were analyzed and processed with the NTA 3.2 Dev Build 3.2.16 Software. The particle number was evaluated for particles with a diameter between 100 and 200 nm.

3.4.10. Dynamic Light Scattering (DLS)

The VLP mean diameter and the homogeneity were measured by DLS using a Zetasizer Nano ZS with the Software version 7.03 (Malvern Instruments, Malvern, UK) at 25°C. VLP suspensions were diluted 100-fold in particle-free PBS prior to the measurement. Each suspension was measured in five replicates and the size distribution was calculated by the intensity report. The homogeneity of the VLP suspension was indicated by the polydispersity index (PDI). The data was processed with the NTA 3.2 Dev Build 3.2.16 Software and gated from 100 to 200 nm in order to get the VLP concentration only.

3.4.11. Bradford Assay

The unpublished protocol was taken from Katrin Reiter and modified.

Material:

- Bradford reagent (BioRad Protein Assay; cold room common shelf 2; 200 µL each well)
- Dilution Plate (96 well plate)
- Reading Plate (transparent 96 well plate)
- Multi-channel pipettes (100 and 300 µL) and tips
- Buffer reservoir for multi-channel pipettes
- Wavelength: 600nm

TABLE 3-25: SCHEMA OF 96 WELL BRADFORD PLATE

	1	2	3-12	Dilutions
A	BSA standard 200 µg/mL	BSA standard 200 µg/mL	Sample	1:2
B	150 µg/mL	150 µg/mL		1:4
C	100 µg/mL	100 µg/mL		1:8
D	75 µg/mL	75 µg/mL		1:16
E	50 µg/mL	50 µg/mL		1:32
F	25 µg/mL	25 µg/mL		1:64
G	12.5 µg/mL	12.5 µg/mL		1:128
H	0 µg/mL	0 µg/mL		1:256

The BSA standards in 1x PBS (or 1x TE or H2O) buffer 200; 150; 100; 75; 50; 25; 12,5; 0 µg/mL were prepared.

TABLE 3-26: BSA STOCK SOLUTION

Standard	Buffer	Mass	Buffer Volume	Note
BSA 200 µg/mL (stock)	1x PBS (or 1x TE or H_2O)	2 mg	10 mL	Filter with 0.22 µm syringe filter and aliquote (400 µL each eppi); Store at -20°C

TABLE 3-27: BSA STANDARD DILUTION PREPARATION

BSA Standard (100 µL) [µg/mL]	Stock solution volume	Buffer volume
200	100	0
150	75	25
100	59	50
75	37.5	62.5
50	25	75
25	12.5	87.5
12.5	6.25	93.75
0	0	100

25 mL of 1:5 Bradford reagent (200µL per well: 19.2 mL full plate) were prepared through mixing 20 mL H2O + 5 mL Reagent. This solution was filtered with 0.22 µm syringe filter and stored in a dark (or covered with aluminum foil) tube (storable for 14 days).

PREPARATION OF THE DILUTION PLATE:

All wells were filled with 150 µL 1x TE Buffer. Well A1 and A2 were left empty because the BSA standard was prepared separately. A3 was used as blank row and therefore 150 µL of 1x TE Buffer were added. 150 µL of sample were pipette in well A4-A12. Then 150 µL from row A were transferred to row B using the multi-channel pipette and mixed properly through pipetting several times up and down. After that 150 µL from B were transferred to well C and the same procedure was repeated until row H.

COLORING REACTION

Into column A1-H1 and A2-H2 10 µL of each diluted BSA standard (see Table 3-27 above) was added. Afterwards 10 µL from the dilution plate were transferred to the measuring plate using the multi-channel pipette, starting from the lowest dilution. Then 200 µL of the diluted and filtered Bradford reagent were added to the sample and standards.

Subsequently the plate was covered so that the coloring reaction takes place in the dark and incubated for 5 min. The plate was shaken periodically and after incubation the plate was measured at a wavelength of 600 nm.

3.4.12. GAG-ELISA

PRINCIPLE

This ELISA is a solid phase sandwich ELISA. A monoclonal antibody specific for human immunodeficiency virus type 1 p24 protein is coated on a 96-well plate. Standards and samples are binding to the immobilized antibody. After a subsequent washing step a horseradish peroxidase conjugated mouse anti-HIV-1 p24 secondary monoclonal antibody is added that binds to the standard/sample and forms an antibody-antigen-antibody “sandwich”. The wells are again washed and OPD substrate solution is added, which is converted into a yellow-orange signal by the conjugated peroxidase. The intensity of the obtained colour is proportional to the amount of HIV-1 p2 present in the standard/sample. To terminate the enzymatic reaction, a stop solution is added and absorbances of the microwells are read at 450 nm and at the reference wavelength of 630 nm for reference.

MATERIAL

- The Gag ELISA was based on the commercially available Human Immunodeficiency Virus type 1 (HIV-1) p24/Capsid Protein p24 ELISA Pairs Set (Sino Biological) which is used to detect p24 proteins in solutions. The protocol was adapted (Katrin Reiter, AG Jungbauer, DBT) for being utilized to measure p24 proteins incorporated inside VLPs
- Standard: One vial contains with 30 ng of lyophilized recombinant HIV-1 p24 was reconstituted with 1 mL detection antibody dilution buffer, aliquoted and stored at -20°C.
- Capture antibody: 0.5 mg/ml of mouse anti-HIV-1 P24 monoclonal antibody (in PBS, pH 7.4) were diluted to a working concentration of 2 µg/mL in PBS before coating. It was aliquoted and stored at -20°C.

- Detection antibody: 0.2 mg/mL mouse anti-HIV-1 p24 monoclonal antibody conjugated to horseradish-peroxidase (HRP) (in PBS, 50 % HRP-protector, pH 7.4) were diluted to a working concentration of 0.1 µg/mL in detection antibody buffer before use. It was stored at 4 °C in the fridge.
- OPD substrate(SIGMA*FAST*™)
- Elisa plate: Nunc MaxiSorp Immuno plate (Thermo Fisher Scientific)
- Dilution plate: Nunc 96F (Thermo Fisher Scientific)

BUFFER PREPARATION

- SNCR buffer

TABLE 3-28: COMPOSITION OF SNCR BUFFER

	For 100 mL
30 mM Tris/HCl pH 7.2	0.36 g
450 mM NaCl	2.63
1.5% (v/v) Triton X-100	1.5 mL
1.5% (w/v) Deoxycholic Acid (Sodium Salt)	1.5 g
0.3% (w/v) Sodium Dodecylsulfate	0.3 g
10 mM EDTA	0.29 g

All substances were first dissolved and then mixed in the right order as written in Table 3-28.

- Stock solution: 10x Phosphate-buffered saline (10x PBS)

TABLE 3-29: COMPOSITION OF 10X PHOSPHATE-BUFFERED SALINE (10X PBS)

pH 7.4, 0.2 µm filtered	**For 1000 mL**
1.37 M NaCl	80 g
27 mM KCl	2 g
100 mM Na2HPO4x2H20	17.8 g
18 mM KH2PO4	2.4 g
RO-Water	Fill up to 1000 mL

This solution can be stored up to 1 month at RT.

- 10x Tris buffered saline *(10x TBS)*

TABLE 3-30: COMPOSITION OF 10X TRIS BUFFERED SALINE (10X TBS)

pH 7.4	For 1000 mL
200 mM Tris	24.22 g
1.5 M NaCl	87.66 g
RO-water	Fill up to 1000 ml

This solution can be stored up to 1 month.

- 10 % Tween20 (1:10 dilution of 10x TBS for Tween20)

TABLE 3-31: COMPOSITION OF 10% TWEEN20

pH 7.2-7.4	For 10 mL
Tween20	1 mL
1 x TBS	Fill up to 10 mL

- Wash buffer: 0.05 % Tween20 in TBS, pH 7.2-7.4

TABLE 3-32: COMPOSITION OF WASH BUFFER

	For 1000 mL
10 x TBS	100 mL
10% (v/v) Tween 20 (in 1x TBS)	5 mL
RO-water	Fill up to 1000 mL

This solution has to be freshly prepared. It is sufficient for 2 plates.

- Blocking buffer: 2 % BSA in wash buffer

TABLE 3-33: COMPOSITION OF BLOCKING BUFFER

	For 200 mL
10x TBS	20 mL
10 % Tween20 (dissolved in 1 x TBS)	1 mL
2 % BSA	4 g
RO-water	Fill up to 200 mL

The solution can be stored for up to one week at 4°C. It is sufficient for 6 plates.

- Sample dilution buffer: 0.1 % BSA in wash buffer, pH 7.2-7.4, 0.2 µm filtered

TABLE 3-34: COMPOSITION OF SAMPLE DILUTION BUFFER

	For 200 mL
10x TBS buffer	20 mL
10 %Tween 20 (dissolved with 1x TBS)	1 mL
0.1 % BSA	0.2 g
RO-Water	Fill up to 200 mL

- Detection antibody dilution buffer: 0.5 % BSA in wash buffer, pH 7.2-7.4, 0.22 µm filtered

TABLE 3-35: COMPOSITION OF DETECTION ANTIBODY DILUTION BUFFER

	For 200 mL
10x TBS	20 mL
10 % Tween20 (dissolved in 1 x TBS)	1 mL
0.5 % BSA	1 g
RO-water	Fill up to 200 mL

- Substrate solution: OPD substrate solution
  One SIGMA*FAST*™ OPD (*o*-Phenylenediamine dihydrochloride) tablet pair was dissolved in 20 mL dH_2O. It was prepared right before and sufficient for 1-2 plates covered in aluminum foil.
- Stop solution: 1 N H_2SO_4 (0.5 M H_2SO_4)

TABLE 3-36: COMPOSITION OF STOP SOLOTION

	For 100 mL
97-99% H_2SO_4	20 mL
dH_2O	1 mL

PROCEDURE

PLATE PREPARATION

IMMOBILIZATION OF COATING ANTIBODY

The capture antibody was diluted to a working concentration of 2 µg/mL by performing a 1:250 dilution of *"mouse anti-HIV-1 P24 monoclonal antibody"* in PBS buffer. For each plate 10 mL of coating solution plus an extra 2 mL for reverse pipetting and 48 µL of the "*mouse anti-HIV-1 P24 monoclonal antibody*" were used. 100 µL of this solution were pipetted with the 12-channel pipette into each well of the MaxiSorp plate. The plate was incubated overnight at 4 °C on a shaker.

PLATE WASHING

The "*Wash buffer*" was prepared every time freshly and the plates were washed three times using the plate washer (Tecan 96PW Microplate Washer).

BLOCKING OF THE MAXISORP PLATE

For blocking 300 µL of the blocking solution (2 % BSA in TBS-T) were pipetted with the 12-channel pipette into each well of the MaxiSorp plate. Afterwards the plate was incubated for 2 hours on a shaker at room temperature.

SAMPLE TREATMENT

150 µL sample and 75 µL of SNCR buffer were incubated for 10 minutes at 70°C for VLP disruption. Then 75 µL 1.5 % Triton X-100 were added and the mixture was incubated for 10 minutes at 100°C.

SAMPLE DILUTION

The samples were diluted with the sample dilution buffer if necessary to ensure that the concentrations were within the standard curve range (0-2000 pg/mL). 170 µL of sample dilution buffer were pipetted into each well and 170 µL of sample dilution buffer in A1 for the negative control. Then 170 µL of standard or sample were added in well A2-A12. Afterwards 1:2 dilutions (Table 3-37) were performed. Starting from row A the samples were mixed 10 times (by pipetting 10 times up and down, reverse pipetting). Then 170 µL of the sample from row A were transferred into row B by reverse pipetting. This procedure was repeated for all other columns and the tips changed after each dilution step.

TABLE 3-37: SCHEME OF THE ELISA PLATE AND SAMPLE DILUTIONS

	1	2	3	4	5	6	7	8	9	10	11	12
A	Negative control (sample diultion buffer)	Standard stock 1:2	Standard stock 1:2	Sample 1 1:2	Sample 2 1:2	Sample 3 1:2	Sample 4 1:2	Sample 5 1:2	Sample 6 1:2	Sample 7 1:2	Sample 8 1:2	Sample 9 1:2
B		Standard stock 1:4	Standard stock 1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4
C		Standard stock 1:4	Standard stock 1:4	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8
D		Standard stock 1:8	Standard stock 1:8	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16
E		Standard stock 1:16	Standard stock 1:16	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32
F		Standard stock 1:32	Standard stock 1:32	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64
G		Standard stock 1:64	Standard stock 1:64	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128	1:128
H		Standard stock 1:128	Standard stock 1:128	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256	1:256

PLATE WASHING

The plate was washed 3 times.

STANDARD DILUTION

The standard and sample dilution was performed on a dilution plate. (Nunc F plate) 170 µL of sample dilution buffer were pipetted into each well of the dilution plate.

A standard curve using 2-fold serial dilutions in sample dilution buffer, and a high standard of 2000pg/mL was prepared.

60 µL of aliquoted and frozen standard were diluted with 390 µL of dilution buffer for a concentration of 4000 pg/mL. Then 170 µL of diluted standard were pipetted in well A2 and A3 for the generation of replicate standard curves with a high standard of 2000 pg/mL.

After the dilution preparation the MaxiSorp plate was washed 3 times as before. Then 100 µL of each sample were transferred from row H of the dilution plate to row H of the MaxiSorp plate by reverse pipetting.

ASSAY PROCEDURE

a. SAMPLE TRANSFER

Starting from the lowest concentration in row H the sample was mixed 3 times by pipetting up and down. 100 µL of the sample from row H of the dilution plate were transferred to row H of the MaxiSorp plate by reverse pipetting. This procedure was repeated for row G, F, E, D, C, B and A. The tips didn't have to be changed in between because the samples were transferred with increasing concentration. The plate was covered with a lid and incubated for two hours at room temperature.

b. PLATE WASHING

The plate was washed three times with the plate washer.

c. INCUBATION WITH SECONDARY ANTIBODY SOLUTION

The detection antibody was diluted to working concentration of 0.1 µg/mL. Therefore a 1:2000 dilution of the "*mouse anti-HIV-1 P24 monoclonal antibody conjugated to HRP"* in antibody dilution buffer was performed. For each plate 10 mL of secondary antibody solution plus an extra of two ml for reverse pipetting were prepared. 12 mL of the sample buffer were pipetted in a 15 mL Falcon tube and 6 µL of the "*mouse anti-HIV-1 P24 monoclonal antibody conjugated to HRP*" were added. 100 µL of the secondary antibody solution were pipetted with the 12-channel pipette to the MaxiSorp plate. The plate was covered with a lid and incubated one hour at room temperature.

d. PLATE WASHING

The plate was washed three times using the procedure given under "*b) Plate washing*"

e. INCUBATION WITH SUBSTRATE SOLUTION

100 µL of the substrate working solution were added to each well. Afterwards it was incubated for 20 minutes at room temperature in the dark.

f. STOP SOLUTION

Then 100 µL of the stop solution were pipetted into each well of the MaxiSorp plate and mixed gently through tapping the plate.

g. MEASUREMENT METHOD

The optical density of each well was determined immediately. The absorbance was measured at 492 nm with the Magellan plate reader. As reference wavelength the absorbance at 620 nm was also measured.

3.4.13. H1-ELISA

MATERIAL

The antibodies from the commercially available Influenza A H1N1 (swine Flu 2009) Hemagglutinin / HA ELISA Pair Set were used in combination with a self-produced recombinant soluble H1 HA as calibration standard.

- **Calibration standard**: Self-made insect expressed soluble H1 HA with a concentration of 56.8 µg/mL was prediluted to a working concentration of 10 ng/mL before use. It was stored at -80°C.
- **Capture antibody**: 1 mg/ml of mouse anti-Influenza A H1N1 (Swine Flu 2009) Hemagglutinin / HA monoclonal antibody (in PBS, pH 7.4) was diluted to a working concentration of 2 µg/mL in PBS before coating. It was aliquoted and stored at -20°C.
- **Detection antibody**: 0.2 mg/mL of rabbit anti-Influenza A H1N1 (Swine Flu 2009) Hemagglutinin / HA polyclonal antibody conjugated to horseradish-peroxidase (HRP) (in PBS, 50 % HRP-Protector, pH 7.4) were diluted to a working concentration of 0.8 µg/mL in detection antibody dilution buffer before use. It was stored at 4°C in the fridge.

Procedure

Plate preparation

Immobilization of coating antibody

The capture antibody was diluted to a working concentration of 2 µg/mL by performing a 1:500 dilution of *"*mouse anti-Influenza A H1N1 (Swine Flu 2009) Hemagglutinin / HA monoclonal antibody*"* in PBS buffer. For each plate 10 mL of coating solution plus an extra 2 mL for reverse pipetting and 48 µl of the "mouse anti-Influenza A H1N1 (Swine Flu 2009) Hemagglutinin / HA monoclonal antibody" were used. 100 µL of this solution were pipetted with the 12-channel pipette into each well of the MaxiSorp plate. The plate was incubated overnight at 4 °C on a shaker.

Plate washing

The "*Wash buffer*" was prepared freshly and the plate was washed three times on the plate washer.

Blocking of the MaxiSorp plate

For blocking 300 µL of the blocking solution (2 % BSA in TBS-T) were pipetted with the 12-channle pipette into each well of the MaxiSorp plate. Afterwards the plate was incubated for 2 hours at a shaker at room temperature.

Sample treatment

225 µL sample and 25 µL of 10% (w/v) Zwittergent in TBS were incubated for 30 minutes at room temperature for VLP disruption.

Sample dilution

The samples were diluted with the sample dilution buffer if necessary so that the concentrations were within the standard curve range (0.0781 ng/mL - 10 ng/mL). 170 µL of sample dilution buffer were pipetted into each well and 170 µL of sample dilution buffer in A1 for the negative control. Then 170 µL of standard or sample were added to well A2-A12. Afterwards 1:2 dilutions were performed. Starting from row A the samples were mixed 10 times (by pipetting 10 times up and down). Then 170 µL of the sample from row A were transferred into row B by reverse pipetting. This procedure was repeated for all other rows and the tips changed after each dilution step.

Plate washing

The plate was washed 3 times.

STANDARD DILUTION

The standard and sample dilution was performed on a dilution plate. (Nunc F plate) 170 µL of sample dilution buffer were pipetted into each well of the dilution plate. Soluble H1 HA was used to prepare a standard curve using 2-fold serial dilutions in sample dilution buffer, and a high standard of 40ng/mL.1.56 µL H1 HA (56.8 µg/mL) were diluted with 998.44µL sample dilution buffer for a concentration of 80 ng/mL. Then 170 µL of diluted standard were pipetted in well A2 and A3 in the dilution plate to get a concentration of 40 ng/mL in well A2 and A3 for double determination.

After the dilution preparation the MaxiSorp plate was washed 3 times as before. Then 100 µL of each sample was transferred from row H of the dilution plate to row H of the MaxiSorp plate by reverse pipetting.

ASSAY PROCEDURE

a. SAMPLE TRANSFER

Starting from the lowest concentration in row H the sample was mixed 3 times by pipetting up and down. 100 µL of the sample from row H of the dilution plate were transferred to row H of the MaxiSorp plate by reverse pipetting. This procedure was repeated for row G, F, E, D, C, B and A. The tips didn't have to be changed in between because the samples were transferred with increasing concentration. The plate was covered with a lid and incubated for two hours at room temperature.

b. PLATE WASHING

The plate was washed three times using the plate washer.

c. INCUBATION WITH SECONDARY ANTIBODY SOLUTION

The detection antibody was diluted to working concentration of 0.8 µg/mL. Therefore a 1:250 dilution of the "*rabbit anti-Influenza A H1N1 (Swine Flu 2009) Hemagglutinin / HA polyclonal antibody conjugated to HRP"* in antibody dilution buffer was performed. For each plate 10 mL of secondary antibody solution plus an extra of two mL for reverse pipetting were prepared. 12 mL of the sample buffer were pipetted in a 15 mL Falcon tube and 30 µL of the "*mouse anti-HIV-1 P24 monoclonal antibody conjugated to HRP*" were added.

100 µL of the secondary antibody solution were pipette with the 12-channel pipette to the MaxiSorp plate. The plate was covered with a lid and incubated for one hour at room temperature.

d. PLATE WASHING

The plate was washed three times using the procedure given under "*b) Plate washing*"

e. INCUBATION WITH SUBSTRATE SOLUTION

100 µL of the substrate working solution were added to each well. Afterwards the plate was incubated for 20 minutes at room temperature in the dark.

f. STOP SOLUTION

100 µL of the stop solution were pipetted into each well of the MaxiSorp plate and mixed gently through tapping the plate.

g. MEASUREMENT METHOD

The optical density of each well was determined immediately. The absorbance was measured at 492 nm and the reference wavelength of 620 nm were measured with the Magellan plate reader. Blank-reduced difference data were used to generate a 4PL calibration curve that served to determine the concentration of the sample.

4. Results and Discussion

4.1. General remarks

The aim of this study was to compare the yield of influenza HA-Gag VLPs in two different insect cell lines and to quantify the VLP-incorporated HA content. In order to verify successful VLP production several analyses were performed, each giving additional information about the produced VLPs. At first, the different baculovirus based constructs were cloned and then expression of the desired proteins was verified by Western Blot. Flow cytometry was performed to give information about the fraction of infected cells in the expression culture. A p24-ELISA and HA-ELISA served to quantify VLP particles and the content of incorporated HA proteins. VLPs were concentrated and purified from soluble proteins through sucrose gradient centrifugation. These concentrated VLP samples were used for further experiments. The amount of VLPs was determined with the NanoSight, the homogeneity was analyzed with the Zetasizer. TEM analysis was performed to verify that VLPs retained their particulate structure and to see whether baculoviruses were still present.

4.2. Expression constructs

4.2.1. Gag_only

The Gag_only construct was used as negative control for all analyses. It consists only of the HIV main structural protein Gag and has no hemagglutinin displayed on the VLP surface. The plasmid was made available by the working group and was used to generate a recombinant baculovirus.

4.2.2. Gag+H1

The Gag+H1 construct was designed for baculovirus surface display of the hemagglutinin of the A/California/4/2009 (H1N1) influenza strain. Due to the transmembrane domain present in the hemagglutinin gene sequence, the protein is transported and anchored into the cellular surface after synthesis and thus, not secreted into the supernatant. The gene sequences and vectors were made available by the working group.

4.2.3. Gag+H1/H1

For the baculovirus surface display construct of Gag+H1/H1 a multiexpression construct with two H1 expression cassettes was generated.

This construct was designed in order to increase the number of hemagglutinin trimers on the cellular surface and to compare it with the Gag+H1 construct. Again, the gene sequences and the vectors were made available by the working group.

4.2.4. H1 SOLUBLE

The H1 soluble construct was designed to produce a secreted form of the hemagglutinin of A/California/4/2009 (H1N1) that can be used as a standard for the H1 ELISA. The transmembrane domain of the H1 gene sequence was removed to prevent its anchorage into the cellular surface. This virus stock was made available by the working group and was used for further experiments.

4.2.5. GAG+H3

The Gag+H3 construct was designed for baculovirus surface display of the hemagglutinin influenza antigen A/Hiroshima/52/2005 (H3N2). Due to the transmembrane domain of the hemagglutinin gene sequence the protein is transported and anchored into the cellular surface after synthesis. The sequences and vectors that were needed for this construct were made available by the working group.

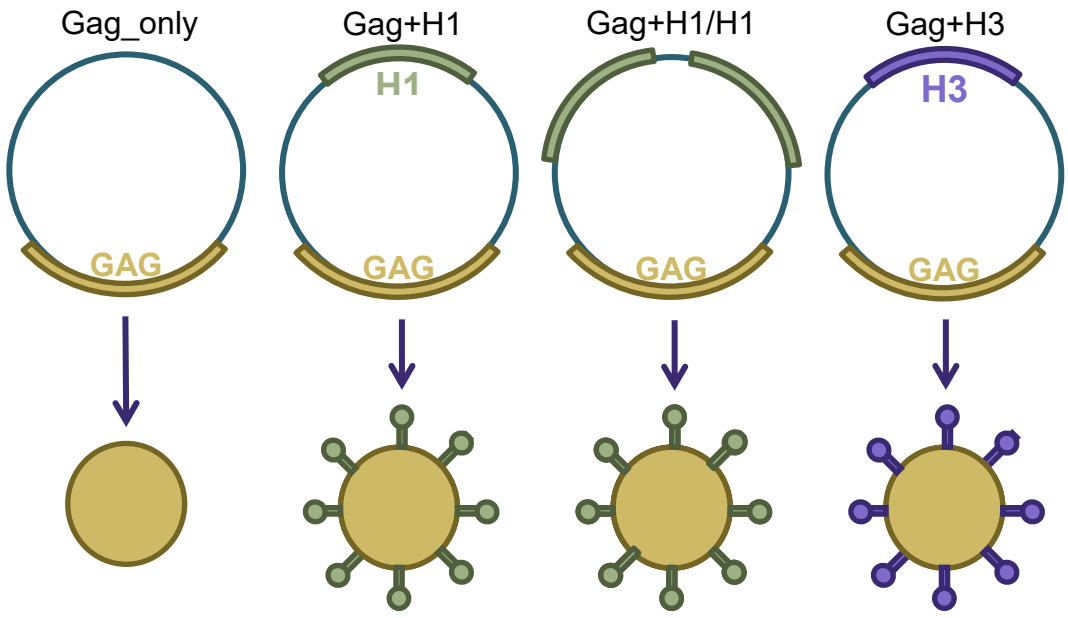
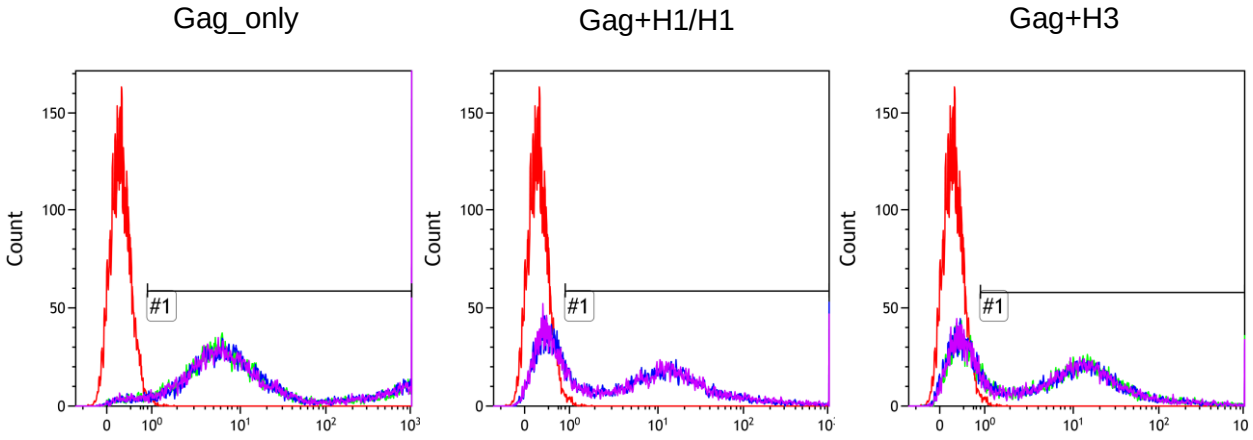


FIGURE 4-1: SCHEME OF DIFFERENT VLP CONSTRUCTS; FROM LEFT TO THE RIGHT: GAG_ONLY CONSISTING OF THE HIV-GAG MATRIX PROTEIN ONLY – GAG+H1 CONSISTING OF THE GAG MATRIX PROTEIN AND THE HEMAGGLUTININ OF THE INFLUENZA H1N1 CALIFORNIA 09 VIRUS – GAG+H1/H1 CONTAINING TWO H1 EXPRESSION CASSETTES – GAG+H3 CONSISTING OF THE GAG MATRIX PROTEIN AND THE HEMAGGLUTININ OF THE INFLUENZA H3N2 HIROSHIMA VIRUS

4.3. Comparison of protein Infectivity

Generated virus working stocks of different expression constructs were used for infection of *Sf*9 and *Tnms*42 cells to compare protein expression and expression characteristics in the two cell lines. The cells were infected in triplicates. For each sample the same amount of cells was infected with a MOI (multiplicity of infection) of 5 and incubated for 4 days. At incubation times longer than 4 days cell degradation and lysis, caused by infection, is already very pronounced. This results in cell debris and cytosolic contents to be released into the supernatant, which are difficult to remove. Furthermore, proteolytic enzymes are able to degrade the produced VLPs. As negative control insect cells infected with an unrelated virus that does not contain YFP (Gag_only without YFP) were used. The cells for the negative control were treated the same way as the samples. After 4 days the cells were counted and the viability was determined. Infection leads to growth stop, cultures with a higher proportion of infected cells show a lower cell density as compared to weakly infected cultures. The amounts of infected cells were first checked under the fluorescence microscope for YFP (yellow fluorescence protein) fluorescence. The flow cytometry was performed in order to get exact data about the infection. Recombinant baculoviruses expressing the VLPs components also harbor the YFP expression cassette to allow for easy monitoring of the infected cell population.



Gag_only				Gag+H1/H1				Gag+H3			
Marker	%Gated	X-Med	X-AMean	Marker	%Gated	X-Med	X-AMean	Marker	%Gated	X-Med	X-AMean
All	100.00	8.39	181.71	All	100.00	3.67	31.67	All	100.00	4.79	25.82
All	100.00	8.85	182.76	All	100.00	3.46	28.69	All	100.00	4.19	23.85
All	100.00	7.99	165.52	All	100.00	3.46	28.69	All	100.00	5.00	26.88
All	100.00	0.27	0.29	All	100.00	0.27	0.29	All	100.00	0.27	0.29
#1	95.44	9.13	190.37	#1	64.63	11.41	48.77	#1	66.08	11.60	38.86
#1	95.87	9.51	190.61	#1	64.71	10.80	44.10	#1	64.98	11.48	36.49
#1	95.76	8.59	172.83	#1	64.71	10.80	44.10	#1	67.29	11.82	39.74
#1	1.09	1.02	1.05	#1	1.09	1.02	1.05	#1	1.09	1.02	1.05

FIGURE 4-2: INFECTED *SF*9 CELLS AFTER 4 DAYS OF INCUBATION. RED – NEGATIVE CONTROL (GAG WITHOUT YFP INFECTED CELLS); PURPLE, BLUE, GREEN – TRIPLICATE SAMPLES; THE GATES WERE SET AT 1% OF THE FLUORESCENCE MINUS NEGATIVE CONTROL

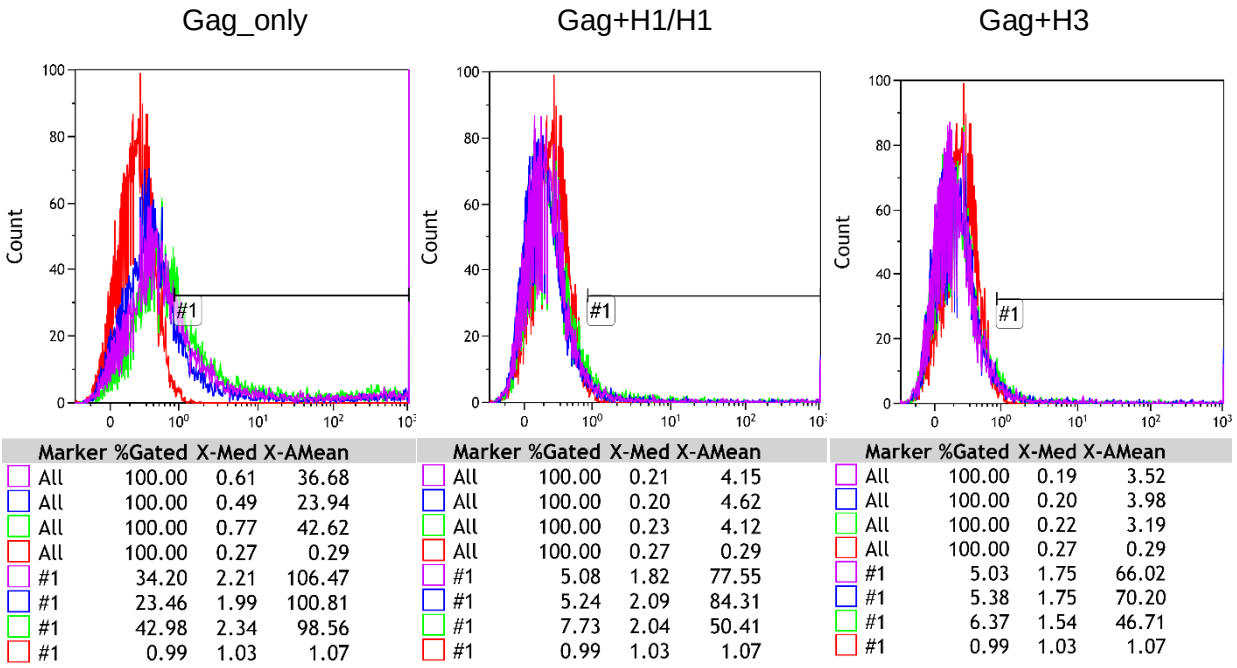


FIGURE 4-3: INFECTED *TNMS42* CELLS AFTER 4 DAYS OF INCUBATION.RED – NEGATIVE CONTROL (GAG WITHOUT YFP INFECTED CELLS); PURPLE, BLUE, GREEN – SAMPLE TRIPLICATES; THE GATES WERE SET AT 1% OF THE FLUORESCENCE MINUS NEGATIVE CONTROL

The flow cytometry results from the *Sf*9 samples are shown in figure 4-2, the *Tnms*42 samples can be seen in figure 4-3. Sample triplicates are given in green, blue and purple and indicate that cells show a similar degree of infection among triplicate samples. The red peak represents the negative control, which are cells infected with a baculovirus not expressing YFP. Figure 4-2 shows that 95% of the *Sf*9 cells were infected at day 4 post infection using the Gag-only construct. In contrast 64-66% of *Sf*9 cells were infected with Gag+H1/H1 or Gag+H3 day 4 post infection. This difference can also be seen in *Tnms*42 cells. The Gag_only infected samples showed an average of 34% infected cells and the viruses encoding for both VLP components (HA and Gag) infected only 5-7% of the cells in the expression culture. It might be that the expression of an additional complex glycoprotein that has to undergo posttranslational modifications and transport to the cellular surface increases the metabolic burden of the cell in a way, that budding of infectious baculovirus as secondary infection are reduced. Furthermore, it is possible that the displayed proteins that are also incorporated on the baculovirus surface hinder the attachment of the gp64 protein to the cellular membrane and thus impede with infection. Moreover, a difference of infectivity can be observed between *Sf*9 and *Tnms*42 cells using the same baculovirus for infection. Because of that reason virus stocks are always made with *Sf*9 cells and not with *Tnms*42 cells.

Because of the low amount of infected *Tnms*42 cells the same experiment was repeated with the Gag+H1/H1 construct. Furthermore, some conditions were changed in order to improve infection of the Tnms42 cells. After 4 days of incubation the infection was monitored under the fluorescence microscope, the cells were counted and the viability determined. Less infected cells were observed in the expression culture without medium change but the infected cells had shown intense fluorescence (data not shown). In contrast, the cells that had undergone a medium change before infection showed higher infection efficiency, but weaker fluorescence (data not shown). The addition of anti-clumping agent had a detrimental effect on the infection in both cases (infection with and without medium change). The anti-clumping agent may be utilized to reduce cell aggregation in suspension. The reagent may modify the cell surface in a way to reduce attachment of the virus to the cell and thereby reduce infection. Cells were analyzed using the flow cytometer to quantify the number of infected cells. The Gag+H1/H1 samples showed 17-20% infected cells whereas in the Gag+H1/H1 + media change samples 41-53% of the cells were infected (Figure 4-4). Using the medium change strategy, we could improve the infection protocol and increase the fraction of infected *Tnms*42 cells by roughly 2-fold.

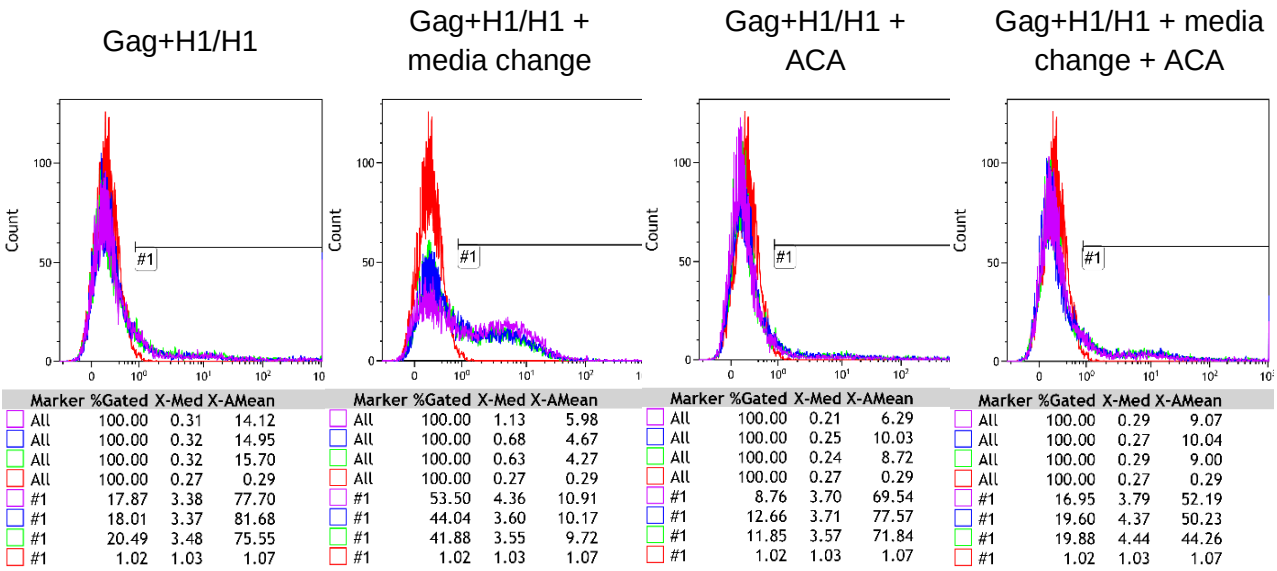


FIGURE 4-4: INFECTED *TNMS*42 CELLS WITH DIFFERENT CONDITIONS AFTER 4 DAYS OF INCUBATION; GATED AT 1% OF THE NEGATIVE SAMPLE

4.4. COMPARISON OF GAG EXPRESSION

After 4 days of incubation, cells were removed by low-speed centrifugation (10 min at 3000 rpm) and the supernatant was centrifuged again 10 minutes at 18000 rpm to remove residual cell debris and aggregates. The resulting supernatant was stored at 4°C for further analyses. In order to determine the number of produced VLPs a p24-ELISA was carried out.

The principle is that it is known that a VLP consists of 2000 Gag-protein molecules (Floderer *et al*., 2018) and these can be detected after dissociation. At first, VLPs were treated with detergents and high temperature to enable dissociation of the VLPs and to liberate the single Gag-proteins into the supernatant. Then the Gag-proteins were bound to the capture antibody immobilized on the surface of a 96-well ELISA plate. After an incubation time of two hours and several washing steps, Gag proteins were detected by a p24-specific antibody conjugated to a horseradish peroxidase (HRP).

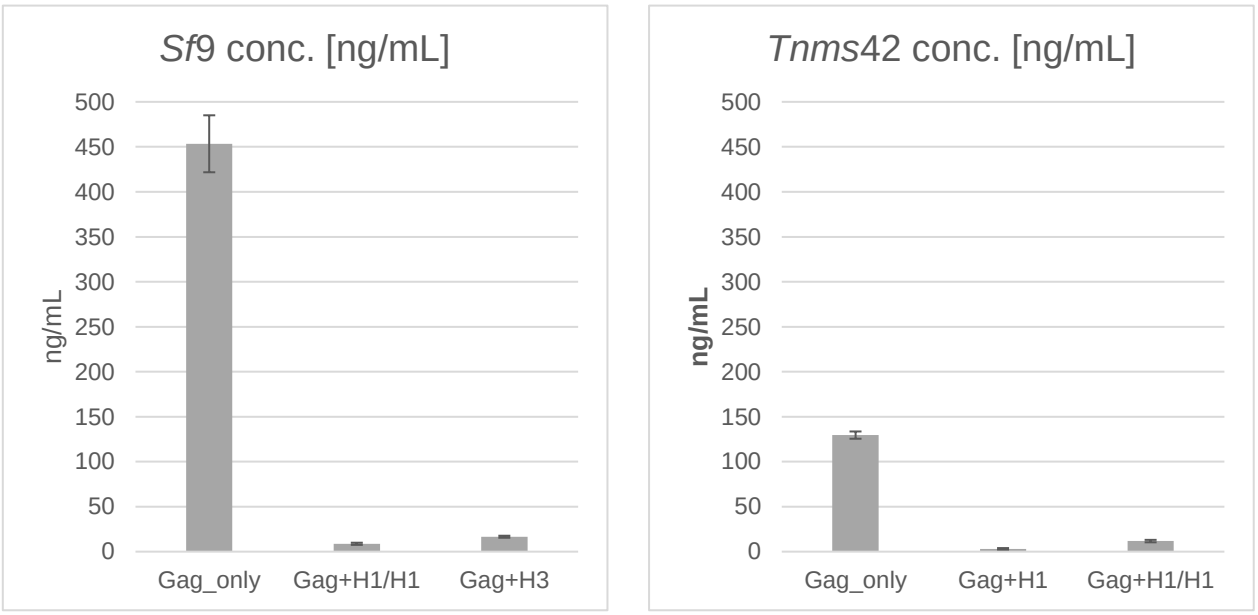


FIGURE 4-5: NG OF GAG PROTEIN PER ML SUPERNATANT PRODUCED IN *SF*9 CELLS (LEFT), PRODUCED IN *TNMS*42 CELLS (RIGHT)

The Gag concentration per mL expression supernatant from *Sf*9 cells can be seen in Figure 4-6. Between 400 and 500 ng Gag-protein were produced in the Gag_only expressing cells. Upon co-expression of the HA protein, Gag concentrations dropped to 20 ng/mL for both expression constructs – Gag+H1/H1 and Gag+H3. One reason for the lower expression level was the lower number of infected cells (see Figure 4-2). The *Tnms*42 samples produced, as expected due to the low amount of infected cells, four fold less Gag-proteins than the *Sf*9 cells. Again, when co-expressing the HA with the Gag protein, Gag concentration dropped 15-fold, a phenomenon also seen in *Sf*9 cells. Also a calculation per infected cell revealed that the *Tnms*42 cells produced less Gag-protein in case of the Gag_only construct than the *Sf*9 cells. However, regarding the hemagglutinin constructs, Gag+H1 and Gag+H1/H1, the *Tnms*42 cells produced more Gag-protein per infected cell than the *Sf*9 cells. Maybe it is easier for the *Tnms*42 cells to display a protein on the cell surface. Therefore, further investigations according VLP-based vaccine production should be done with the *Tnms*42 cell line. Normalizing the results with respect to infection level enables us to compare the expression levels on a per infected cell basis.

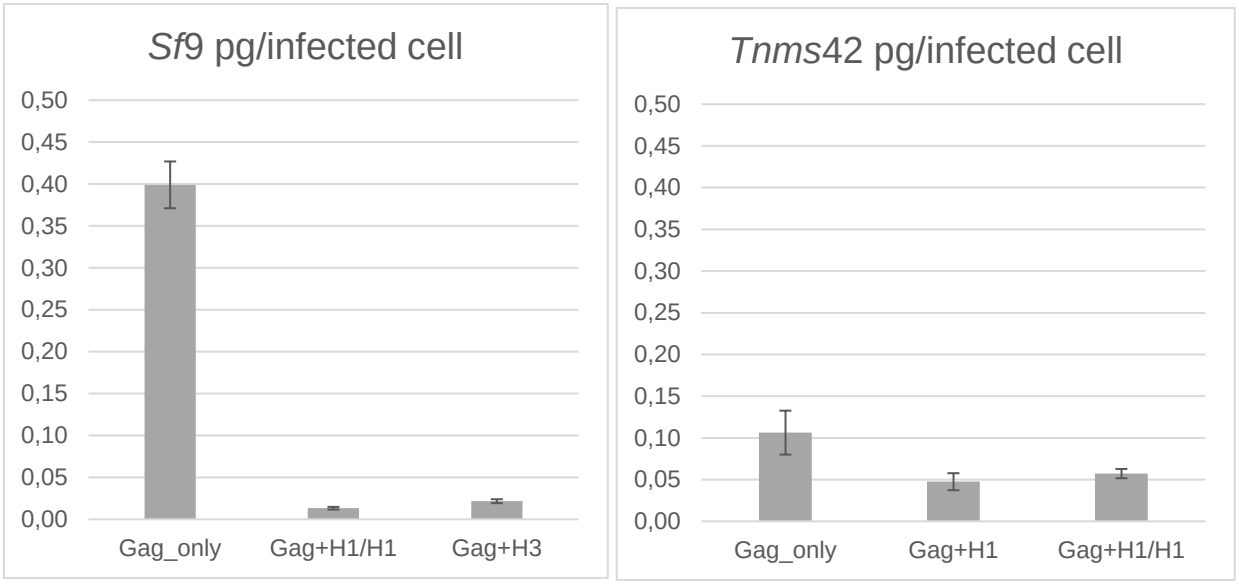


FIGURE 4-6: MEAN PRODUCTIVITY PER INFECTED CELL. LEFT - *SF*9 CELLS; RIGHT - *TNMS*42 CELLS

Gag_only samples had produced around 0.4 pg Gag-protein per infected cell and the HA-Gag-infected samples around 0.02 pg Gag-protein per infected cell (20-fold reduction). That suggests that cells expressing an additional complex protein, such as hemagglutinin glycoprotein, on the surface may experience additional metabolic burden, which is reflected in a drop in protein expression of the Gag component. This phenomenon can be seen for cells expressing both HA-Gag VLPs (Figure 4-6 LEFT).

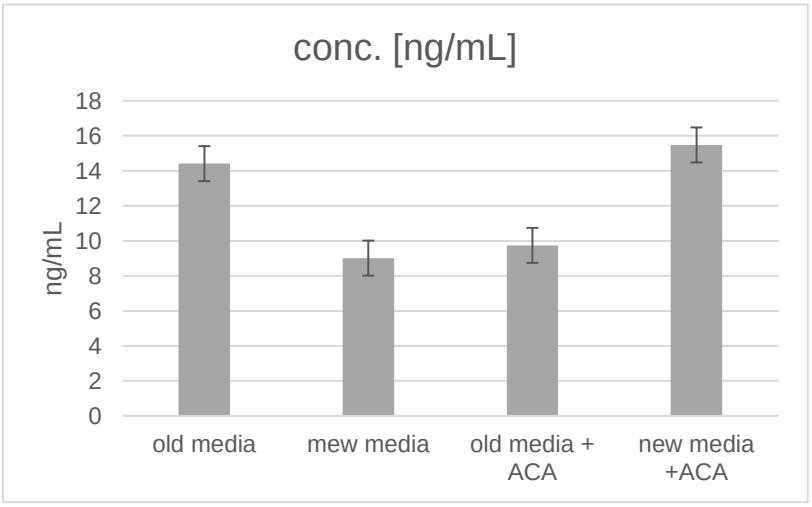


FIGURE 4-7: NG OF GAG PROTEIN PER ML SUPERNATANT USING DIFFERENT INFECTION PROTOCOLS (OLD MEDIA = CELLS WERE INFECTED IN THE SAME MEDIUM AS THEY WERE GROWN; NEW MEDIA = MEDIA EXCHANGE BEFORE INFECTION OF CELLS; ACA = ADDITION OF ANTI-CLUMPING AGENT)

As we aimed to improve our infection protocol for *Tnms*42 cells to yield a higher proportion of cells being infected, we tested the influence on old/fresh medium and the effect of the addition of anti-clumping agent on virus infectivity. As visualized in Figure 4-4, only 18% of the cells were infected at day 4 post infection, without performing a medium change. We could improve the infection by performing a medium change step before infection (45% of infected cells). However, when comparing Gag expression in cells infected without performing a medium change, the yield of Gag-protein was roughly 1.6-fold higher. When normalizing the data in respect to the infected cell population, we saw that per infected cell, roughly 2-fold more Gag protein was produced using an infection protocol without performing a medium change (Figure 4-8). About 0.45 pg Gag-protein per cell was produced after 4 days of infection, whereas only 0.25 pg Gag-protein was produced with medium change.

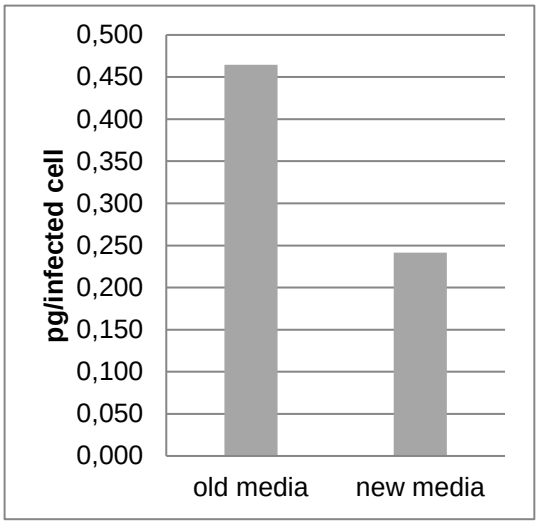


FIGURE 4-8: PG OF GAG PROTEIN PER INFECTED *TNMS*42 CELL WITH DIFFERENT CONDITIONS

4.5. H1 ELISA

HA-ELISA was performed using the commercially available HA-antibody pair (Influenza A H1N1 (swine Flu 2009) Hemagglutinin / HA ELISA) with some modifications. However, due to the fact that the hemagglutinin in the samples was present in the conformation of a trimer, a self-produced standard was developed and used. Soluble hemagglutinin containing a His-tag was produced in insect cells using the baculovirus expression system and split into two fractions. The construct was made available by members of the research group. We aimed at testing whether we could improve recombinant HA yield after His-tag purification by increasing the pH value during the binding step. Nine parts of expression supernatant (150 mL) were mixed with one part 10x PBS to slightly increase the pH from 6. to 7. We could not increase the pH to pH 8.0 (as recommended by the manual), as a precipitate was formed (probably by anorganic salts). Afterwards the protein was captured by binding to nickel-beads and was purified over a column. Finally, the protein was concentrated using Vivaspin20 tubes (cutoff: 30 kDa) and the concentration was determined with the Bradford assay and UV-VIS spectrometry and were 22.2 µg/L and 56.8 µg/L expression supernatant for the pH-adjusted and non-pH-adjusted purification respectively. The 260/280 results were in the same range as the results from the Bradford Protein Assay.

The SDS-PAGE gels and Western Blots from the purification, elution and concentration steps are shown in Figure 4-9.

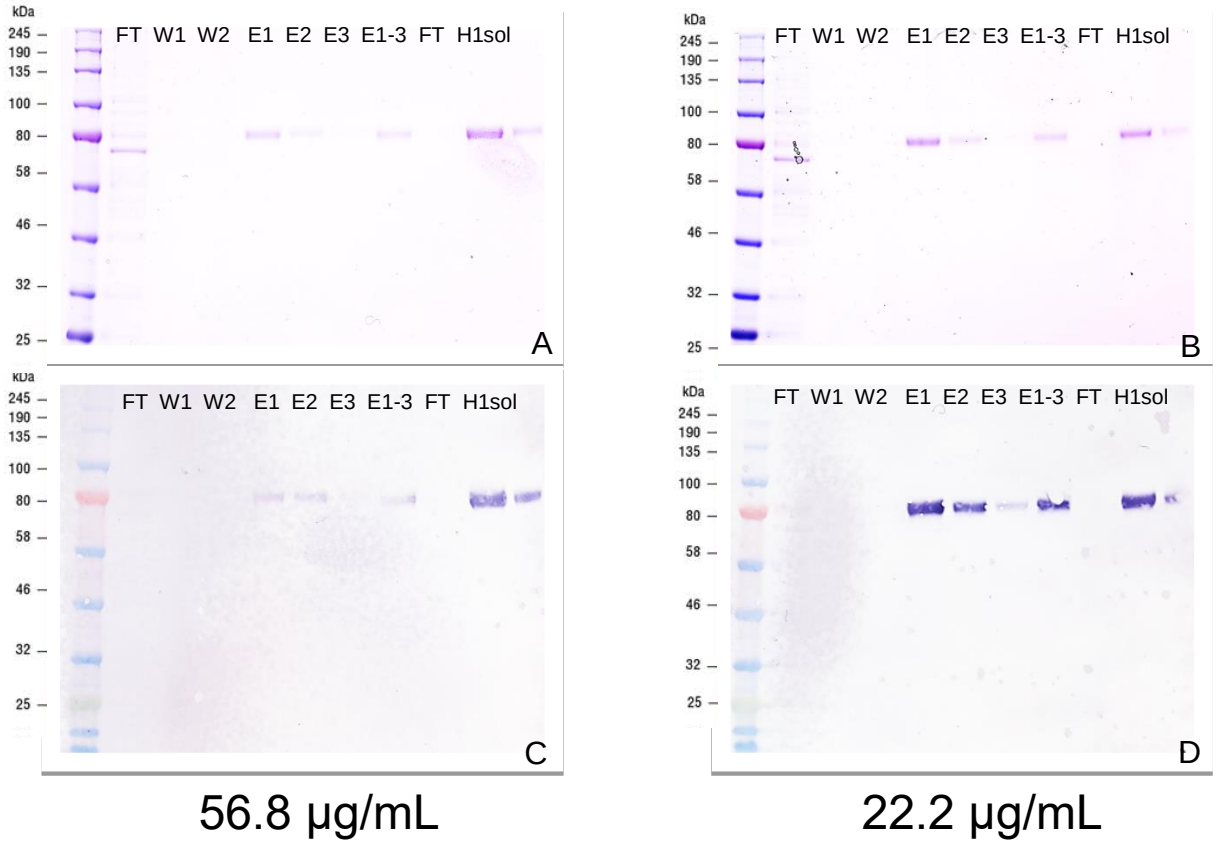


FIGURE 4-9: A AND B - SDS-GELS; C AND D - WESTERN BLOTS; A AND C – PURIFICATION WITHOUT PH ADJUSTMENT; B AND D – PURIFICATION WITH PH ADJUSTMENT (FT=FLOW THROUGH; W1=WASH STEP 1; W2= WASH STEP 2; E1= ELUTION STEP 1; E2= ELUTION STEP 2; E3= ELUTION STEP 3; FT = FLOW THROUGH; H1SOL= CONCENTRATED PROTEIN)

In the end the fraction without PBS had a higher protein concentration than the fraction with PBS. This purified soluble HA was utilized as calibration standard for the H1-ELISA.

As it was shown that quantification of HA incorporated in a virus or virus-like or even as soluble protein is highly underestimated by ELISA due to the particulate structure or the formation of rosettes respectively (Bottcher *et al*., 1999), detergent-treatments are usually employed for quantification of HA in influenza virus preparations or samples of soluble trimeric HA (Johannsen et al., 1983). We tested commonly employed methods like Triton X-100, SNCR-buffer and Zwittergent 3-14 with different incubation time and temperatures and compared them to the treatment methods we are using for the p24-ELISA. Different treatments were tested for their potency in disrupting VLPs and thereby increasing ELISA readouts.

The detergents Zwittergent 3-14 and Triton X-100 (in combination with SNCR buffer, see chapter 3-4-13) were tested and the resulting OD ratio of blank to sample was calculated (Figure 4-10). Treatment 3 (1% Zwittergent 3-14 for 30 minutes at RT) resulted in highest OD readings of the HA-Gag expression supernatant sample and gave the best blank to sample ratio of 1:27. Zwittergent ratios (Treatment 3 and 4) were 5.5-fold higher than compared to Triton X-100 (Treatment 1) and Triton X-100 in combination with SNCR-buffer (Treatment 2). Treatment 3 was used for further experiments. In order to determine if Zwittergent 3-14 interferes with the assay we tested, whether we get the same results if samples were diluted 1:10 pre or post treatment (1% Zwittergent 3-14 for 30 minutes at RT). The resulting identical curves in Figure 4-11 show that there is no interference of the detergent as the calculated concentrations of HA present in the sample are 12000 ng/mL. Moreover, it was analyzed if the treatment of the calibration standard has an impact on recoveries of back-calculated concentrations of the calibration curve. Therefore, an ELISA was performed with the soluble HA with and without Zwittergent 3-14 treatment spanning a concentration range of 40 ng/mL - 0.02 ng/mL. Figure 4-12 shows that the two standard curves are identical and that the treatment has no impact on the analyses.

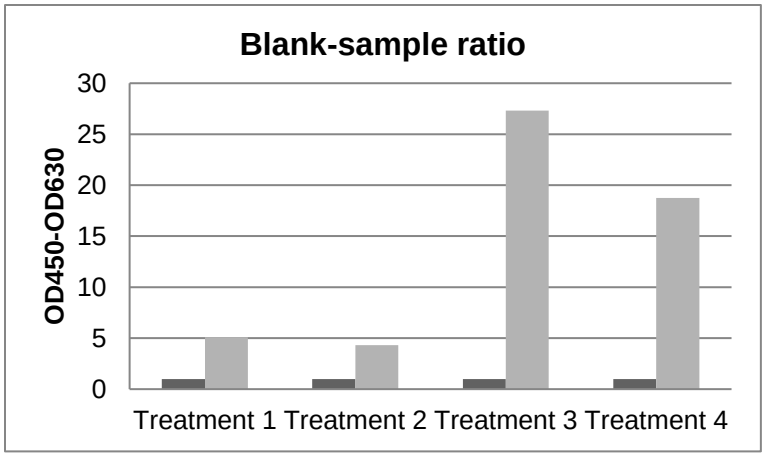


FIGURE 4-10: BLANK-SAMPLE RATIO BETWEEN DIFFERENT SAMPLE TREATMENTS; X-AXIS: BLANK AND SAMPLE TREATED IN 4 DIFFERENT WAYS; Y-AXIS: DIFFERENCE OF OD630 VAUES MINUS OD450 VALUES

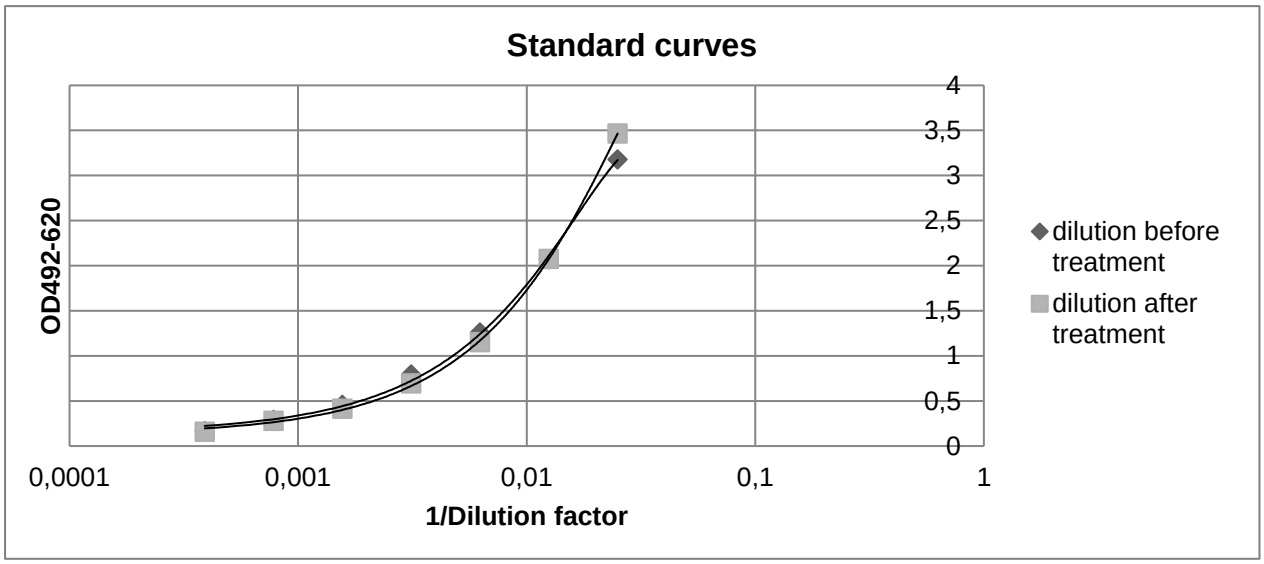


FIGURE 4-11: TWO STANDARD CURVES WITH DILUTION BEFORE AND AFTER TREATMENT WITH ZWITTERGENT 3-14; X-AXIS: 1/DILUTION FACTOR; Y-AXIS: DIFFERENCE OF OD620 VALUES MINUS OD492 VALUES

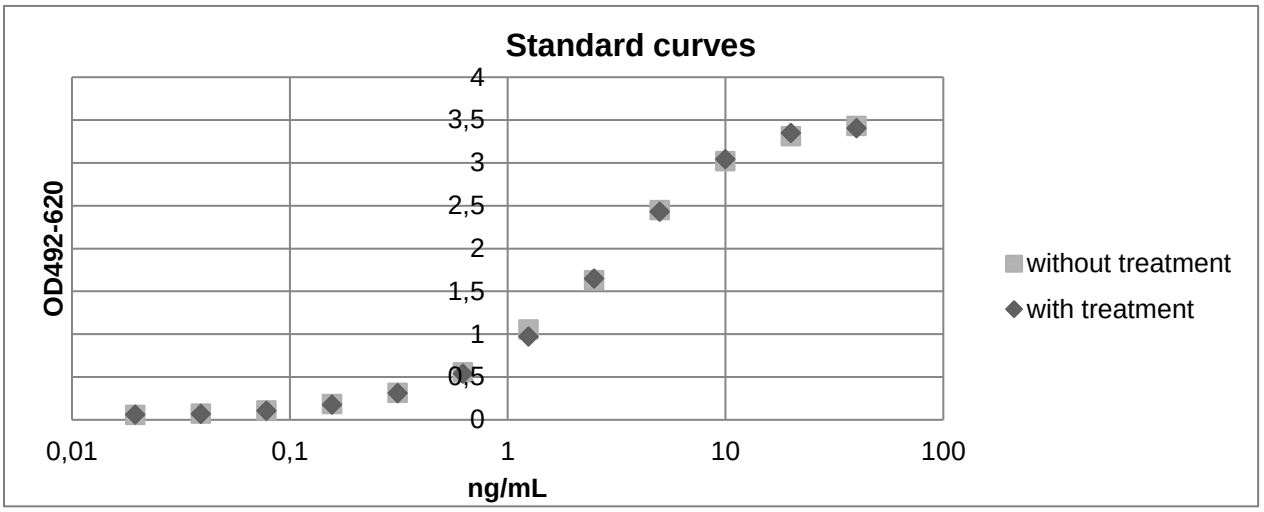


FIGURE 4-12: STANDARD CURVE WITH AND WITHOUT TREATMENT OF THE STANDARD; X-AXIS: NG/ML STANDARD; Y-AXIS: DIFFERENCE OF OD620 VALUES MINUS OD450 VALUES

TABLE 4-1: RECOVERY AND RSD OF THE STANDARD CURVES WITH AND WITHOUT TREATMENT

concentration [ng/mL]	0.005	0.010	0.020	0.039	0.078	0.156	0.313	0.625	1.25	2.5	5	10	20	40
conc. calculated without treatment [ng/mL]				0.031	0.099	0.203	0.367	0.660	1.224	2.493	4.981	11.372	24.137	35.212
Recovery (without treatment) [%]				80%	126%	130%	118%	106%	98%	100%	100%	114%	121%	88%
conc. calculated with treatment [ng/mL]				0.033	0.100	0.207	0.375	0.667	1.282	2.314	5.051	10.878	21.433	32.354
Recovery (with treatment) [%]				85%	128%	133%	120%	107%	103%	93%	101%	109%	107%	81%
RSD [%]				5%	1%	1%	2%	1%	3%	5%	1%	3%	8%	6%

Furthermore, different concentrations of the capture and detection antibody were tested and the resulting OD ratio of blank to sample was calculated (Figure 4-12). Condition 1 resulted in the highest OD readings of the HA-Gag expression supernatant sample and gave the best blank to sample ratio of 1:7. The dynamic range of the HA-ELISA is from 0.0781 ng/mL to 10 ng/mL HA, the upper limit of quantification is at 20 ng/mL HA because there the recoveries start to be off the 80%-120% limit (Table 4-1). After establishing the conditions for the H1-ELISA the samples Gag+H1 and Gag+H1/H1 were analyzed, in order to determine if we see an increase in HA yield when having a second HA expression cassette on the baculovirus. The samples were diluted 1:8 to fall within the dynamic range of the HA-ELISA. Figure 4-13 shows that we find 3-fold more HA in the supernatant when having a second HA expression cassette on the virus (435 ng/mL versus 1201 ng/mL).

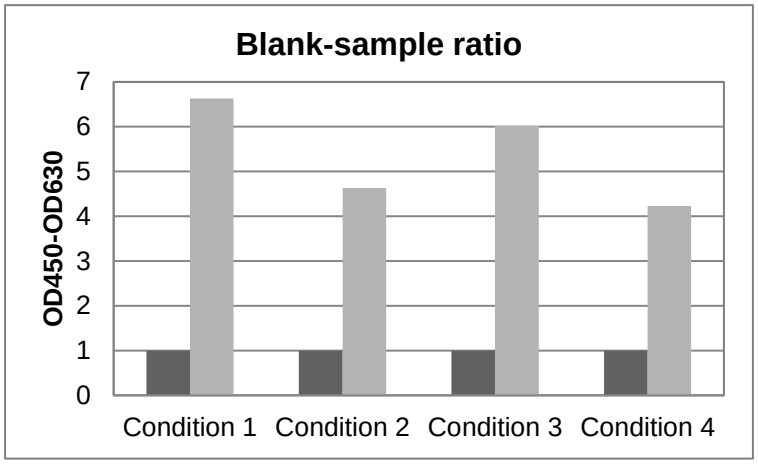


FIGURE 4-12: BLANK-SAMPLE RATIO BETWEEN DIFFERENT ANTIBODY CONCENTRATIONS

Assuming that there is no baculovirus, containing HA incorporated into its virion, in the expression supernatant this would translate to an almost 3-fold higher abundance of H1 HA on VLPs expressed using the baculovirus with two H1 expression cassettes. The results suggest that more H1 protein was produced and displayed on the VLP surface using two expression cassettes. As already mentioned a fraction of the HA may be incorporated onto baculovirus virions present in the supernatant but we do not know to which degree. It would be of interest if there whether we could still increase the HA incorporation in HA-Gag VLPs by including further HA expression cassettes. At this point, however, we do not know if it is desirable to have a higher abundance of HA on the VLP surface or whether this hinders accessibility to certain HA epitopes and therefore decrease antigenicity when administered as vaccine.

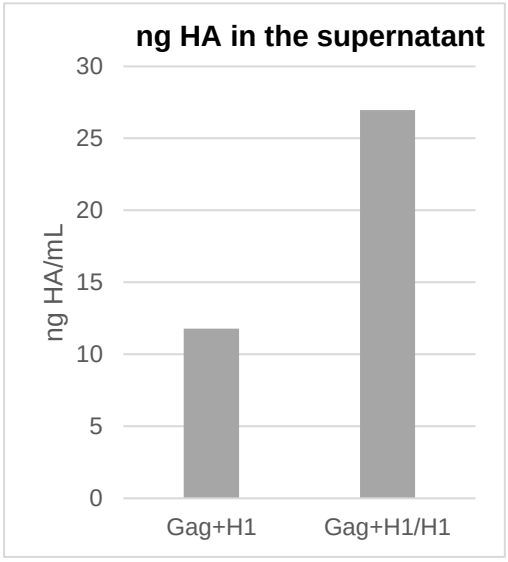


FIGURE 4-13: AMOUNT OF H1 TRIMERS ON THE SURFACE OF ONE VLP

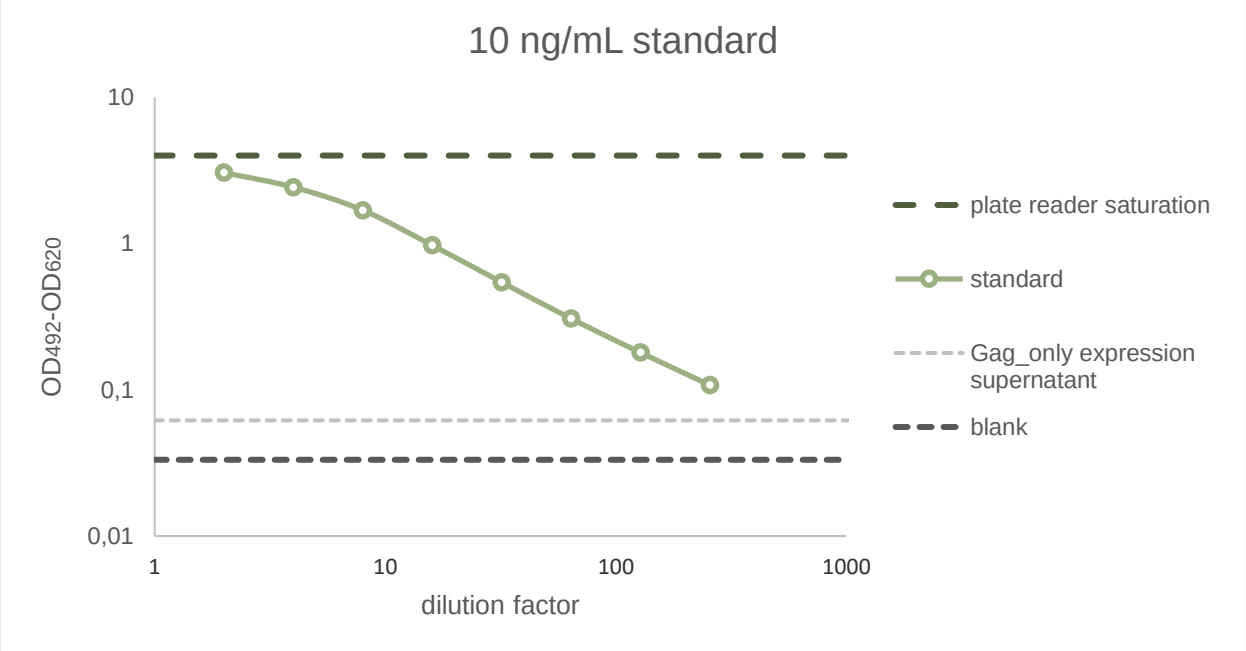


FIGURE 4-14: STANDARD CURVE OF THE APPLIED HA-ELISA STARTING WITH A CONCENTRATION OF 10 NG/ML; GAG_ONLY EXPRESSION SUPERNATANT WAS USED AS NEGATIVE CONTROLX-AXIS: 1/DILUTION FACTOR; Y-AXIS: DIFFERENCE OF OD492 VALUES MINUS OD620 VALUES

4.6. Ultracentrifugation

VLPs were generated by infection of *Sf*9 and *Tnms*42 cells with recombinant baculovirus encoding HIV-1 Gag and Hemagglutinin H1 or H3. As a control, cells were infected with a baculovirus, solely expressing HIV-1 Gag matrix protein, resulting in VLPs lacking H1/H3 on the surface. VLPs were harvested from supernatant of infected cells by ultracentrifugation and were taken up in a small volume (500 µL) to concentrate the samples. Subsequently, sucrose density gradient ultra-centrifugation was performed to remove viral impurities, cell debris or bigger aggregates. Baculoviruses were removed partially because of different weight and size compared to the VLPs. The gradients were separated into 12 fractions each and were analyzed by Western Blotting.

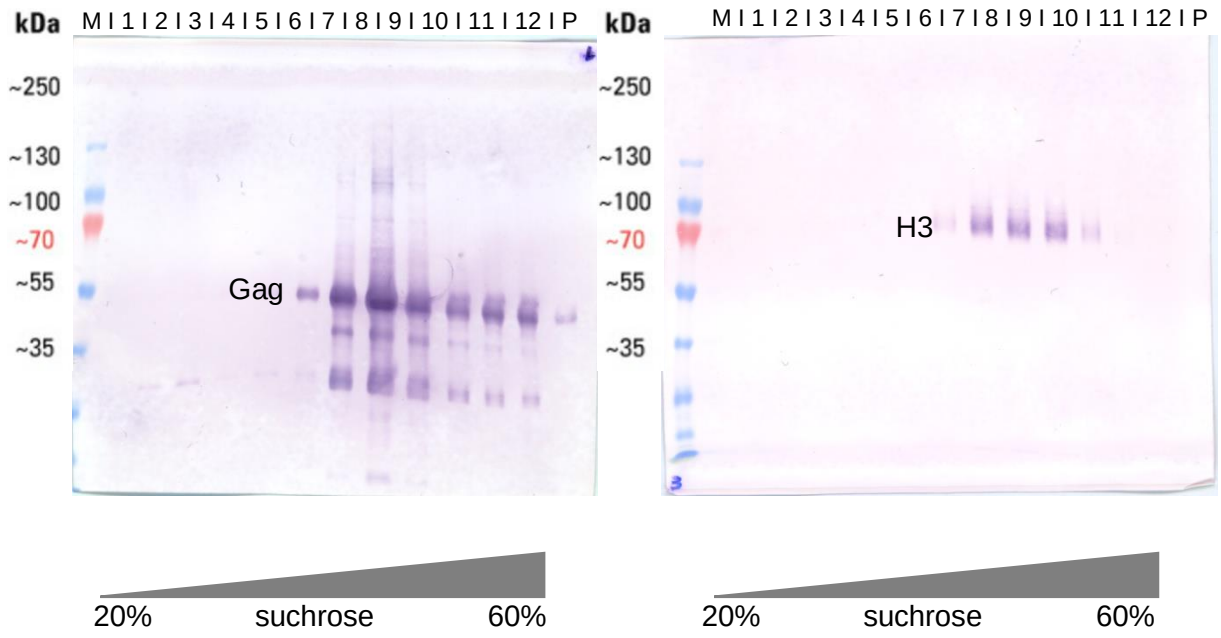


FIGURE 4-15: WESTERN BLOTS OF SUCROSE GRADIENT FRACTIONS AFTER ULTRACENTRIFUGATION. LEFT - *SF*9 GAG_ONLY; RIGHT - GAG+H3; M: PAGERULER™ PRESTAINED PROTEIN LADDER USED AS MASSRULER; 1-12: FRACTIONS OF THE SUCROSE GRADIENT; P: PELLET OF SUCROSE GRADIENT

In figure 4-15 the Western Blots of the sucrose gradient fractions are shown. On the left blot, the matrix protein HIV-1 Gag is visualized at a size of around 55 kDa. Hemagglutinin H3 is visible on the right blot at around 60 kDa. H3 protein was present in sucrose gradient fractions 8 - 10. In contrast, HIV-1 Gag protein was present in fractions 5 - 12 and in the pellet. That indicates that a higher amount of Gag protein was produced, resulting in more intense signals on the Western Blot. Fractions containing the most VLP material (Gag only: fraction 7-9, Gag+H3: fraction 8-10) were pooled to reduce the sucrose concentration. High sucrose concentrations are not desirable for further analysis. The resulting pellets were taken up in appropriate amounts of HEPES buffer and were further examined for their particle size, VLP concentration, homogeneity and purity.

Alternatively, to the sucrose gradient, ultracentrifugation with sucrose bed could be performed. This way VLPs are decelerated or stopped before crashing against the ultracentrifugation tube and damage of the particles could be reduced. A sucrose bed with appropriate sucrose density could probably also separate impurities and baculovirus particles from VLPs. This method is less labor intensive compared to the sucrose gradient centrifugation, although dialysis needs to be performed afterwards to remove sucrose. In general, it is very difficult to separate baculovirus particles from VLPs due to their similar surface protein composition and size. For a higher purification grade, more sophisticated methods including separation via chromatographical methods (Steppert *et al*., 2016) must be applied.

4.7. Zetasizer Malvern

In order to determine the homogeneity and the VLP mean diameter the samples were analyzed by dynamic light scattering (DLS) using a Zetasizer Nano-ZS. This technique measures the diffusion of particles moving under Brownian motion and converts this to size distribution using the Stokes-Einstein relationship. A single and narrow peak and a polydispersity index (PDI) lower than 0.2 indicate homogeneity and a low amount of impurities. The samples in figure 4-16 showed a peak at around 200 nm reflecting the size of HIV-1 VLPs of 100 - 200 nm (Steppert *et al*., 2016).

*Sf*9 *Tnms*42

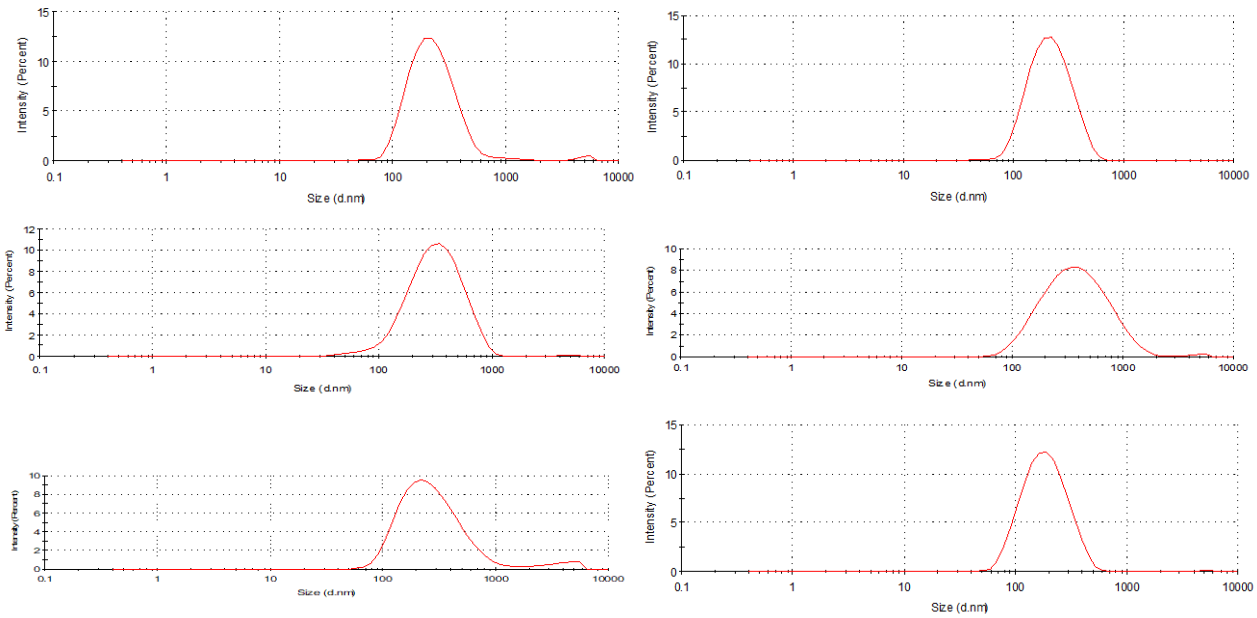


FIGURE 4-16: ZETASIZER-RESULTS FROM TOP TO THE BOTTOM: GAGONLY; GAG+H1/H1; GAG+H3; X-AXIS: SIZE OF THE PARTICLES (D.NM); Y-AXIY: INTENSITY (PERCENT)

Some samples showed an additional peak at higher size indicating VLP aggregates. In general, slight differences between the VLP preparations can be seen, which might result from the different fractions that were pooled after sucrose gradient purification. Fractions closer to the bottom of the tube contain impurities of bigger size including cell debris and aggregates compared to fractions closer to the top of the tube. All samples purified by sucrose gradient had a polydispersity index lower than 0.2. Moreover, no great differences between the VLP preparations in different cell lines were obtained.

Additionally, VLP preparations that were not purified by sucrose gradient ultracentrifugation were analyzed using the Zetasizer. These samples were not evaluable because of strong inhomogeneity, which implies the importance of a sucrose gradient purification to remove cell debris, aggregates or other impurities.

4.8. NanoSight

The NanoSight LM-10 uses Nanoparticle Tracking Analysis in order to obtain size distribution and concentration of nanoparticles in suspension. Serial dilutions of the samples were measured and the average particle concentration per mL was calculated. To determine the concentration of VLPs, particles with a diameter between 100 and 200 nm were gated. A comparison between the H1 and H3 constructs and the *Sf*9 and *Tnms*42 cell lines was not possible as corresponding VLPs were previously purified using different methods. Moreover, no double or triple determination was made to ensure statistical accuracy. The number of particles per mL with a diameter between 100 - 200 nm are listed in Table 4-2 and were in a range of around 2 x 10^11 particles/ml, except for *Sf*9 Gag+H3 where the concentration was lower with 0.42 x 10^11 and for *Sf*9 Gag_only where the concentration was a bit higher with 6.39 x 10^11 VLPs/mL.

TABLE 4-2: NANOSIGHT RESULTS; CONCENTRATION OF VLPS PER ML

Sample name	Concentration VLPs/mL
*Sf*9 Gag_only	6.39 x 10^11
*Sf*9 Gag+H1/H1	2.19 x 10^11
*Sf*9 Gag+H3	0.42 x 10^11
*Tnms*42 Gagonly	1.97 x 10^11
*Tnms*42 Gag+H1/H1	1.19 x 10^11
*Tnms*42 Gag+H3	2.35 x 10^11

4.9. Transmission Electron Microscopy (TEM)

To confirm the presence of budded VLPs and to investigate their size and morphology, sucrose purified VLP preparations were analyzed by transmission electron microscopy. Both, Gag_only VLPs and H1/H1 VLPs appeared spherical and ranged in a diameter size between 100 - 200 nm (figure 4-18). As the overall diameter of the VLPs usually depends on the size of the incorporated matrix protein, present VLPs are a bit bigger than wild-type influenza VLPs that are in the range of 80 - 120 nm (Krammer *et al*., 2010). Moreover, it can be seen that the VLPs were hollow inside and that they consisted of two layers. The inner comprising of the self-assembled Gag protein and the outer derived from the insect cell membrane through the budding process. The darker regions around the VLPs (Figure 4-17, right) could be the incorporated hemagglutinin. In both VLP preparations also rod shaped baculovirus particles are visible (Figure 4-17). The cloud like structures at the end of the rods could be comprised of the major baculovirus envelope protein gp64 (Wang *et al*., 2016). The baculovirus is double as big as the VLPs with a length of 300 nm.

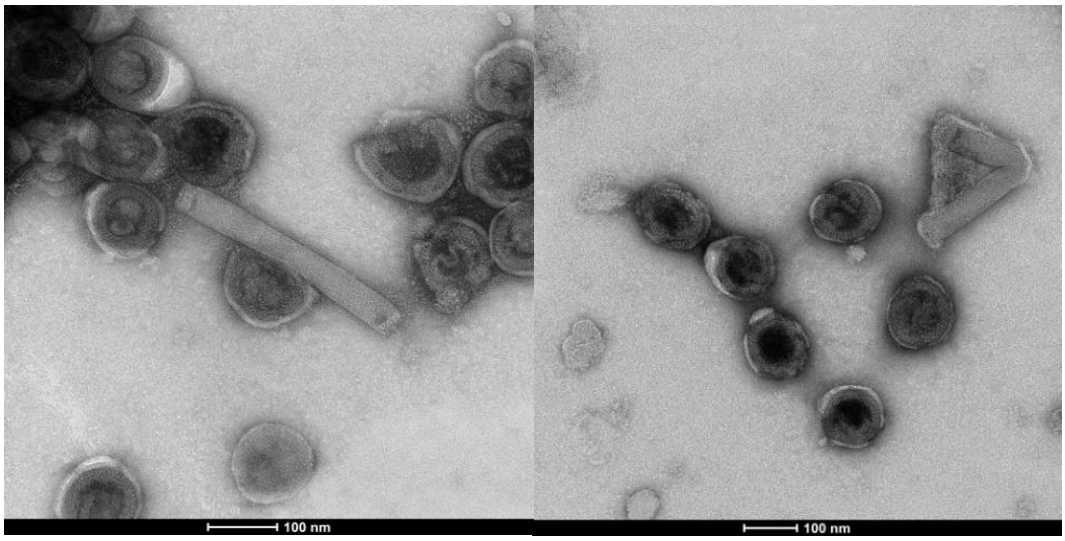


FIGURE 4-17: IMAGE OF TEM MEASUREMENT OF *SF*9 GAGONLY (LEFT) AND *SF*9 GAG+H1/H1 (RIGHT)

5. CONCLUSION

The aim of this study was to compare the cell lines *Spodoptera frugiperda Sf*9 and *Tnms*42 cells derived from the *Trichopulsia ni* BTI-TN5B1-4 "HighFive™ in terms of VLP production and protein surface display. VLPs consisted of the HIV-1 Gag-protein and the influenza hemagglutinin. The results from the HIV-1 p24 ELISA revealed that *Tnms*42 are advantageous for the production of protein displaying VLPs because of higher expression rate per infected cell as compared to *Sf*9 cells. No differences in terms of quality of the VLPs could be observed during analysis with different methods. However, the main bottleneck of *Tnms*42 cells turned out to be the variable degree of infection efficiency we obtained during our experiments. To quantify the amount of HA in the expression supernatant, a commercially available H1 A/California/4/2009 (H1N1) ELISA was modified to suit our applications. A recombinant secreted H1 was produced with the baculovirus expression system in order to have a standard that resembles the conformation of the H1 trimers present on the VLP surface. We found 11.7 ng/mL HA in the expression supernatants of *Tnms*42 cells. Supposing that there are only VLPs present in our expression supernatant, this would mean that we have around 400 H1 trimers displayed on one VLP using the Gag+H1 expression construct. The Gag+H1/H1 construct contained a second HA expression cassette and therefore, produced more H1 protein resulting in about 1200 H1 trimers per VLP (27 ng/mL expression supernatant of *Tnms*42). The VLPs were purified with a sucrose gradient ultracentrifugation and subsequently concentrated. Afterwards analysis with the Malvern Zetasizer was performed whereby increased purity of the samples could be confirmed. TEM images showed that VLPs in the right size range (100-200 nm) had been produced. However also baculoviruses were present which indicated that baculovirus could not completely be removed using sucrose gradient ultracentrifugation. Further research should be conducted in the separation of baculovirus from the VLPs or inactivation has to be applied. Moreover, the limit of the expression machinery of the cell and the most efficient amount of hemagglutinin present on the VLP surface regarding immunogenicity should be determined. Therefore, constructs with a different number of pACEBac1_H1 sequences should be generated and with the resulting VLPs mouse studies could be performed.

6. APPENDIX

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