UNIVERSIDADE NOVA DE LISBOA

EXPLORING INSECT CELLS VERSATILITY FOR PRODUCTION OF INFLUENZA VIRUS-LIKE PARTICLES

DANIELA FILIPA POLICARPO SEQUEIRA

THESIS SUMBITTED FOR GRANTING OF THE DEGREE OF MASTER IN MEDICAL MICROBIOLOGY

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Abstract

A potential strategy to produce safer and broadly protective influenza vaccines is to co-express, in the same cell host, multiple hemagglutinins (HA) with a matrix protein (M1) which self-assemble in virus-like particles (VLPs). This study demonstrates the suitability of combining stable expression and the baculovirus-expression vector system (BEVs) in insect Hi5 cells for production of such multi-HA Influenza VLPs. Stable pools of Hi5 cells expressing two HAs were generated and later infected with a M1-encoding baculovirus at two cell concentrations (CCIs; 2×10^6 cells/mL and 3×10^6 cells/mL). The HA concentration in culture supernatant was followed over time, with more productive infections observed at higher CCIs. To extend the culture time, a re-feed strategy was implemented based on the identification of key nutrients which were exhausted during cell growth. Afterwards, supplemented cultures infected at a CCI of 4×10^6 cells/mL allowed a 4-fold increase in HA concentration, at harvest, when compared to cultures infected at a CCI of 2×10^6 cells/mL. The production of multi-HA influenza VLPs using the aforementioned strategy could be successfully scaled-up to 2L bioreactor cultures with even higher volumetric (1.5-fold) HA yields.

To surpass the unpredictability of gene expression promoted by the random integration strategy mentioned above, the recombinase-mediated cassette exchange (RMCE) technology was explored. The feasibility of having two cassettes flanked by distinct pairs of flippase recognition target sites (FRTs) was evaluated. Unfortunately, significant cross-interaction was observed between the selected pairs. To circumvent this bottleneck, a backup strategy consisting in the co-expression of two genes from the same locus after implementation of one cassette system, in insect Sf9 cells, was attempted. However, the isolated clones showed low expression of both M1 and HA proteins. Ongoing work focuses on the isolation of clones tagged in high expression loci by fluorescence activated cell sorter technology.

This work demonstrates how the versatility of insect cell expression technology can be explored to produce Influenza VLPs as vaccine candidates.

Keywords: Influenza vaccines; virus-like particles (VLPs); multivalent HA vaccines; insect cells; BEVS; RMCE.

Resumo

A co-expressão de várias hemaglutininas (HA) e proteína da matriz (M1), no mesmo hospedeiro, formando partículas semelhantes a vírus (VLPs), constitui uma importante estratégia para desenvolver vacinas contra o vírus da gripe. Este trabalho mostra a combinação de uma linha celular estável de células de insecto com o sistema de expressão mediada por baculovírus para a produção deste tipo de VLPs. Foram estabelecidas duas populações de células de insecto Hi5, expressando duas HAs, posteriormente infectadas com um baculovírus contendo a proteína M1, a duas concentrações celulares diferentes (CCI; 2 e 3×10^6 cells/mL) sendo que a mais elevada demostrou ser a mais produtiva. De seguida, implementou-se uma estratégia baseada na adição de nutrientes específicos para prolongar o tempo de cultura. As culturas previamente suplementadas e infectadas a uma CCI de 4×10^6 células/mL produziram 4x mais HA comparativamente às culturas infectadas a uma CCI de 2×10^6 células/mL, não suplementadas. Esta estratégia foi também aplicada num biorreactor de 2L permitindo 1,5x mais produção, volumétrica, de HA comparativamente a experiências em pequena escala.

De forma a ultrapassar a imprevisibilidade de uma integração aleatória, foi explorado o sistema de troca de cassete mediado por recombinase (RMCE). A viabilidade de um sistema com duas cassetes integradas flanqueadas por diferentes locais de reconhecimento (FRTs) foi avaliada, tendo sido observada a interação entre ambos os pares selecionados. Como segunda estratégia, foi implementado um sistema com uma cassete para co-expressão de dois genes em simultâneo, em células de insecto Sf9. Porém, os clones isolados mostram fraca expressão de M1 e HA, pelo que uma estratégia de isolamento de clones com expressão génica mais forte está em desenvolvimento utilizando uma tecnologia de sorteamento.

Assim, este trabalho demonstra a versatilidade da tecnologia aplicada em células de insecto, que pode ser explorada para produzir VLPs multivalentes, com potencial para se tornar a próxima geração de vacinas para o vírus da gripe.

Palavras-chave: Vacinas para a; partículas semelhantes a vírus (VLPs); vacinas de HA (hemaglutinina) multivalentes; células de insecto; sistema de expressão mediada por baculovírus; sistema de troca de cassete mediada por recombinase.

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List of Acronyms

AcMNPV	*Autographa californica* multicapsid nucleopolyhedrovirus
Asn	Asparagine
BEVS	Baculovirus expression vector system
BVs	Budded virions
CCI	Cell concentration at infection
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DSBs	Double strand-breaks
DSP	Downstream processing
ECL	Enhanced chemiluminescence
eGFP	Enhanced green fluorescent protein
FBS	Fetal bovine serum
Flp	Flippase
FRT	Flippase recognition target site
Glc	Glucose
Gln	Glutamine
GOI	Gene-of-interest
HA	Hemagglutinin
hFlpe	Humanized Flpe
HR	Homologous recombination
HRP	Horseradish peroxidase
iFlp	Insect cells codon-optimized flippase
IR	Illegitimate recombination
MDCK	Madin-Darby Canine Kidney cells
MTT	Thiazolyl blue tetrazolium bromide solution
NA	Neuraminidase
NEP	Nuclear export protein
NP	Nucleocapsid protein
ODVs	Occlusion-derived virions

PBS	Phosphate buffered saline
PDT	Population doubling time
PTMs	Post-translation modifications
rBACs	Recombinant baculovirus
RBC	Red blood cells
RdRp	RNA-dependent RNA polymerase complex
recHA	Recombinant hemagglutinin
recNA	Recombinant neuraminidase
RMCE	Recombinase-mediated cassette exchange
SSR	Site-specific recombinases
TALENs	Transcription activator-like effector nucleases
TIVs	Trivalent inactivated influenza vaccines
TTBS	Tween tris buffered saline
VLPs	Virus-like particles
vRNPs	Viral ribonucleoproteins
ZFNs	Zinc-finger nucleases

1 Introduction

1.1 Influenza virus

Influenza viruses are responsible for annual epidemics and, occasionally, pandemics, responsible for acute febrile respiratory tract disease commonly known as "flu". They belong to *Orthomyxoviridae* family and are divided into three genera (A, B and C), being genus A the most threatening due to its potential to cause global pandemics (Lowen et al., 2007; Steinhauer and Skehel, 2002). Influenza A viruses are divided into several subtypes depending on their surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Eighteen different HA subtypes and eleven different NA subtypes have been characterized so far, but only H1, H2, H3, N1 and N2 subtypes have been found to cause human pandemics (CDC, 2014).

Influenza viruses are enveloped containing a segmented, negative single stranded RNA genome and bud from the apical domain of epithelial cells. They have a standard nomenclature which includes the virus type, the species from which it was isolated (if not human), respective location, strain number and year of isolation as well as the hemagglutinin (HA) and neuraminidase (NA) subtype in case of influenza A viruses. Subdivision can also be done into serotypes in terms of antibody responses. The genome contains 8 ssRNA segments, existing as ribonucleoproteins (vRNPs), that can code for 11 proteins, including the M1 matrix protein (bridge between the envelope and the viral core) and the surface glycoproteins HA and NA (virus envelope) (Nayak et al., 2004). The viral core also contains the nucleocapsid protein (NP), the nuclear export protein (NEP) and three different polymerase proteins, PB1, PB2 and PA forming the RNA-dependent RNA polymerase complex (RdRp). The envelope is also composed by M2 ion channel plus host cell's lipids (Nayak et al., 2004).

The replication cycle of influenza viruses begins with the viral recognition and subsequent binding to the *N*-acetylneuraminic (sialic) acids of host's surface, preferentially α-2,3- or α-2,6-carbon linkages (Figure 1). After the binding step, internalization of virus particles occurs via receptor-mediated endocytosis. HA is cleaved by internal proteases and

in the acidic environment of the endosome, cleaved HA undergoes conformational changes leading to the fusion of viral and endosomal membranes (Steinhauer, 1999). M2 ion channel opens and allows the release of vRNPs from M1 into the cytoplasm. After this, eight vRNPs that include NP-nuclear transport signals are imported to the nucleus through nuclear pores (Neumann et al., 2000).

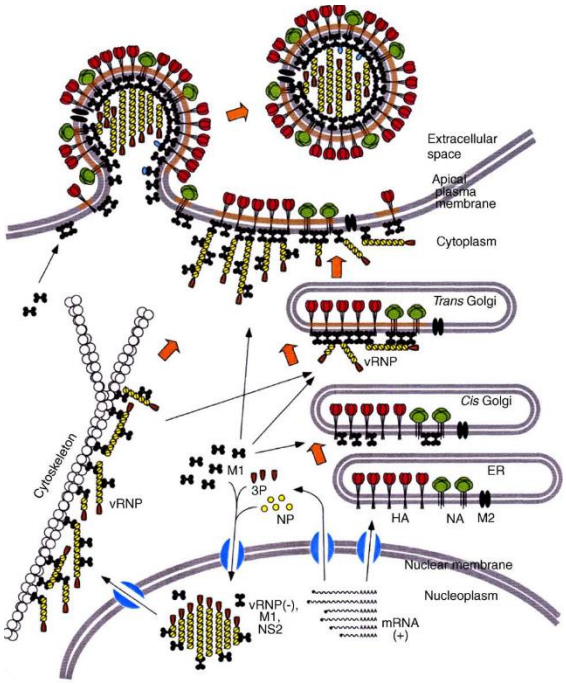

Figure 1 - Replication cycle of Influenza virus (Nayak et al., 2004).

After DNA replication and translation, 11 viral proteins are produced of which HA, NA and M2 undergo post-translation modifications (PTMs) in the *cis*-Golgi apparatus and rough endoplasmic reticulum (Nayak et al., 2004). These glycoproteins are then transported to the budding site together with eight vRNPs and other viral proteins in order to form virions. Budding occurs with the involvement of host and viral components, being HA, NA and M1 key players in this process. M1 is responsible for the encapsidation of the vRNPs into the membrane and for the budding process. On the other hand, NA plays a critical role in the release of the viral particles due to its syalidase activity, cleaving the binding of HA to host sialic acids (Nayak et al., 2009).

In human respiratory epithelium, α-2,6-carbon linkages are more abundant than α-2,3-carbon linkages. Due to the existence of α-2,3-carbon linkages in duck gut epithelium humans can be infected by avian influenza virus. When this transmission occurs, it leads to a more severe infection because α-2,3-linkages are more prevalent in the lower respiratory tract (e.g. lungs) (Couceiro et al., 1993; Matrosovich et al., 2004). Pigs contain both carbon linkages, meaning they can be infected by avian and human strains. In case such double infection occurs, strains may undergo reassortments and a novel strain capable of infecting humans is generated. On the other hand, different strains within the same subtype can also reassort and thus generate a new strain in a phenomenon named antigenic shift. The infections arising from such reassortments are usually severe because people are not immunized against the new strain, as it was the case of 2009 H1N1 pandemics (Steinhauer and Skehel, 2002). In addition, natural mutations can occur during viral genome replication due to errors in the RdRp polymerase enzymes leading to antigenic drift of a given strain (Steinhauer and Skehel, 2002). This is very likely to occur in influenza viruses because their polymerase enzymes do not perform proofreading as they lack a 3'-5' exonuclease activity that would enable them to repair small errors during DNA replication. Regardless of being minor changes, these mutations can lead to a loss of immunogenicity and thus are held responsible for the renewal of influenza vaccines annually (Steinhauer and Skehel, 2002).

1.1.1 Egg-based influenza vaccines

The market of Influenza vaccines were estimated at $2.9 billion in 2011 and thought to accomplish $3.8 billion by 2018 (Conferences series, 2015).

The most commonly used platform for production of influenza vaccines is hen's eggs. Production starts by infecting the allantoic fluid of the eggs with influenza viruses. After several rounds of replication, virions are harvested and chemically inactivated (e.g. with formaldehyde) or attenuated (e.g. serial passages at sub-optimal conditions). From this process, a whole virion preparation, a split vaccine or either a subunit vaccine can be achieved (Cox et al., 2008). Due to the fact that HA is the key surface glycoprotein in influenza viruses, triggering an immune response, its presence in a vaccine against influenza infection is

essential. Trivalent inactivated influenza vaccines (TIVs), composed by two influenza A viruses (H1N1 and H3N2) and one influenza B virus, are produced with this system. They are standardized in order to contain the same amount of HA of each virus strain, being the most commercialized influenza vaccine (Cox et al., 2008). However, this platform is very laborious, time consuming and costly, requiring large numbers of chicken eggs to produce one shot of vaccine and up to 9 months of production time. Besides this, the presence of eggs' proteins can trigger allergies in humans which leads to an impairment of biosafety (Zeiger, 2002). In addition, the ability of some strains to replicate to high yields in hen's eggs is unpredictable and when dealing with a very pathogenic strain the embryos can be killed without producing any virus.

1.1.2 Cell-based influenza vaccines

Mammalian cell lines represent today a robust platform for influenza vaccine production. First results on the effectiveness of continuous cell lines, such as Madin-Darby Canine Kidney cells (MDCK) on influenza virus replication were reported 40 years ago (Meguro et al., 1979; Tobita et al., 1975), providing evidence that mammalian cells could represent a robust platform for influenza vaccine production. A few years later, an inactivated influenza vaccine produced in MDCK cells showed to be more efficient in neutralizing antibody induction in ferrets than egg-grown vaccine (Katz and Webster, 1989). This culminated with the recent FDA approval of Flucelvax (Novartis, 2015), a trivalent inactivated influenza vaccine manufactured using MDCK cell culture technology.

Another continuous cell line used for influenza vaccines production is Vero cells. Vero cells are the most widely accepted continuous cell line by regulatory authorities and have been used for the production of viral vaccines such as for polio and rabies virus (Montagnon, 1989). They enable higher-titer growth of wild-type H5N1 strains (Barrett et al., 2009), which in case of a pandemic is of great importance particularly if a short supply of eggs occur or if the embryos are killed by the highly pathogenic virus strain. Although continuous cell lines like MDCK and Vero have raised some safety questions due to their potential oncogenic properties, regulatory authorities are becoming more receptive given the

improved screening technologies to analyse their biosafety. The use of cell-culture grown virus proved to be efficient for influenza vaccine production in a short period of time and with higher antigens yields as well as being capable of inducing neutralizing antibodies (Ehrlich et al., 2008; Kistner et al., 2007).

Despite having several advantages over the egg-based platform, MDCK and Vero cells still have their downsides. They can be transformed over several passages, have oncogenic potential and require a solid matrix to support their growth in bioreactors. The human cell line – PER.C6 (derived from primary cultures of human fetal retinoblast)– has also been showing to be efficient in producing high titers of influenza virus of a variety of subtypes (Pau et al., 2001). The advantage of this cell line in relation to MDCK or Vero is its ability to grow to high cell densities in suspension culture without the need for serum or solid matrix.

Overall, despite the advantages of the cell-based platform for production of influenza vaccines (e.g. higher titers of antigen in a short period of time), isolation of the virus is still required thus leading to the need for biosafety laboratory conditions. Besides this, inactivation or attenuation of the offspring also represents a major shortcoming. Furthermore, adaptation of the virus strains can occur during virus propagation, which can lead to a lower antigenicity of the vaccine.

1.1.3 Subunit vaccines

Recombinant influenza vaccines

Given the downsides of egg-based and cell-based influenza vaccines, efforts have been conducted into the development of safer and more flexible vaccine candidates profiting from recombinant DNA technology.

Recombinant hemagglutinins (recHA) have been shown to be highly immunogenic, inducing the production of broadly reactive neutralizing antibodies representing a potential vaccine candidate against influenza virus infection. One example is FluBlok (Protein Sciences Corporation), which contains three full-length recombinant HA proteins, two from influenza A virus (H1N1 and H3N2) and one from influenza B virus and it was the first recombinant protein based influenza vaccine, approved by FDA in 2013 (Corporation, 2015).

The strains included in this vaccine are updated on an annual basis so that it resembles as much as possible the circulating strains thus leading to a more efficient immunization. Also, it contains three times the amount of HA in the TIVs, thus inducing higher antibody titers and has proved to be immunogenic and well tolerated (Cox et al., 2008). Besides this, it is safer because it is a purified antigen free of host or other viral proteins (Cox et al., 2008; Cox and Anderson, 2007). More recently, it was shown that a specific region of hemagglutinin – the stem region – can be recognized by antibodies and is able to stimulate cross-reactive immunization leading to protection against many H1 subtype influenza strains in mice (Yassine et al., 2015).

Neuraminidase, the second most abundant envelope glycoprotein of influenza viruses, naturally forms tetramers and helps in the release of virions from cells. However, after challenge with recombinant neuraminidase (recNA) in mice, immune protection was only shown when coupled with adjuvants, and clinical trials in humans showed no significant vaccination effect with this antigen (Cox, 2008). That being said, a recNA-based vaccine does not represent a good alternative on its own.

Virus-like particles

Virus-like particles (VLPs) are protein structures that self-assemble naturally, mimicking the structure of a native virion lacking the viral genome which is a major advantage in terms of biosafety for implementation as human vaccines. Consequently, several types of VLPs from enveloped and non-enveloped viruses have been explored to become vaccine candidates (Crisci et al., 2012; Kushnir et al., 2012).

Numerous studies have addressed the immunogenicity of VLPs as vaccines reporting their efficacy in mice and in humans (Klausberger et al., 2014; Krammer and Grabherr, 2010). VLPs can be a more effective strategy to induce immunity over inactivated virions (Bright et al., 2007) because during the inactivation process native epitopes lose their folding thus decreasing their ability to stimulate a strong immune response. For example, Cervarix™ (GlaxoSmithKline) is a VLP-based vaccine approved by the FDA for vaccination of women

against cervical cancer (Monie et al., 2008). It is composed by two viral proteins of human papillomavirus and produced using the insect cells-baculovirus system.

The use of influenza VLPs as vaccine candidates against influenza virus infection has been widely explored. Several reports exist today showing the efficacy of influenza VLPs in generating immune responses in mice after lethal virus challenges (Galarza et al., 2005; Pushko et al., 2005; Quan et al., 2007). Influenza VLPs are traditionally composed by the four major influenza proteins (HA, NA, M1 and M2) (Latham and Galarza, 2001) and their morphology resembles the native influenza virus with spikes on the surface and sizes between 80-120nm (Pushko et al., 2005; Quan et al., 2007). However, it was found that HA and M1 combined are sufficient to generate well assembled and functional VLPs with immunogenic properties (Quan et al., 2008). It was reported that M1 has the ability to colocalize with HA during its exocytic transport to the membrane and in the membrane (Ali et al., 2000; Barman et al., 2001) by association with its cytoplasmic tail and transmembrane domain (Chen et al., 2007). This protein is involved in the budding as it accommodates beneath the lipid bilayer interacting with it, causing its asymmetry and bending, facilitating the initiation of the budding process. However, it was also suggested that it may take a certain amount of M1, like a threshold, for the budding to occur (Bourmakina and García-Sastre, 2005). Depending on the diversity of proteins found in an influenza VLP, it can be monovalent or multivalent. Sometimes a monovalent VLP may not be enough to counteract a disease and there is the need for a multivalent (Pushko et al., 2011). For example, authors showed that a bivalent influenza VLP induced immunity against two viral strains decreasing the viral titers in the lungs (Quan et al., 2008) demonstrating that VLPs are a flexible way of producing candidate vaccines for specific and correlated virus strains.

One major concern when producing influenza VLPs is that protein post-translation modifications (PTMs) such as glycosylation and sialylation resemble as much as possible the *in vivo* pattern of the native product as they deeply affect biological functionality and antigenicity. It is known that insect cells do not have the same glycosylation pattern as humans cells (Marchal and Jarvis, 2001). Therefore, several studies have been conducted to address the functionality of influenza VLPs produced in insect cells (Bright et al., 2007; Pushko et al., 2005; Quan et al., 2007) and results are clearly positive suggesting that insect

cells can perform as good as mammalian cells. Several influenza VLPs produced in insect cells are already being subjected to preclinical trials (Table 1).

The fact that well assembled and functional VLPs can be produced in insect cells, coupled with their efficiency in triggering immune responses and displaying antigens for a number of applications has significantly increased the popularity of these cells in the industrial field.

Table 1 - Insect-cell derived VLPs in preclinical studies (adapted from Krammmer et.al 2010).

Influenza subtype	Influenza proteins	Animal model	Results	Comments	Year
H3N2	HA,NA,M1,M2			First report of Influenza VLPs	2001
H3N2	HA,NA,M1,M2	Mice	Protection from challenge	Interleukin-12 tested as an adjuvant	2005
H9N2	HA,NA,M1	Mice	Protection from challenge		2005
H3N2	HA,NA,M1	Mice and ferrets	High HAI antibody titers	Compared with inactivated whole virus and rHA	2007
H1N1	HA,M1	Mice	Protection from challenge	First report of a cytotoxic T-cell responde	2007
H1N1 and H3N2	HA,M1	Mice	Protection from challenge	Bivalent vaccine, comparison with inactivated whole virus	2008
H5N1	HA,NA,M1	Ferrets	Protection from challenge		2008
H5N1	HA,NA,M1	Mice	Protection from challenge	Bivalent vaccine, comparison with inactivated whole virus	2008
H5N1	HA,NA,M1	Mice	Protection from challenge		2009
H1N1	HA,M1	Mice	Protection from challenge	Focus on dose-dependence of protection, bacterial toxins tested as adjuvants	2009
H1N1	HA,NA,M1	Mice and ferrets	Protection from challenge	VLPs from the 1918 pandemic strain	2009
H1N1, H3N2 and B	HA,NA,M1	Mice	Protection from challenge	First trivalent approach, compared with split vaccine Fluarix	2009
H5N1	HA,NA,M1	Mice	Protection from challenge	Focus on long-term protective immune responses	2009
H1N1v	HA, M1	Mice	HAI titers of 1:2048	Alternative insect cell line, fast reaction to 2009 pandemic	2010
H1N1v	HA, M1	Mice	Protection from challenge	Fast reaction to 2009 pandemic, single-shot strategy	2010

1.2 Insect cells

The increasing interest in insect cells led to the generation of a cell line from the ovarian tissues of the cabbage looper - *Trichoplusia ni* (Hink, 1970). From this cell line, BTI-TN-5B1-4 clones were patented in 1994 (Granados et al., 1994) and Invitrogen then commercialized a more productive clone of this cell line under the name of High-Five™ cells (Hi5). The most important *Spodoptera frugiperda* insect cell lines - Sf9 and Sf21 - were characterized in 1977 (Vaughn et al., 1977) and were derived from the pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda*.

Insect cells can be cultivated in static (e.g. T-flasks) and in suspension (e.g erlenmeyer, shake flasks and bioreactors) systems. They grow at 27ºC, in serum free media to high cell densities (Rhiel et al., 1997). They can be sub-cultured for serial passages and do not require CO_2 for growth. Besides this, insect cells are typically more resistant to temperature (Gerbal et al., 2000) and osmolarity (Yang et al., 1996) fluctuations than mammalian cells which constitutes a major advantage for their biotechnological application.

It has been shown that insect cells are very efficient at producing recombinant proteins (Cox, 2012) and their scale-up has been successfully implemented and being improved (Bédard et al., 1997; Kioukia et al., 1996; Maranga et al., 2004).

1.2.1 Baculovirus expression vector system (BEVS)

The baculovirus expression vector system (BEVS) was firstly used in 1983 to produce a recombinant protein in insect cells (Smith et al., 1983a). Since then it has proved to be a reasonable platform to express recombinant proteins in insect cells and one of the great advantages of using this platform relies on the good yields of expression that can be achieved with similar eukaryotic PTMs (Harrison and Jarvis, 2007).

The BEVS relies on the infection of insect cells by recombinant baculoviruses that were genetically modified to carry genes of interest. Baculovirus is a rod-shaped (30-60 nm ×250–300 nm) with double-stranded DNA genome and infects insects and other arthropods (Jehle et al., 2006). The wild type baculovirus replication cycle is biphasic giving rise to two types of virions: occlusion-derived virions (ODVs) and budded virions (BVs), as shown in

Figure 2. Virus's life cycle comprises three phases concerning gene expression: immediate early/early, late and very late (Passarelli and Guarino, 2007). In the very late phase of infection polyhedrin is expressed by a very strong promotor due to its importance in viral ODVs assembly (Volkman, 1997). Given that in biotechnological applications the infection is done with BVs there is no need for polyhedrin protein which gives the opportunity to change this gene for a gene-of-interest (GOI; recombinant protein) (Merrington et al., 1997). Thereby, this allows high productivities that can reach more than 25% of total cell proteins (Caron et al., 1990) although only at the very late stage of infection. Likewise, the p10 protein gene expression is also driven by a very late strong promoter (Smith et al., 1983b) and this gene can also be replaced by a GOI in recombinant baculoviruses not affecting the replication cycle.

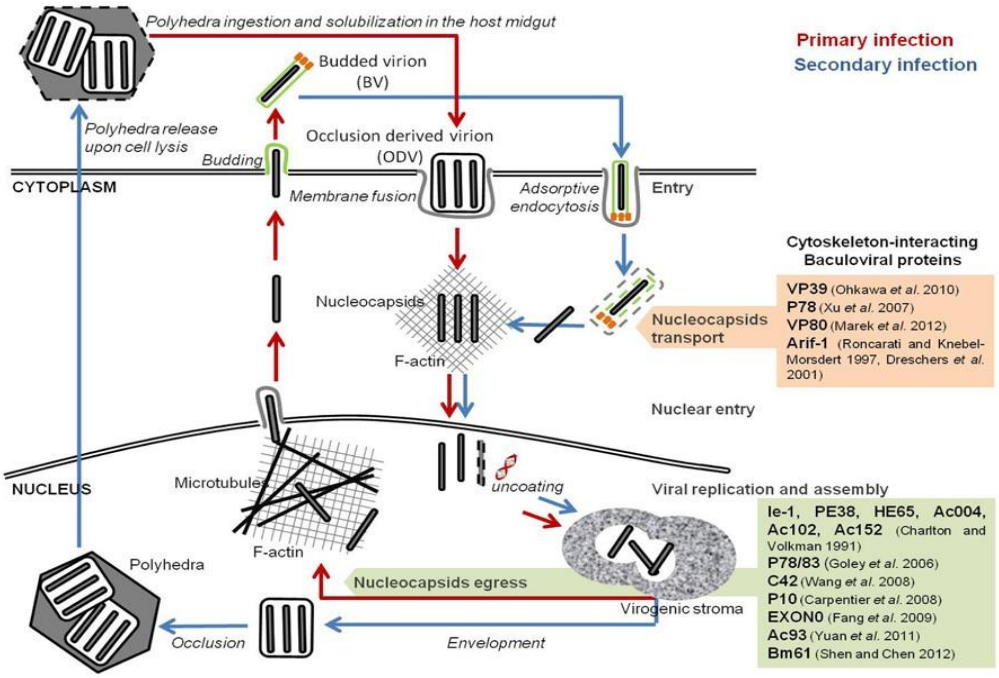

Figure 2 - Wild-type baculovirus replication cycle (Monteiro et al., 2012).

The most commonly used baculoviruses are *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) named after its ability to encapsidate multiple nucleocapsids in the occluded particle (polyhedron) and owns a genome of approximately 134kbp (Ayres et al., 1994). It is widely used in lepidopteran derived insect cell lines mentioned above (Sf9, Sf21 and Hi5).

The BEVS has become very popular in the biotechnology field, with numerous commercially available kits. For example, the MultiBac^{TM} system is able to generate multiprotein co-expression which is an evolution over the polycistronic vectors. It was further optimized by eliminating the baculoviral genes *v-cath and chiA* that encode proteases, abrogating their function (Bieniossek et al., 2012). Subsequently to bacmid transfection, recombinant baculovirus are assembled and released from cells following infection and propagation of the viruses which also leads to recombinant protein production.

To address a potential insect cell PTMs issue, the SweetBac™ system was designed to integrate N-acetylglucosaminyltransferase and β1,4-galactosyltransferase enzymes in the viral genome to generate humanized glycosylation patterns on recombinantly expressed proteins (Palmberger et al., 2012; Palmberger et al., 2015). Another issue is the accumulation of inactive forms in host cells and protein degradation by the ubiquitin proteasome pathway, for example. By co-expressing chaperones (e.g. calnexin and calreticulin chaperones) and folding factors along with the desired protein, authors have managed to enhance the surface expression and protein folding in insect cells (Kato et al., 2005).

In relation to baculovirus vector stability, it may be affected by tandem repetition of promoter sequences in polycistronic constructs (Belyaev and Roy, 1993) and to by-pass this problem identical promoters have been separated into different transcription directions. Moreover, improved stability can also be accomplished by producing a bicistronic mRNA including the GOI coupled to an essential baculoviral gene - gp64 – hence placing a positive selection pressure upon the entire mRNA, thereby ensuring the expression of the protein of interest (Pijlman et al., 2006).

Comparing the two most used cell lines, Hi5 cells have shown to be better recombinant protein producers than Sf9 cells (Davis et al., 1993; Krammer et al., 2010) which are better at producing infectious viral particles (Monteiro et al., 2014). Both cell lines have

been proved to undergo oxidative stress during baculovirus infection, resulting in loss of cell viability and consequently cell death (Wang et al., 2001). Despite the popularity of BEVS, many things remain unclear concerning the impact of infection on the cell host, which is a critical subject when the aim is to scale-up the process as efficiently as possible (Maranga et al., 2004; Monteiro et al., 2014).

Although BVs are considered safe because they cannot replicate in mammalian cells, its genome is able to integrate in the human genome (Merrihew et al., 2001) and the consequences of it still remains uncleared. Therefore, BVs and host's cell DNA contamination are a concern when the goal is to generate a product for human use. Efforts to develop better purification processes have been conducted (Rueda et al., 2000; Vicente et al., 2009) though it is very challenging because in this system virions are co-produced with the desired protein. In order to circumvent this issue, the Geneva Biotech's ManuBac™ system is being developed which is a virion free protein production platform that uses an induction protocol to turn off virions production at the same time VLP production is turned on (Biotech, 2015). Likewise, to eradicate the presence of baculovirions, a non-replicative baculovirus was engineered by removing the *vp80* gene which is implicated in viral protein cleavage, maturation, assembly and release of virions from cells (Marek et al., 2011).

1.2.2 Advantages/disadvantages for production of Influenza vaccines

Considering influenza vaccines, BEVS-based production has proved to be as efficient as more traditional strategies like egg- and cell-based (Bright et al., 2008) with great cultivation benefits compared to mammalian hosts and easily scaled-up. Furthermore, the construction of recombinant baculoviruses (rBacs) is becoming more rapid and versatile, allowing to easily obtain multi-gene expression. Flexibility in rBac construction gives the opportunity of combining genes of different influenza strains, including the most prone to diverge and suffer mutations like HA, allowing the production of broader vaccines. Thus, it offers the great opportunity to renew a vaccine much more rapidly without the need of isolating the circulating influenza virus strain which holds its own biosafety and laborious issues. Hereupon, some shortcomings seen in other vaccines-platforms are overcome such as

the virus adaptation to cells, the inability of propagating more pathogenic strains that leads to host death, lack of proteins prone to cause allergies contained in eggs as well as being much more cost effective and faster.

The main bottlenecks of the insect cells-baculovirus system for influenza VLPs production reside in the downstream processing (DSP) (unable to separate rBac from VLPs) and viral stock maintenance (infectious particles titer decreases with time). On top of that, for influenza VLP vaccine candidates that do not contain the M1 protein, a more complex purification process is needed to carefully extract the membrane-anchored HAs without comprising protein integrity.

1.3 Cell line development

The issues associated with BEVS lead to an increasing effort to develop stable systems to produce recombinant proteins in insect cells without the need of using viral infection. Cell line development consists on engineering cells to stably express the GOI and it should be a rapid and standardized process. Stable cell lines are obtained thanks to genetic modifications and one of the main goals is transcriptional efficiency of the GOI where strong promoters, enhancers elements and *cis* and *trans*-acting elements play key roles (Nehlsen et al., 2009; Nehlsen et al., 2011). Besides the amount of protein produced, its quality and maintenance of its functionality is very important.

Stable cell lines can be obtained by allowing a GOI to integrate into the genome based on random integration or locus-specific integration. A number of options have been reported for the maintenance of a vector integrated in the genome and most frequently cells are positively selected with a drug (antibiotic for instance) (Fernandes et al., 2012). However, regarding industrial purposes, it is preferable if the process does not need a selection agent because it causes instability in cells and increases the cost of the process (Qiao et al., 2009; Schiedner et al., 2008).

Although offering many advantages, the establishment of stable cell lines still encloses major shortcomings that need to be addressed such as (1) long timeline needed to generate stable, high expressing clones, (2) product yield and quality and (3) flexibility of the cell line.

1.3.1 Random integration

Random integration requires a laborious screening process to identify stable and high expressing clones because expression of the GOI greatly depends on the chromosomal elements nearby the integration site, a phenomenon called "position effect", which makes the integration process irreproducible (Nehlsen et al., 2011; Siegal and Hartl, 1998). Furthermore, random integration can i) lead to the interruption of cellular housekeeping genes, ii) give rise to epigenetic silencing, iii) affect cell's stability and iv) induce mutagenic effects by inhibiting protective genes or causing gene mutations.

Nevertheless, it offers an advantage over targeted integration as it does not requires knowledge of the genome sequence and chromosomal sites characterization which is not available for some transformed cell lines, being the case of Sf9 and Hi5 cells.

1.3.2 Locus-specific integration

Locus-specific integration is advantageous in the way that if good locus/loci have been previously identified it/they can be tagged and then exchanged for the GOI without the need of screening. When deep knowledge of the working cell line exists, characterized genomic sites can be exploited and reused by homologous recombination (HR) leading to a precise, predictable and reproducible process. Nevertheless, HR is quite inefficient due to dominance of illegitimate recombination (IR) with a ratio of HR/IR of 1:1000 which hampers its broader applicability in transformed cell lines (Turan et al., 2013). Due to this low efficiency, efforts have been made to develop methods capable of achieving higher site-specific integration frequencies.

ZFNs, TALENs and CRISPRs

Genome editing methods have expanded and artificial enzymes such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been designed to stimulate HR and they rely on the introduction of double strand-breaks (DSBs). Both enzymes have a nuclease activity and a customizable DNA-binding domain which enables to direct them to any target sequence (Mani et al., 2005; Vanamee et al., 2001). In order to broaden their applicability, efforts are being made to design more gene targets in different cell types and to improve the delivery method, targeting specificity and avoiding cytotoxicity. In addition, clustered regularly interspaced short palindromic repeats (CRISPRs) coupled with Cas endonucleases are being used in genome engineering and include programmable RNA-guided DNA endonucleases with ability to modify genomes (Mali et al., 2013; Ronda et al., 2014).

Despite their advantages, concerns are related to potential unspecific cleavage of endogenous genes (Miller et al., 2007). Moreover, such systems rely on genome sequence knowledge of the working cell line.

Recombinase-mediated cassette exchange (RMCE)

RMCE was firstly introduced by Schalke and Bode (1994) and it is a process in which a tagging cassette, flanked by a pair of heterologous recombinase target sites, can be exchanged by a target vector after being integrated into the genome (Oumard et al., 2006). Not only these target sites have to be non-compatible so that the exchange process is accurate but they must also be the same in the tagging and target cassettes.

Typically, the anchored cassette (tagging) encodes a reporter protein and a given selective marker (antibiotic resistance gene, for example), and then it is exchanged for a GOI by means of a site-specific recombinase (SSR), as depicted in Figure 3. The recombinase can be provided in the tagging, the target vector or in a separate vector.

Even though after the tagging step an intensive screening of the best locus is required, the RMCE system enables the reuse of the same locus, decreasing the time spent in further screening process (Gama-Norton et al., 2010; Nehlsen et al., 2009). Moreover, it has been shown that this method offers stable and high levels of gene expression (Coroadinha et al.,

2006; Schucht et al., 2006). This system is flexible enough to be used in many applications ranging from the biotechnology field for the establishment of producer cell lines (Coroadinha et al., 2006; Rose et al., 2013), for antibody production (Wiberg et al., 2006) or in the genetic area by allowing a more efficient analysis of gene function in mice (Seibler et al., 1998).

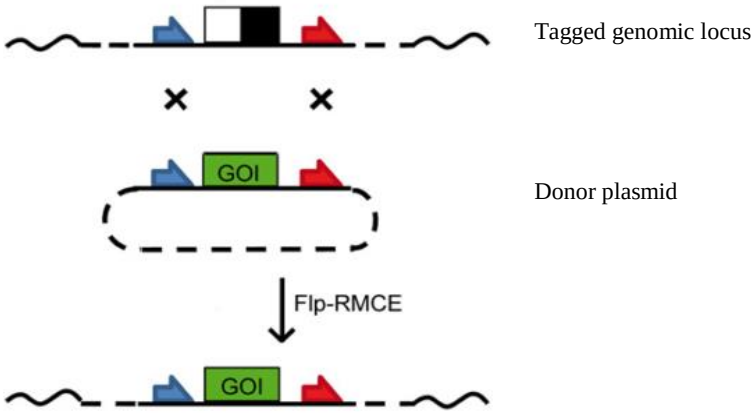

Figure 3 - RMCE principle: tagging a locus with a cassette flanked with heterospecific target sites into the genome and then exchanging it for the GOI. Adapted from Turan et al. 2010.

Site-specific recombinases (SSRs)

The most commonly used site-specific recombinases belong to two distinct families according to the structure of their active site: the Tyr-class such as Cre and Flp (Nunes-düby et al., 1998) or the Ser-family like ΦC31 (Smith and Thorpe, 2002) (Table 2).

Concerning ΦC31, it comes from *Streptomyces* bacteria where its role is to allow the integration of a phage into the bacterial chromosome which occurs via *attP/attB* sites (Smith and Thorpe, 2002). However, the system cannot be applied to RMCE neither to multiplexing protocols owing to the lack of recombinase efficiency of the enzyme over the integrase efficiency. The Cre enzyme (for "causes recombination") is a bacteriophage (P1) encoded integrase and was firstly described in bacteria whose function relies on a target site called loxP (locus of crossover in P1). This site is a 34bp sequence that consists of two inverted 13bp repeats separated by an 8bp spacer (Sternberg et al., 1986). Since its discovery, this system has been applied in mammalian cells aiming to be a powerful tool for deeper

understanding of genomic phenomena in eukaryotes (Sauer and Henderson, 1988) and efforts have been made to improve its efficiency (Koresawa et al., 2000). Despite having some benefits, Cre-induced toxicity as well as impairment of the host's DNA integrity have been reported (Fernandes et al., 2015; Schmidt et al., 2000).

Flp was identified in the 2µm circle plasmid of *Saccharomyces* cerevisiae where it is involved in site-specific recombination (Andrew et al., 1985; Mcleod et al., 1986) and its use in mammalian cell lines was firstly reported by O'Gorman (O'Gorman et al., 1991). The Flp enzyme induces a double-reciprocal crossover between two pair of target sites (FRTs) each one consisting on an 8bp asymmetric spacer flanked by a 13pb repeat at one side and two 13bp repeats on the other side, completing a 48bp FRT site. Although the spacer sequence determines the orientation of the site it does not contact directly with the enzyme (Turan et al., 2010). Even though in some cases the Flp/FRT system is less efficient than the Cre/loxP its use has been increasing significantly (Fernandes et al., 2012; Whiteson et al., 2007). Examples are the production of viral vectors for gene therapy (Coroadinha et al., 2006), study of genetic phenomena (Nehlsen et al., 2011; Seibler et al., 1998), recombinant protein production (Kim MS and Lee, 2008; Nehlsen et al., 2009; Wilke et al., 2011) or engineering strains (Cesari et al., 2004). There are various sets of flippase recognition target sites (Flp/FRTs) that were designed by mutagenesis (Schlake and Bode, 1994) and these have different recombination efficiencies and probability of cross-recombination events (Schlake and Bode, 1994; Turan et al., 2010).

Given the need of using Flp enzyme in animal cells, there were several efforts into improving its efficiency. For instance, the wild type Flp (wt Flp) was extremely inefficient at 37ºC because its optimum activity temperature is 30ºC (Buchholz et al., 1996) and efforts were made in order to improve this characteristic. Buchloz was able to construct Flpe successfully which is more termostable at 37ºC (Buchholz et al., 1998). Later on, Flpe enzyme was mouse-codon optimized into Flpo (Raymond and Soriano, 2007) and hFlep (humanized Flpe) also with great success (Kondo et al., 2009). So far toxicity of Flp has not been reported.

Fernandes *et al.* developed a Sf9 master cell line making use of RMCE and flippase enzyme with the purpose of being a good alternative to BEVS. Firstly, the authors were able

to produce the same amount of enhanced green fluorescent protein (eGFP) as in the BEVS system (Fernandes et al., 2012) and then the same principle was successfully applied to the production of more complex proteins such as rotavirus-like particles (Fernandes et al., 2014). This resulted in increased quality and yield of production, which is often compromised in the baculovirus-expression system due to proteolysis in late stages of infection (Monteiro et al., 2012). Stable expression does not compromise the host at such a level as it is seen for BEVS and it can also be adapted to bioreactor strategies for industrial purposes. Thus, it is predicted that this system will be robust enough to outpace the BEVS once some difficulties are overcome like the longer period of time taken to have the product and the lack of chromosomal loci characterization in insect cells.

Table 2 - Most used SSR in mammalian cell lines. Adapted from (Wirth et al., 2007).

SSRs	RTs employed	Cell lines
Cre	LoxP/Lox511	MEL
		NIH3T3
		mES
		Fertilised mouse oocytes
		K562
		J558L
	LoxP/Lox2272	mES
	LoxP/Inverted LoxP	mES
	Lox66/71 and Lox2272	mES
	Lox511/InvertedLox511	MEL
	LoxP/LoxP257	Mouse B hybridoma cells
		HeLa/CHO cells
		Primary MEF/mES cells
	LoxP/Lox5171	mES
Flp	FRT/FRT3	BHK
		mES
		NIH3T3
	FRT/FRT5	NIH3T3
		HEK293
		mES
		BHK
ΦC31	attB and attP	Primary epidermal progenitor cells
		mES
Cre and Flp	LoxP and FRT	mES

Aim of the thesis

This thesis aims at developing robust insect cells based platforms for production of complex products such as Influenza virus-like particles (VLPs) as vaccine candidates.

In order to achieve such goal two strategies have been designed (Figure 4). The first one consists in combining stable expression of two HAs in Hi5 cells, based on random integration of the GOIs, with baculovirus-mediated expression of M1 and additional HA proteins to produce multivalent VLPs. This approach minimizes the potential risk for instability caused by the addition of many genes in a single baculovirus vector, when developing a production process for multivalent HA VLPs. To by-pass the expression unpredictability of HA random integration, the second strategy consists in generating stable insect cell lines based on our in-house developed flippase-recombinase mediated cassette exchange (Flp-RMCE) platform, to be able to then re-use pre-characterized genomic loci to integrate multiple HA. The feasibility of having two genomic cassettes flanked by different pairs of flippase recognition target sites (FRTs) (double-RMCE platform) will be evaluated.

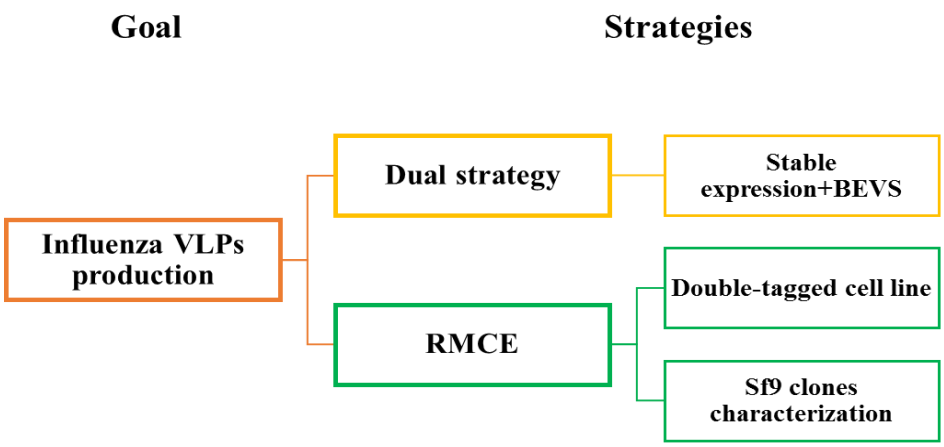

Figure 4 – Aim of the thesis and strategies adopted.

2 Materials and Methods

2.1 Molecular Biology

2.1.1 Plasmid design and construction

Primers sequences are listed in Appendix A.1.

Stable HA expression

pIZT/HA$_{1,2}$ vector: HA_1 and HA_2 vectors were kindly provided by RedBiotech AG (Switzerland). Each HA gene was amplified by PCR and cloned into a KpnI or NotI (respectively) excised pIZT/V5-His (Invitrogen, Carlsbad, USA) resulting in pIZT/HA_1 and pIZT/HA_2 vectors. OpIE2 promoter and HA_2 genes were amplified by PCR from pIZT/HA_2 vector and cloned into pIZT/HA_1 vector opened by inverse PCR.

pIZT/HA$_{2,3}$ vector: HA_3 vector was kindly provided by RedBiotech AG (Switzerland) and amplified by PCR into a SacI excised pIZT/V5-His (Invitrogen, Carlsbad, USA) resulting in pIZT/HA_3 vector. OpIE2 promoter and HA_2 genes were amplified by PCR from pIZT/HA_2 vector and cloned into a ClaI excised pIZT/HA_3 vector.

Double-RMCE system associated vectors

pTaggF$_{13}$/F$_{14}$: The tagging cassette based on the F_{13} and F_{14} FRT sites and containing OpIE2 and OpIE1 promoters (pTagg) was designed by us and synthetized by GenScript (USA). This cassette was then digested with NheI and PsiI. iCherry and hygromycin marker genes were amplified by PCR from an in-house vector and cloned in the previous excised vector.

pTargetF$_{13}$/F$_{14}$: To construct the target vector, OpIE2 and OpIE1 promoters were eliminated from pTaggF$_{13}$/F$_{14}$. eGFP and neomycin marker genes were amplified by PCR from an in-house vector and cloned in the promoterless construct excised with NheI and NotI.

pOpIE2 M1/HA: an in-house vector containing F_w and F_5 sites, eGFP and hygromycin resistance genes was opened by inverted PCR (peGFP/Hygro). OpIE2 promoter and HA genes were amplified by PCR from an in-house construct and cloned in the previous opened peGFP/Hygro vector (pOpIE2 M1/HA+eGFP). eGFP was then eliminated by digestion with BamHI and NotI and M1 (previously amplified by PCR from an in-house vector) was cloned in the excised site.

2.1.2 Techniques supporting plasmid construction

General PCR-protocol

The oligonucleotides used for PCR were custom-made by Sigma Aldrich (St.Louis, USA). A typical PCR-reaction included 4µl of 5x polymerase buffer (Thermo Scientific), 0.4µl of 10mM dNTPs (NZYTech), 0.4µl of 25µM primers (Sigma), 20ng of template DNA and 1 to 5 U of Phusion® High-Fidelity DNa polymerase (Thermo Scientific). RNAse-free water (Sigma) was also added to the final volume of 20µl. The PCR-amplification program started with a 30s denaturation step at 98ºC, followed by 30 cycles of 10sec denaturation at 98ºC, primer annealing for 30s performed up to 5ºC below the melting point of the primer, and extension at 72ºC according to the fragment size. The next step in the cycle was final extension at 72ºC for 10 min.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate DNA-fragments. The concentration of each gel varied according on the size of the fragments in question. Agarose (Lonza) was melted in 1x TAE buffer (Promega) and stained with GelRed or RedSafe (Biotium; iNtRON Biotechnology). Before loading, samples were mixed with loading buffer (NEB; #B7024S) and a standard ladder was used according to the range of fragment sizes expected. For purification of bands, when needed, Illustra GFX kit (GE Healthcare) was used. Gels were photographed using GelDoc^{TM} system (Bio-Rad) and DNA quantification was used using Nanodrop ND-2000c (Thermo Scientific).

Transformation and vector isolation

Competent *E.coli* cells were transformed according to the manufacturer's protocol (NZYTech, ref. MB00401 or Clontech, ref. 636763). Transformed cultures were spread on LB-agar plates containing ampicillin or zeocin and grown overnight at 37 °C. The next day, several isolated colonies were picked and grown separately, in falcon tubes, using 5mL of TB antibiotic supplemented culture medium at 37ºC and 190rpm. After 16-18h, 2mL of cell culture was harvested by centrifugation and DNA was extracted and purified with the miniprep kit (Thermo Scientific) following the manufacturer's protocol.

To identify whether transformants contained the gene of interest, PCR screening and vector digestion were followed by agarose gel electrophoresis analysis.

Digestion of DNA

DNA-digestion of PCR-fragments or vector-DNA was performed with the appropriate restriction endonucleases according to the manufacturer's specifications (NEB). When digestion of a vector was desired, further excision and purification from agarose gel was performed with Illustra GFX purification kit (GE Heathcare).

Ligation with In-Fusion

For the ligation of DNA-fragments the In-Fusion® HD Cloning kit was used following the instructions of the manufacturer (Clontech; ref. 638910). The ligated vector-DNA mix was used to transform bacterial cells, as previously described.

2.2 Cell line development

2.2.1 Transfection

Foreign DNA was inserted into cells using lipotransfection based on Cellfectin® II reagent (Invitrogen). 8µl of Cellfectin and 100ul of Grace's Insect Medium (Gibco) were used to $1x10^6$ cells (unit of transfection, UT). Transfections were conducted in 125mL shake flasks in 10mL working volume.

For the tagging step in the RMCE strategy, parental Hi5 and Sf9 were transfected at cell concentrations of $0.3x10^6$ cells/mL and $0.5x10^6$ cells/mL, respectively, using 0.3µg/UT of DNA. Selection was performed with hygromycin (0.2mg/mL; Invivogen) or zeocin (0.1mg/mL; Invivogen) depending on the expression vector resistance marker.

For the establishment of stable Hi5 pools expressing HA genes, parental Hi5 cells were transfected at $0.5x10^6$ cells/mL and selection was performed with zeocin (0.1mg/mL; Invivogen).

2.2.2 Cassette-exchange

To perform RMCE, 0,1µg/UT of target cassette and 0,3µg/UT of iFlp-expressing vector were used and selection was performed with hygromycin (Invivogen) or neomycin (Invivogen) depending on the expression vector resistance marker. Cassette-exchange was performed at a cell density of $1x10^6$ cells/mL for Sf9 cells. When viabilities dropped to 50%, cells were transferred to T-flasks ($75cm^2$). After 24h, the medium was replaced by conditioned medium supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) and the respective antibiotic, and then changed every four to five days. Fluorescence intensity and cell colonies' growth were evaluated by visual inspection (DMI 6000, Leica). When confluent, cells were transferred back to suspension and cultured with the routinely used medium plus antibiotic.

2.2.3 Sorting procedures

Cells were sorted in a MoFlo high speed cell sorter (BeckmanCoulter) equipped with a 488 nm laser (200 mWair-cooled Sapphire, Coherent) for scatter measurements and a 561nm laser (50 mW DPSS, CrystaLaser) for iCherry excitation. iCherry was detected using a 630/75 nm bandpass emission filter. As a special requirement for insect cells, cells were resuspended in phosphate buffer saline (PBS) supplemented with Pluronic acid F-68 (PF68; Sigma) (Vidigal et al., 2013). PBS was used as sheath fluid and run at a constant pressure of 207 kPa with a 100µm nozzle and a frequency of drop formation of approximately 30 kHz. Cells were collected into 1 mL of PBS, also supplemented with PF68, and maintained at 4ºC. After sorting, cells were pelleted (200g for 10 min) and seeded in 6-well plates. They were kept for one week in culture medium with antibiotics–antimycotics (Invitrogen).

2.2.4 Cloning

Cloning by limiting dilution is a procedure to separate cells through serially dilutions of the culture suspension until the amount of 1 cell in 100 µl of final solution is reached. The medium is composed by 50% conditioned (the supernatant of exponentially growing parental cells) and 50% fresh medium and G418 (an analogous of neomycin). Then, 100 µl of this mixture was transferred into a separate 96-wells plate so each well receives one cell. When confluency was achieved, each clone was transferred to a 48-well, 24-well, 12-well and then to a 6-well and finally to 10mL suspension culture. From one cell per well to suspension cultures it took about 2-3 months.

2.3 Cell culture

Sf9 cells were purchased from Invitrogen and Hi5 cells were kindly provided by RedBiotech. For suspension cultures, cells were routinely cultured in 125mL or 500 shake flasks (working volume of 10-20mL or 30-50mL, respectively) at 27ºC in orbital shakers at 100rpm. Sf900 II serum-free medium (Gibco) and Insect X-press (Lonza) were used for Sf9 and Hi5 cultures, respectively. Cells were sub-cultured every 3-4 days when cell density reached 2-3x10^6 cells/mL. Hi5 cells expressing HA genes were supplemented with lipids when needed (Chemically Defined Lipid Concentrate; ref. 11905-031) (Gibco). Cell

concentration and viability were calculated by haemocytometer counting (Brand, Wertheim, Germany) using trypan blue exclusion dye (Merck, Darmstadt, Germany). For adherent cultures, cell cultivation was maintained in T-flasks ($75cm^2$) with conditioned medium supplemented with 10% (v/v) of FBS (Gibco) and sub-cultured when confluency was reached.

2.3.1 Freezing and thawing cells

Cells at exponential growth phase ($2\text{-}3x10^6$ cells/mL) were centrifuged at 200g, 4ºC for 10min, and cell pellets were ressuspended in cryopreservation media (CryoStor®, Sigma) to obtain a concentration of $1\text{-}2x10^7$ cells/mL. Aliquots were frozen using a freezing container (Mr. Frosty) (Thermo Fisher Scientific) and stored at -80ºC until further use. Thawing was performed by centrifuging cells with 12mL of fresh medium at 200g for 10 min to eliminate cryo preservation medium. After this, the cell pellet was re-suspended in medium, according the volume to the cell density desired. Suspension culture was then performed in standard conditions.

2.4 Baculovirus

2.4.1 Virus amplification

Recombinant baculoviruses were kindly provided by RedBiotech AG (Switzerland) and virus titters determined using the Virocyt virus counter (Virocyt, USA). Whenever needed, virus amplification was performed by infecting Sf9 cells at a CCI of $1x10^6$ cells/mL using a virus (V0 stock) dilution of 1:500, in 2L shake flasks with 300mL of working volume. After reaching a viability of 70-80%, cultures were harvested by centrifuging at 200g and 4ºC for 10 min and supernatant was collected and stored at 4ºC in the dark until further use.

2.4.2 Virus titration

Virus titration was performed using the MTT method (Mena et al., 2003; Roldão et al., 2009). Briefly, 100 µL of $0.5x10^6$ cells/mL of Sf9 cells were seeded into 96-well plates (Nunc, Roskilde, Denmark) and allowed to attach to the plate for at least one hour at 27°C in the dark. Then, culture supernatant was removed and cells were infected with serial dilutions

of baculovirus. Both positive (non diluted baculovirus stock) and negative (virus free culture media) controls were added to the assay. Plates were incubated for 6 days at 27ºC in the dark. After this period, 10µl of 0.5 mg/mL MTT solution was added to each well and plates were incubated for 4h at 27ºC. After removing the supernatant, the formazan crystals were solubilized with dimethyl sulfoxide (Sigma–Aldrich) (150µl/well) and incubated for additional 20min under constant shaking. The absorbance (570/690nm wavelength) was measured using a plate reader (Tecan, Switzerland). The collected data was analyzed using GraphPad Prism 4 (Graph-Pad Software, Inc., La Jolla, CA).

2.4.3 Infection of insect cells with baculovirus

Hi5 cells were inoculated at $0.3x10^6$ cells/mL and were allowed to grow until the time of the infection. Infections were performed at different CCIs of 2, 3 and $4x10^6$ cells/mL though always with the same MOI of 10 total viral particles/cell.

2.5 Production of influenza VLPs in 2L bioreactors

Bioreactor culture was performed in BIOSTAT® B-DCU 2L vessels (Sartorius, Goettingen, DE). The pH, temperature and dissolved oxygen were monitored on-line. The dissolved oxygen was set to 30% of air saturation and was maintained by automated stirring and air/oxygen supply on demand. The gas flow rate was set to 0.02 L/min. The inoculum was $0.5x10^6$ cells/mL and 40h later a supplement mixture was added, as described above. Infection was performed when cells reached a concentration of $4x10^6$ cells/mL with a MOI of 10 total viral particles/cell. Samples were taken daily to analyze cell viability and density as well as the HA titer. Medium additions and sampling proceedings were performed aseptically in a moveable flow chamber (Cruma 670 FL, Spain).

2.5.1 Downstream processing of influenza VLPs

After cells reached viabilities around 50-60%, the bioreactor bulk was centrifuged at 200g, for 10min at 4ºC. Supernatant was collected and then supplemented with 50 U/mL of benzonase (Merck Millipore, Germany) for 15min at room temperature to digest any host and/or viral DNA in solution. Supernatant was then filtered using a Sartopore 2 membrane

capsule (Sartorius Stedim Biotech, Germany) comprising a 0.45µm prefilter and a 0.2µm filter. Influenza VLPs were purified using anion-exchange chromatography with a SartoBind Q capsule (Sartorius Stedim Biotech, Germany) and concentrated using ultrafiltration/diafiltration by tangential flow filtration (300kDa cassette regenerated cellulose). This solution was sterile filtered using a Whatman cellulose regenerated membrane filter and stored in aliquots at −80ºC.

2.6 Analytical methods

2.6.1 Supplementation

A mixture containing Insect Medium Supplement 10x, 5mM glutamine, 10mM asparagine (all from Sigma-Aldrich) and 20mM glucose (Merck, Millipore) was added to small-scale experiments and bioreactor, corresponding to 10% of the working volume. This mixture was added when cell density reached $2x10^6$ cells/mL.

2.6.2 Negative staining transmission electron microscopy

The morphology and size of influenza VLPs were evaluated by negative staining transmission electron microscopy (TEM). Briefly, 10µl of sample were fixed for 1min in a copper grid coated with Formvar-carbon (Electron Microscopy Sciences, Ft. Washington, PA, USA). After this, the grids were washed with H_2O and then stained with 1% uranyl acetate for 2min and left to air dry. Samples were then observed in a Hitachi H-7650 Transmission Electron Microscope (JEOL, Tokyo, Japan).

2.6.3 Hemagglutination assay

The assay used is a plate-based assay in which the concentration of HA in bulk and purified VLP samples can be determined by comparing the hemagglutination profile of these samples with that of a standard of known HA concentration. Briefly, samples were 2-fold serially diluted in PBS and incubated at 4ºC for 30min with 25µL of 1% chicken red blood cells (RBC) (Lohmann Tierzucht GMBH, Germany). Hemagglutination of RBC was identified by the formation of a network (lattice structure) of interconnected RBC and HA

(positive results); if there is not enough HA to bind to RBC, they settle to the bottom of the well (negative result). As standard, an influenza vaccine with a known HA concentration was added to each assay experiment. The HA titer of a sample was determined by calculating the maximum dilution that gave a positive outcome and comparing it to the one obtained for the standard.

2.6.4 Exometabolome analysis

^{1}H-NMR was performed in a 500MHz Avance spectrometer (Bruker, Billerica, MA) equipped with a 5-mm QXI inversed probe. Spectra were acquired using a NOESY-based pulse sequence with water presaturation, performing 256 scans with 4s acquisition time, 1s relaxation delay and 100 ms mixing time at 25ºC. DSS-d6 (Cat.No. 613150, Isotec, Sigma-Aldrich) was used as internal standard for metabolite quantification in all samples. In order to maintain a constant pH between samples, these were mixed with phosphate buffer (pH 7.4) prepared in DH_2O (Cat.No. 151882, Sigma-Aldrich) at a 2:1 ratio. Before spectra acquisition, the spectrometer was calibrated by determining the 90º pulse and the water chemical shift center of each sample. Each spectrum was phased, baseline corrected and integrated using Chenomx NMR Suite 7.1 (Chenomx, Inc.). Most metabolites are defined by several clusters at different chemical shifts, which in some cases may overlap or be affected by the damping effect caused by water suppression, inducing an underestimation of their concentrations. Therefore, after automatic fitting of each metabolite, the best resolved and farthest peak from the water region was chosen for manual adjustment and metabolite quantification.

In bioreactor culture, lactate concentrations were routinely determined using an YSI 7100 Multiparameter Bioanalytical System (YSI Life Sciences, Dayton, OH).

2.6.5 Immunofluorescence

In order to detect HA in Hi5 cells membrane, a protocol of immunofluorescence was performed. Briefly, $2x10^6$ cells of each population were centrifuged at 300g for 5min, cell pellets were collected and washed with PBS twice before incubation with 50µl of anti-HA antibody solution (dilution 1:20 in PBS) for 1h at 4ºC in the dark. After this, samples were

centrifuged and washed twice with PBS and incubated with 50µl of secondary antibody (dilution 1:200 - ref.A11015; Thermo Fisher Scientific) for 30min at 4ºC in the dark. This secondary antibody was labelled with GFP. After two washing steps with PBS, samples were ressuspended in 0.5mL of PBS and fluorescence microscopy analysis was conducted to detect eGFP (DMI 6000, Leica).

2.6.6 Cell sonication

After centrifugation, buffer containing 50mM Hepes, 300mM NaCl and 15% trehalose was added to cellular extracts to a final concentration of $4x10^6$ cells/mL. Cells were then sonicated on ice during 1min with 10% amplitude 15s-On-15s-Off pulses (Branson Digital Sonifier). The sonicated cells were centrifuged at 10 000g at 4ºC for 10-15 min, after which the supernatant was collected and used in further experiments.

2.6.7 Flow cytometry

CyFlow® space (Partec GmbH) was used to evaluate recombination efficiencies as well as to characterize tagging and target populations and clones in terms of eGFP or iCherry fluorescence intensity and percentage. Samples were collected and diluted in PBS. eGFP was detected using FL1 channel (emission filter: 520 ± 5 nm) and iCherry by using the FL4 channel (emission filter 590 ± 50 nm). Analysis from 30 000 events per sample was done using FlowJo software.

2.6.8 Western blot

Samples were denatured with a reducing agent (Novex® NuPAGE®, USA), heated to 70ºC for 10min, and loaded on a NuPAGE® Novex® 4-12% Bis-Tris Gel 1.0mm (Thermo Fisher Scientific) for protein separation through gel electrophoresis using MOPS running buffer (50min at 200V). Molecular weight markers SeeBlue®Plus 2 prestained standard 1x (Invitrogen) and Magic mark (Magic mark XP western protein standard, Novex, USA) were used. Proteins were then transferred to a nitrocellulose membrane using iBlot® Transfer Stack (Thermo Fisher Scientific). The membrane was blocked for 1 hour at room temperature using a solution consisting of 5% skim milk (Merck) in tris buffered saline pH 8.0 (Sigma-

Aldrich, USA) with Tween 20 (Merck, USA) (TTBS). Incubation with primary antibodies was done overnight at room temperature; for the secondary antibodies only 1h was used. Detection was performed with the enhanced chemiluminescence detection system (ECL) (Amersham Biosciences).

Analysis of samples from bioreactor by western blot

To detect HA and M1 protein in bulk samples by western blot, culture samples were centrifuged at 200g for 10min and the supernatant was collected. For HA detection the primary antibody was diluted 1:1000 whereas for anti-M1 a 1:2000 dilution was used. Secondary IgG antibodies used were conjugated with horseradish peroxidase-labeling (HRP). For HA detection, a 1:5000 dilution secondary antibody was used (Ref sc-2473; Santa Cruz Biotechnology) whereas for M1 a dilution of 1:200 (ref. 81-1620; Life Technologies) was used.

Detection of M1 in Sf9 clones

To detect M1 by western blot, samples were centrifuged at 200g for 10min and pellets and supernatants were collected. Pellets from $2x10^6$ cells/mL were sonicated to allow extraction of M1. A monoclonal IgG1-mouse antibody (ref. ab22396; Abcam) was used in a dilution of 1:500 and polyclonal secondary antibody linked to HRP was used in a dilution of 1:5000 (ref. NA931; GE Healthcare).

2.6.9 RNA extraction and RT-PCR

For evaluation of gene expression mRNA levels were assessed by PCR. RNA was extracted from $8x10^6$ cells using the RNeasy kit (Qiagen) and quantified using Nanodrop ND-2000c (Thermo Scientific).1µg was converted to cDNA which was synthetized using the First Strand cDNA Synthesis kit (Roche Diagnostics, Manheim, Germany). PCR was executed using 20ng of cDNA and amplification was performed as described above with primer annealing at 69ºC and extension step at 72ºC for 13 sec. Agarose gel electrophoresis was performed in a 2% agarose concentration for 1h at 90V and the Quick-Load® 100 bp DNA ladder was used (ref.N0467G). Primers sequences are listed in Appendix A.2.

2.6.10 Genomic DNA extraction

To confirm the presence of the tagging cassette in clones, genomic DNA was extracted using an in-house protocol. Briefly, $8x10^6$ cells were pelleted and then added to a solution of 1mL of Bradley's solution, 5µl of proteinase K and 2 µl of RNase, followed by overnight incubation at 55ºC. Bradley's solution is composed by 10mM Tris/HCl (pH 7.5), 2mM EDTA, 10mM NaCl and 0.5% SDS. The next day, 250µl of 5mM NaCl was added for 5min on ice and the solution centrifuged for 15min at 10 000 rpm. Isopropanol was added to the resulting supernatant to precipitate DNA. After centrifugation at 10 000 rpm for 10min, supernatant was discarded, the pellet was washed with 1.2mL of 70% cold ethanol and allowed to air-dry. Pellet was dissolved in nuclease-free water.

PCR was performed using 100ng of DNA and amplification was done as described above with primer annealing at 69ºC and extension step at 72ºC for 2min and 51s. Primers sequences are listed in Appendix A.3. Agarose gel electrophoresis was performed in a 0.7% agarose concentration for 1h at 90V and the NZY DNA Ladder III (NZYTech; ref. MB04402) was used.

3 Results

3.1 Combining stable insect Hi5 cell line with the baculovirus expression system for production of multi-HA influenza VLPs

There are two main strategies to produce recombinant proteins in insect cells: i) infection of parental cells with baculovirus, which results in transient expression, and ii) transfection of parental cells with a plasmid harbouring the GOI, which leads to stable and continuous production after appropriate selection. Also, transient expression after transfection (before selection) is also a platform in stable production. However, both systems enclose a major limitation that is the number of genes one can express in the same virus or plasmid without compromising their stability. By using a stable cell line in combination with a virus-based expression system one can rationally modulate the number of genes to express in each building block (cells and virus) and thus mitigate such risk. This strategy will be herein used to generate multi-HA influenza VLPs.

3.1.1 Establishment of stable insect cell lines by random integration

An initial screening of insect cell lines (Sf9 and Hi5) was performed in order to identify the most suitable for stable expression of HA protein(s). Parental Sf9 and Hi5 cell lines were transfected with a plasmid harbouring two HA genes, represented by construct #1 in Figure 5. The weaker OpIE1 promoter was used to drive expression of the zeocin resistance gene and the best promoter was chosen to drive expression of HA genes. Then, 48h (for Hi5 cells) and 72h (for Sf9 cells) after transfection, cells were infected with a rBac encompassing three HA genes (being expressed by the polyhedrin promoter) using a CCI of $2x10^6$ cells/mL and MOI of 10 virus/cell, and the concentration of HA in culture supernatant was followed for two days (Figure 6). Authors have shown that Hi5 are better at recombinant protein production (Monteiro et al., 2014). In this work, results show that Hi5 cells are able to secrete more HA protein than Sf9 at the end of day 2 post-infection, with a 4-fold increase in HA concentration. Based on these results, Hi5 cells were selected for the establishment of a stable insect cell line (by random integration) that could assist the production of (multi-HA) influenza VLPs.

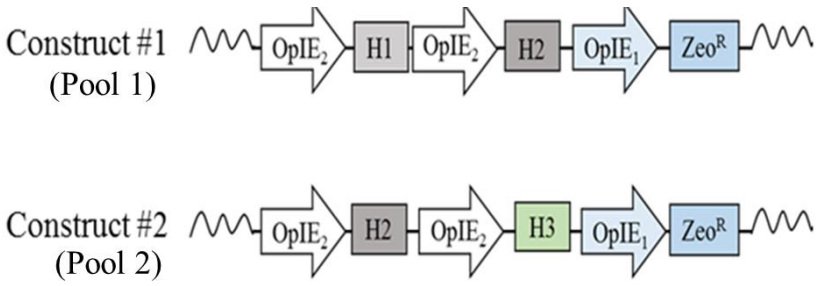

Figure 5 - Scheme of the two expression vectors used to transform parental cells. OpIE2 and OpIE1 promoters were selected to drive HA and zeocin resistance gene expression, respectively.

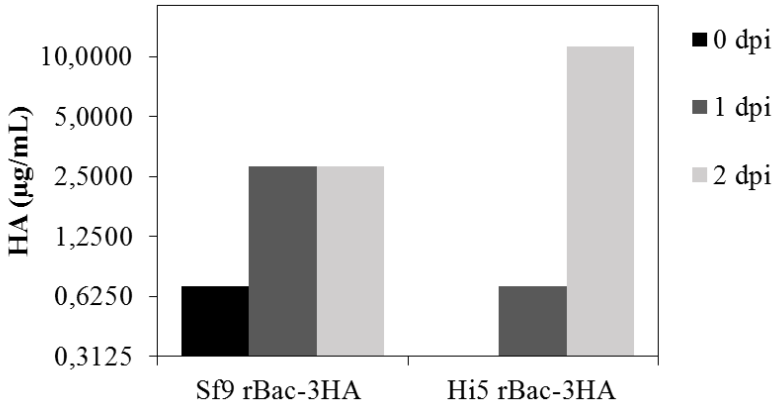

Figure 6 - HA concentration in culture supernatant. Parental Hi5 and Sf9 cells transfected with construct #1 (Figure 5) were infected with a rBac containing three HA genes at a CCI of $2x10^6$ cell/mL and MOI of 10 total viral particles/cell 48h (for Hi5 cells) and 72h (for Sf9 cells) after transfection. Dpi means days post-infection.

Two stable insect Hi5 cell pools were established by transfecting parental cells with the two constructs represented in Figure 5. Upon two to three weeks in zeocin selection cells achieved lower and stable duplication times and stable pools were obtained with cell viabilities above 90% and population duplication times (PDT) around 23h in shake flask cultures. These PDT are slightly higher than those of parental Hi5 cells (18h-20h), as expected. Cell growth and protein production profiles for both pools were followed along passages as represented in Figure 7. For pool 1, there are no significant differences between passages in terms of cell growth kinetics and PDT (data not shown) until passage 27. Only at passage 30, cells start decreasing their growth rate so this cell passage was not used for further experiments. For pool 2, a lipid supplementation helped on the growth kinetics of cells, with PDT around 21h instead of 24h, and to extend the peak of cell density from $2.4x10^6$

cells/mL to 3.7x10^6 cells/mL. Although expression of HA genes represent a burden to stable pools, the cell viability along passages was continuously above 90% up to the end of exponential growth phase (day 4 after passage) (data not shown).

Immunofluorescence microscopy of cultured cells allowed the detection of HA protein in both pools and results show that it localizes at the cell membrane (Figure 8), as expected (Ali et al., 2000). Therefore, in order to evaluate if the expression of the target protein is impaired throughout the adaptation period, the concentration of HA in cellular extracts was assessed by hemagglutination assay (the same cell concentration was sonicated in all samples – 4x10^6 cells/mL). Results show that HA concentration in cell pellets did not vary significantly along passages for both pools (Figure 9), suggesting that protein expression is not negatively affected during the adaptation period.

After the characterization of stable pools, both populations were further used in process optimization to enhance VLPs production.

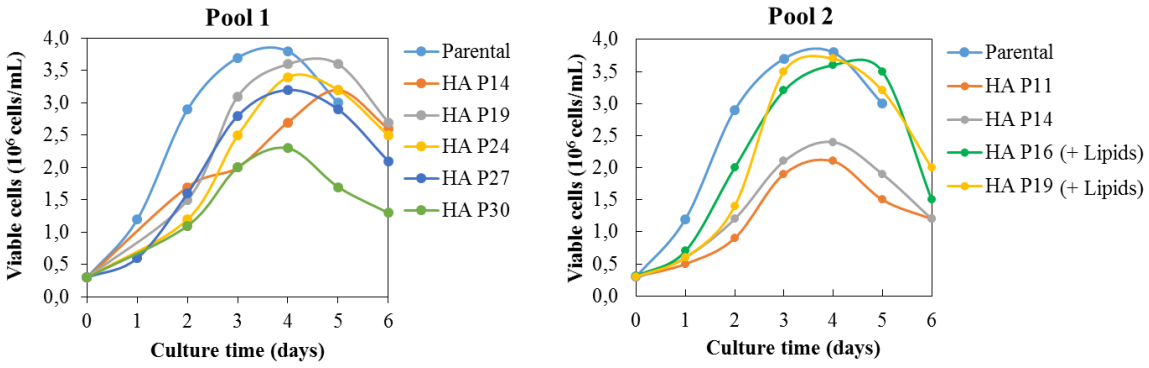

Figure 7 - Growth profiles of stable Hi5 pools expressing two HA genes, along passages.

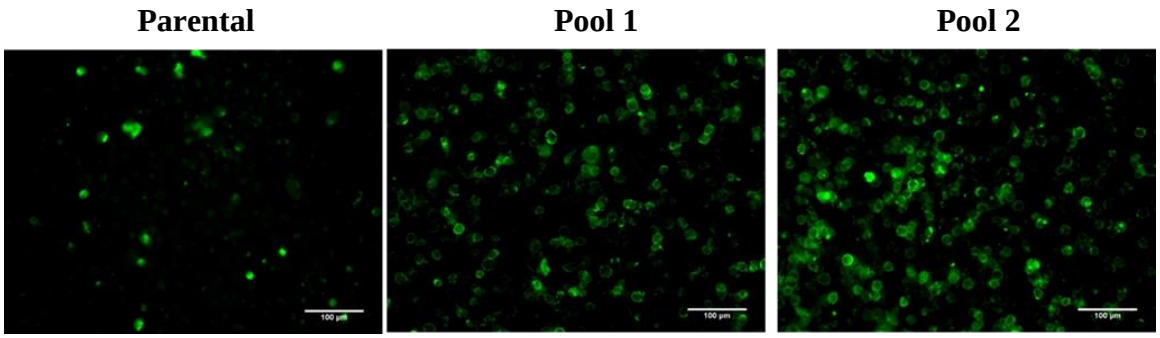

Figure 8 - Immunofluorescence detection of HA in the membrane of two stable Hi5 pools. Negative control (parental Hi5 cells) was added to evaluate unspecific binding of the antibody used. Secondary antibody was labelled with GFP. Scale bars indicate 100µm.

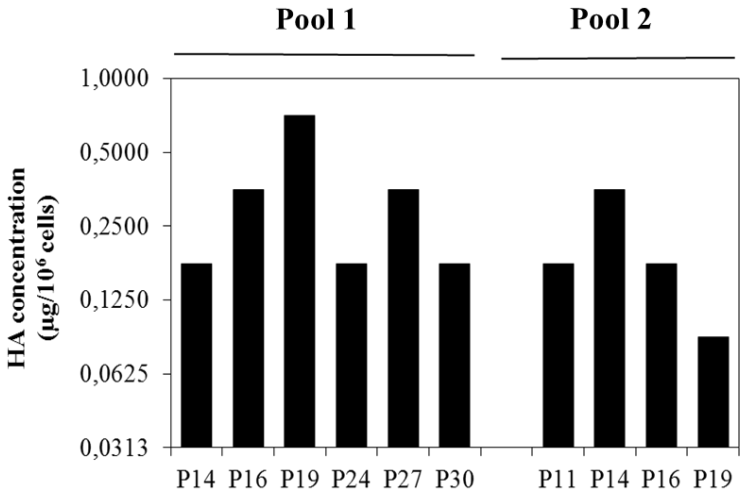

Figure 9 – Concentration of HA in cellular extracts along passages for two stable Hi5 pools.

3.1.2 Optimizing HA production in Hi5 pools

Aiming at increasing HA protein production, two strategies were followed: (1) re-feed of key nutrients to the culture and (2) optimizing the CCI.

In small-scale experiments, the maximum cell concentration one could reach for both stable Hi5 pools with the routinely culture medium used was around 3.5×10^6 cells/mL. Based on these results, it was decided to investigate the performance of both pools for HA production when infected at two different CCIs, $2x10^6$ cells/mL as standard condition and $3x10^6$ cells/mL as alternative. Both stable pools were infected with a baculovirus encoding M1 at an MOI of 10 total viral particles/cell. Growth profiles during infection (Figure 10A)

and HA productivity levels (Figure 10B) were evaluated. For pool 1, although increasing the CCI had no effect on the maximum concentration of HA achieved in culture supernatant, it was achieved one day earlier compared to infection at a CCI of $2x10^6$ cells/mL (Figure 10B). For pool 2, using a CCI of $3x10^6$ cells/mL allowed a 4-fold increase in HA production when compared to CCI of $2x10^6$ cells/mL (Figure 10B).

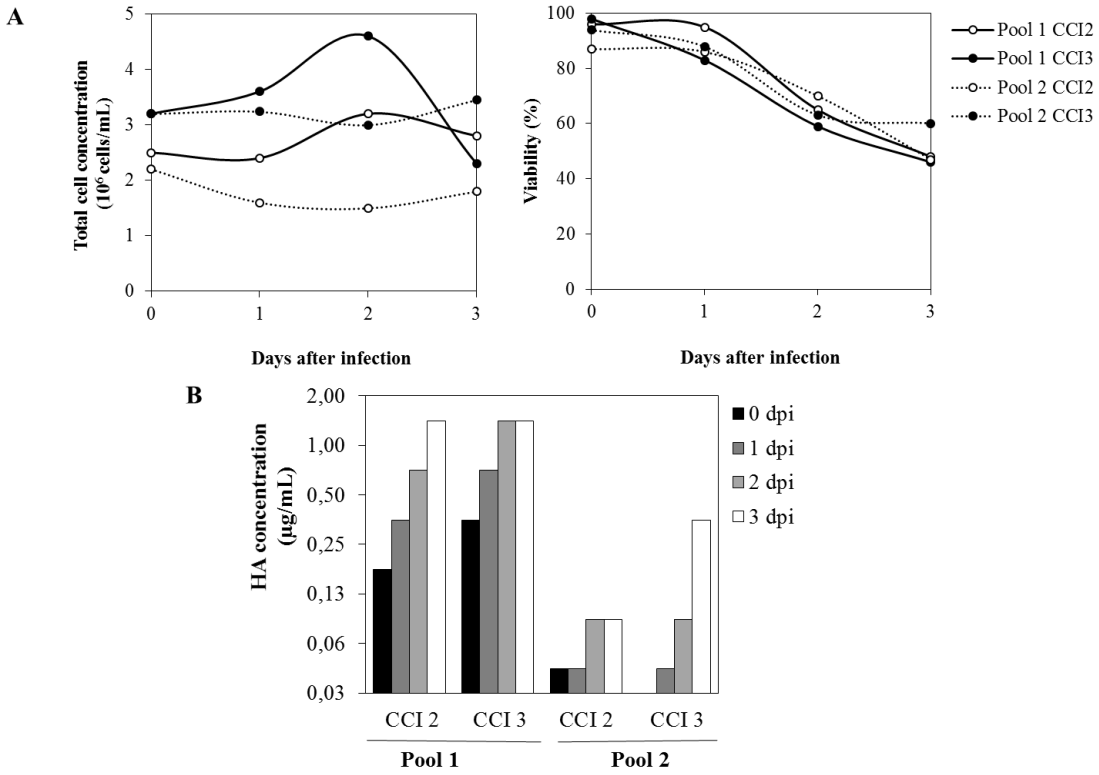

Figure 10 – A) Growth profiles for infections at CCI $2x10^6$ cells/mL and CCI $3x10^6$ cells/mL for both pools. B) HA concentration in culture supernatant during infection for both CCIs and pools. Dpi means days post-infection.

The next step was to investigate the feasibility of increasing cell growth performance to allow infections at higher cell densities, which will potentially translate into enhanced protein production. A stable Hi5 pool was cultured in small shake flasks where a maximum cell density of $3.6x10^6$ cells/mL was reached by 83h of culture and then cell viability started to decrease (Figure 11A). Supernatant samples were collected along culture time and analysed by ^{1}H-NMR (Figure 11B) in order to identify eventual exhaustion of key nutrients contributing for the onset of death phase. ^{1}H-NMR data showed that these cells avidly

consume asparagine (Asn), exhausting the 8.5mM available at inoculation, in less than 48h (Figure 11B). However, Asn seems not to be essential for these cells as they continue to grow upon its exhaustion. At the same time, cells increase uptake rate of aspartate (Asp) most likely to compensate Asn depletion. Glutamine is the second most consumed amino acid, being completely exhausted by 130h of culture (Figure 11B). Interestingly, the small levels of accumulated lactate (5mM) started to decrease once glucose concentration was limiting (Figure 11C). Based on these results, a re-feed strategy was designed in order to supplement the culture 72h after inoculation with the three key nutrients identified above (Asn, Gln and Glc) plus a mixture of lipids, proteins and vitamins for insect cells (Fernandes et al., 2014). Glutamine and glucose concentrations were restored to values near those found at the inoculation time (Figure 11C). Asparagine concentration only increased to 3mM after the supplementation but rapidly became depleted again. Noteworthy is the increase in lactate concentration after glucose addition reaching values between 11-14mM (Figure 11C). Importantly, the strategy herein adopted led to an increase in the maximum cell concentration achieved (4.9×10^6cells/mL vs $3.6x10^6$cells/mL in non-supplemented cultures) and in culture time (23h more than in non-supplemented cultures) without compromising cell viability (Figure 11A).

That being said, in order to increase cell density, a re-feed strategy using Asn, Glc, Gln and a supplementation mixture will be used after 72h of inoculation.

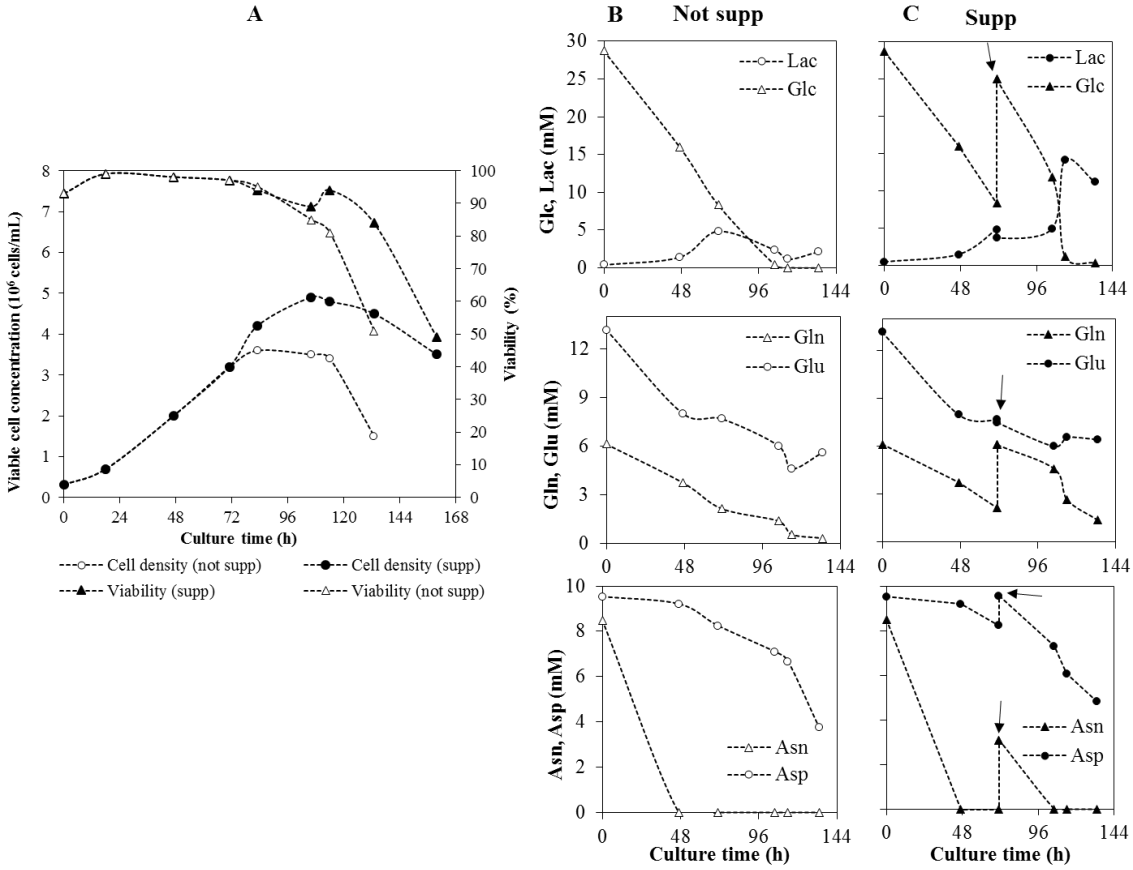

Figure 11 - A) Growth profiles of supplemented and non-supplemented cultures. Metabolites analysis by ^{1}H-NMR: glucose (Glc), glutamine (Gln), glutamate (Glu), asparagine (Asn), aspartate (Asp) and lactate (Lac) before (B) and after supplementation (C). The arrows indicate the time of the re-feed.

3.1.3 Production of multi-HA influenza VLPs

Afterwards, the goal was to implement this feeding strategy in shake flasks to increase the CCI and, probably, protein production.

Hi5 pool 1 was infected with two different recombinant baculovirus, one enclosing the M1 gene (rBac-M1) and another enclosing three HA proteins (rBac-3HA). These two baculovirus were used to address if a possible HA enrichment in the supernatant with the highest CCI was due to improved stable production or a boost in HA from baculovirus replication. Cultures infected at a CCI of $4x10^6$ cells/mL were supplemented 72h after inoculation and infected 24h after the supplementation.

The same MOI of 10 total viral particles/cell was used in both experiments and growth profiles during infection (Figure 12A) and HA productivity levels (Figure 12B) were

evaluated. Infection at the highest CCI (around $4x10^6$ cells/mL) resulted in a 4-fold increase in HA concentration in the supernatant with rBac-3HA and in a 2-fold increase when using rBac-M1, as shown in Figure 12B. Also, cell concentration profiles for both infections show a stronger impact in cells infected with a rBac-3HA where cells reached lower viabilities earlier (Figure 12A).

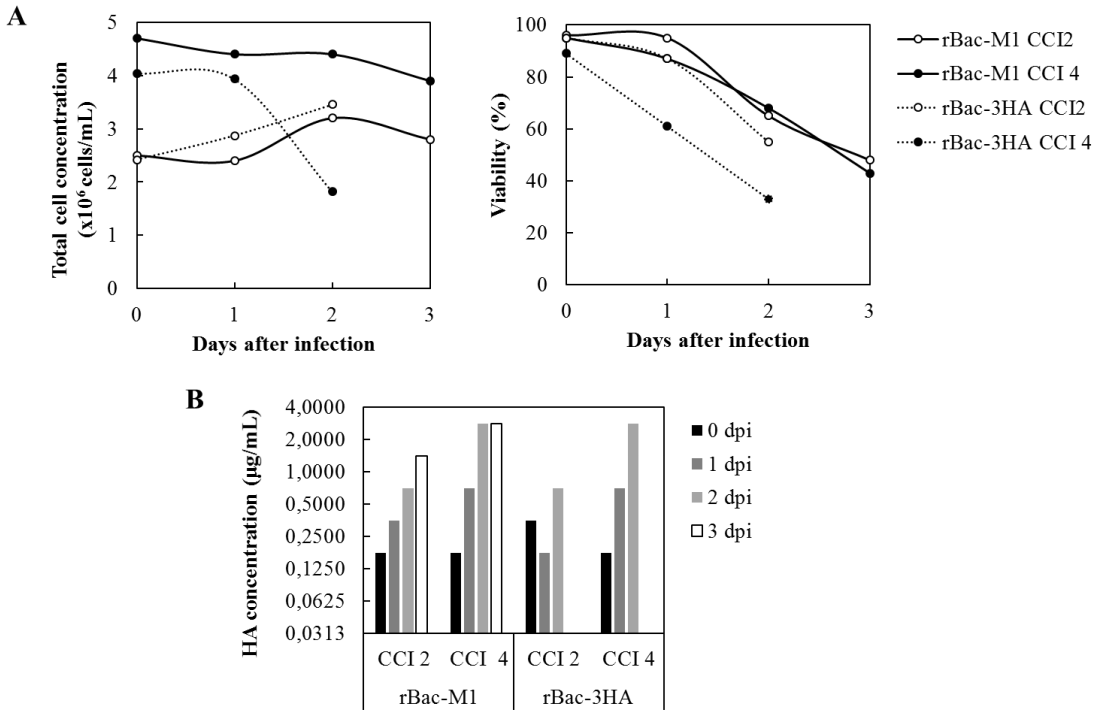

Figure 12 – Multi-HA VLP production in small-scale shake flask cultures. A) Cell concentration profiles for infections of pool 1 with rBac-3HA and rBac-M1 at CCIs $2x10^6$ cells/mL and $4x10^6$ cells/mL. B) HA concentration in the supernatant for both baculovirus infections at CCI $2x10^6$ cells/mL or $4x10^6$ cells/mL (culture was supplemented 72h after inoculation, i.e 24h before infection). Dpi means days post-infection.

3.1.4 Scale-up production of multi-HA influenza VLPs

In order to prove the scalability of multi-HA influenza VLPs production with the best strategy studied, the same experiment was conducted in a 2L stirred-tank bioreactor with dissolved oxygen and temperature control. Once cells reached $2x10^6$ cells/mL supplementation was performed. Infection was done with a rBac-3HA, to produce a pentavalent VLP, at a CCI of $4x10^6$ cells/mL. Bioreactor culture conditions maintained cells with higher viability and total cell density during infection, extending it from 2 to 3 days compared to small scale experiments, as seen in Figure 13A. Lactate concentration was kept to lower levels (3.5mM) than in shake flask cultures (23mM) (Figure 13B) most likely due to oxygen supply. Together, these conditions allowed to increase the volumetric and specific productivity of HA by 1.5-fold at the end of infection (Figure 13C). Western blot shows the increase in HA (64kDa) and M1 (28kDa) in the supernatant with the course of infection (Figure 13D).

To confirm VLPs production, negative staining transmission electron microscopy allowed the visualization of VLPs with the expected size range (80nm-120nm) (Figure 13E). Therefore, production of multi-HA influenza VLPs could be successfully scaled-up with increased HA titer and higher viabilities.

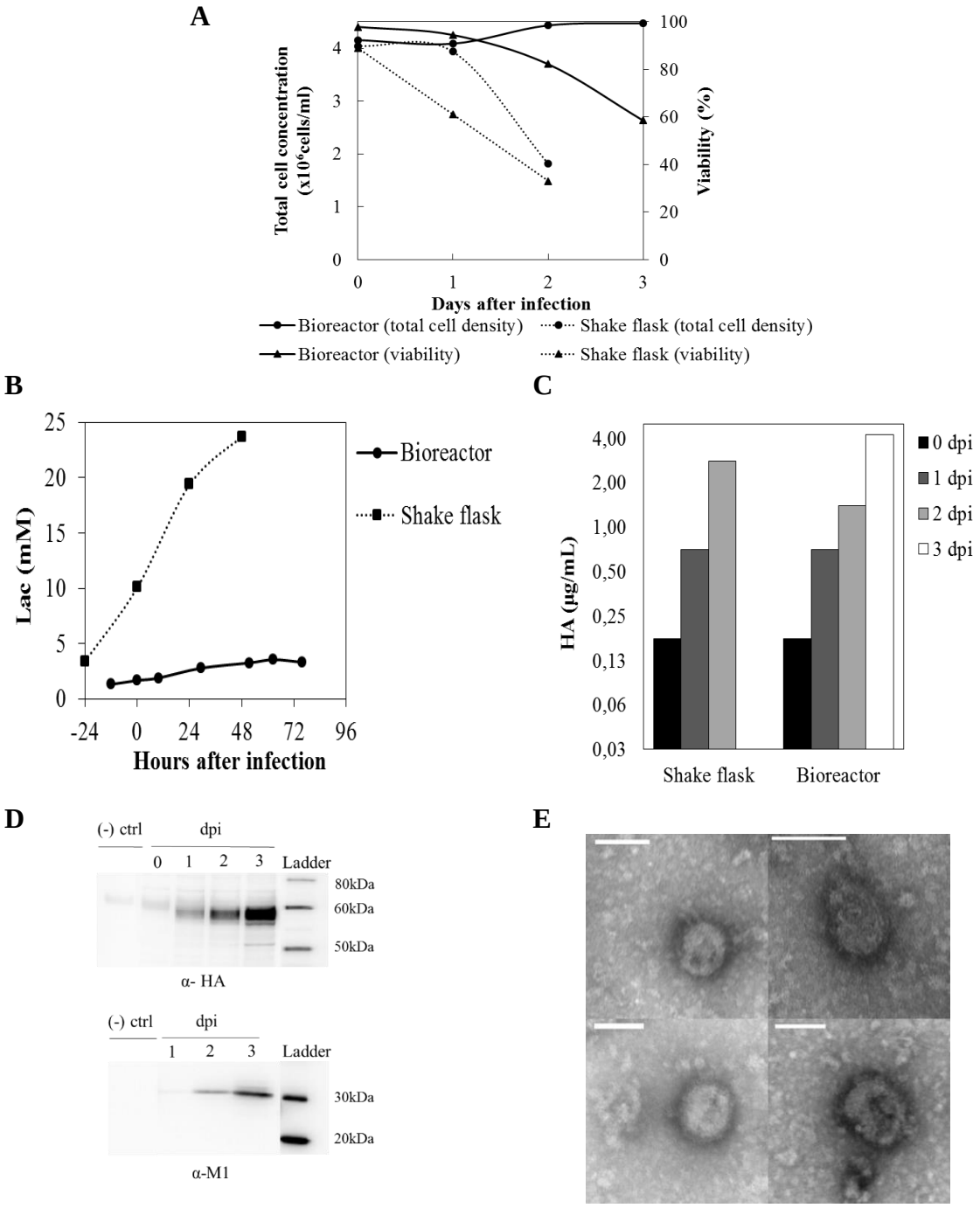

Figure 13 - Production of pentavalent VLPs in supplemented bioreactor culture by infection of Hi5 pool 1 at a CCI of $4x10^6$ cells/mL. Comparison between shake flask and bioreactor experiments regarding A) cell concentration profiles and viability; B) lactate (lac) concentration during infection and C) HA concentration in supernatant during infection. D) Western blot of culture supernatant for detection of HA and M1 along infection. Dpi stands for days post-infection. E) Electron microscopy analysis of VLPs by negative staining; scale bars represent 100nm.

3.2 Establishing a double-RMCE insect cell platform

In this part of the work the goal was to develop a cell platform that could be re-used to express several genes simultaneously, allowing the production of multi-protein complexes, such as influenza VLPs. The strategy adopted consists in tagging parental cells with two expression cassettes flanked by different pairs of FRTs. This means that two genomic loci would be targetable and more genes of interest can be replaced at the same time, contributing to a more flexible stable cell platform. In addition, the cell platform could also be used to produce monomeric products by exploring the potential of having two loci for integration of multiple copies of the same gene, contributing to improved production yields.

In order to make such system reliable and robust, some features need to be taken into consideration: i) the two pairs of target sites cannot cross-react with each other, keeping the system directional and predictable, and ii) two selective agents are needed in order to select cells that contain both constructs in the genome. In this section, the feasibility of such system will be addressed for Hi5 cells.

3.2.1 Vector design and FRT sites

A cassette system flanked by the pair of FRT sites wild-type (F_w) and mutant 5 (F_5) was previously implemented in-house in insect Sf9 cells (Fernandes et al., 2012). The second pair of mutant FRT sites chosen was the $F_{13}F_{14}$, which showed to be successful at multiplexing RMCE and better at recombination than the F_w/F_5 pair in mammalian cells (Turan et al., 2010).

Two Hi5 cell pools were established by transfecting parental cells with one of two tagging cassettes, both encoding iCherry and hygromycin as marker genes driven by OpIE2 and OpIE1 promoters, respectively, only differing in the flanking FRT sites (Figure 14A). The fluorescence intensity of each tagging population upon selection is represented in Figure 15. In order to address the question of cross-interaction between both pairs of target sites, two promoterless target cassettes were constructed, both encoding eGFP and neomycin as reporter and resistance genes, respectively (Figure 14B). This cassette design allows the monitoring of RMCE efficiency using eGFP expression as readout, which only occurs if the

target cassette replaces the flanked region in the cell genome. In addition, only cells where cassettes have been exchanged will be resistant to G418 (an analogous of neomycin).

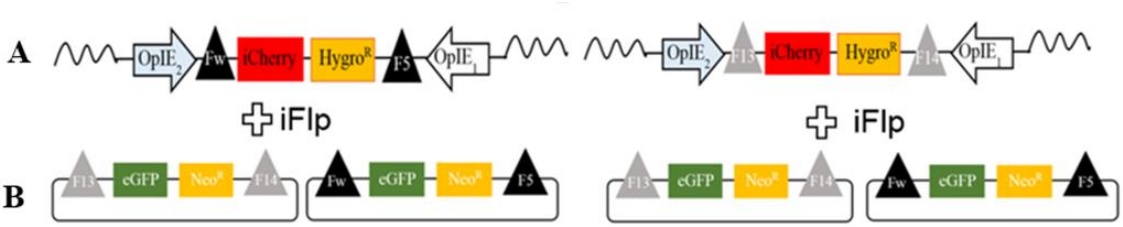

Figure 14 - Scheme of tagged populations (A) and target vectors (B) used to address cross-interaction between F_wF_5 and $F_{13}F_{14}$.

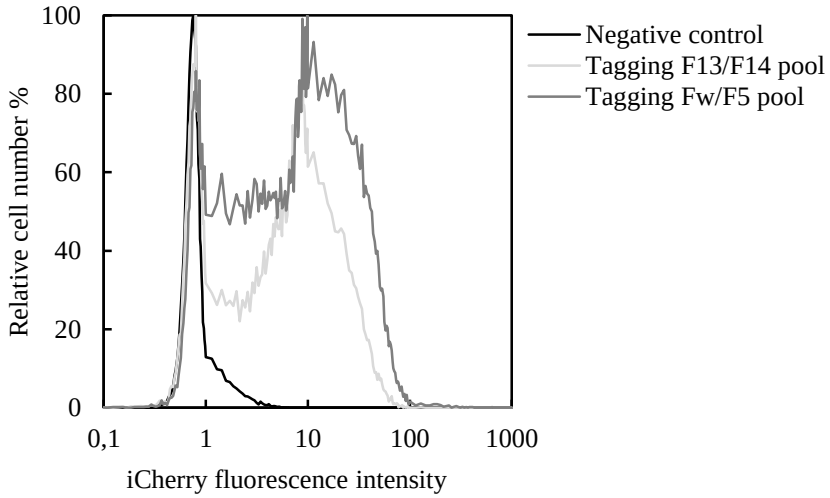

Figure 15 - Fluorescence intensity profiles of tagging pools at the day of transfection with target vectors.

3.2.2 Feasibility analysis: evaluation of target sites' specificity

In order to address the feasibility of a double-tagged cell line, both Hi5 tagging populations previously established were independently submitted to cassette exchange with each target vector in the presence of a Flp-encoding plasmid. The flippase used in this work was codon-optimized for insect cells (iFlp), thus recombination efficiency was significantly higher than using Flpe (unpublished data). The presence of eGFP positive cells was assessed via flow cytometry and fluorescence microscopy (Figure 16A and B). Figure 16A shows results in a transient phase of expression (48h post-transfection) evidencing eGFP positive cells in tagging populations transfected with vectors that differed in target sites (0,31% in $F_{13}F_{14} \times F_wF_5$ and 0,27% in $F_wF_5 \times F_{13}F_{14}$). In order to eliminate the hypothesis that eGFP expression could be the result of a random integration of the promoterless target vectors near

endogenous promoters, transfections were repeated without adding iFlp. Flow cytometry analysis in transient phase of expression shows the absence of eGFP positive cells in both transfections (Figure 16A), thus supporting the cross-reactivity of the two pairs of FRT sites.

Two days post-transfection, G418 was added in order to initiate the selection process of cells that had exchanged cassettes to evaluate the potential use of $F_{13}F_{14}$ enriched populations in RMCE. Figure 16C shows flow cytometry analysis of all target populations after this period. Two different inoculums were tested trying to identify the best selection strategy to efficiently enrich these populations in eGFP positive cells. The selection with a lower inoculum was, sometimes, more efficient (Figure 16C).

After two weeks in G418 selection, the presence of eGFP positive cells in $F_{13}F_{14} \times F_wF_5$ and $F_wF_5 \times F_{13}F_{14}$ populations corroborated the data obtained in transient phase. Based on these results, it is proved the cross-interaction between F_wF_5 and $F_{13}F_{14}$ target sites making them inapplicable for the implementation of a double-locus system of RMCE.

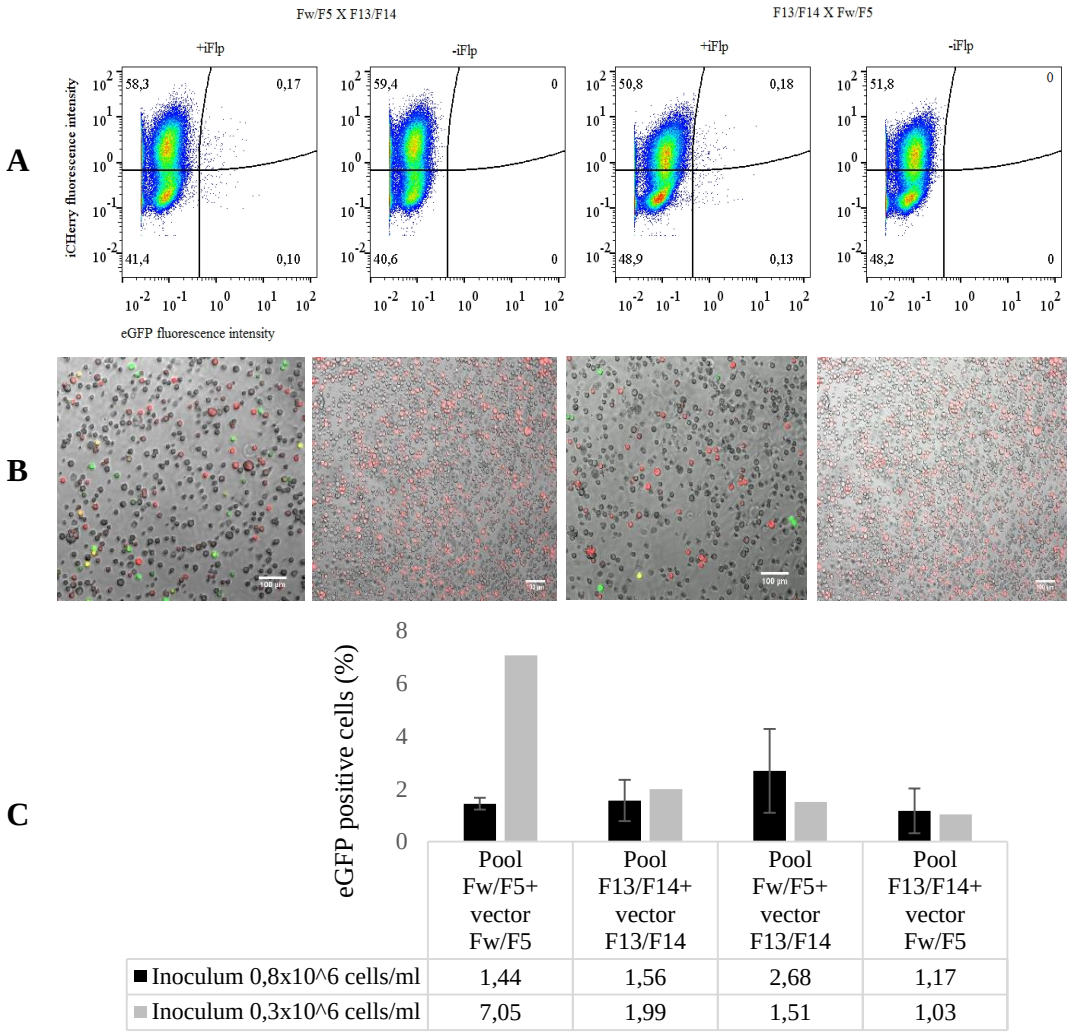

Figure 16 - Evaluation of cross-interaction between target sites Fw/F5 and F13/F14. (A) Flow cytometry and (B) fluorescence microscopy results 48 hours after transfection. Scale bars indicate 100µm. (C) eGFP positive cells (%) in different target populations after two weeks in selection with neomycin. Black bars represent the average of two experiments with cell passage at a high inoculum and grey bars are representative of a third experiment with cell passage at a lower inoculum.

3.3 Production of Influenza VLPs using RMCE

The double-RMCE insect cell lines developed above (with two tagged loci using the F_wF_5 and $F_{13}F_{14}$ cassettes) showed to be unfeasible to express multi-gene products. Therefore, it was decided to evaluate the capacity of stable insect cell lines (Figure 17A) to produce influenza VLPs by co-expressing two influenza viral proteins – M1 and HA - from the same locus. As represented in Figure 17B, the same promoter was used to drive the expression of both proteins, OpIE2, the strongest insect cell promoter available for stable expression.

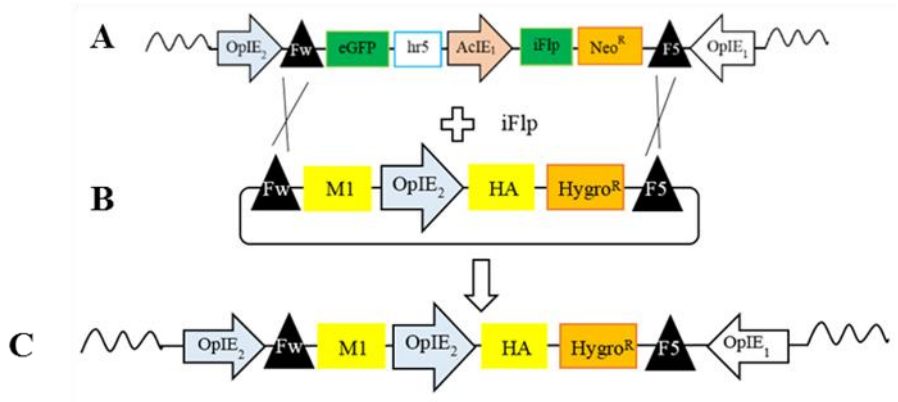

Figure 17 - A) Construct integrated in clones. B) Target vector encoding M1 and HA genes and C) Resulting population expressing M1 and HA from the same locus after RMCE.

3.3.1 Cell line development

To generate RMCE cell platforms singly tagged in high expressing and exchangeable loci, with lower screening efforts, the sequence of steps outlined in Figure 18 were followed. Firstly, Sf9 parental cells were tagged with an iCherry-containing vector, then enriched with the 30% strongest iCherry-expressing cells by FACS, and finally submitted to RMCE to exchange for an eGFP reporter cassette. Promoters driving expression of the target genes were placed outside the region flanked by the FRTs (Figure 18) to allow a faster selection of cells that have exchanged cassettes. This occurs because the promoterless genes in the incoming cassette will only be expressed if it replaces the FRT flanking region in the cell genome. Upon selection with G418, the resulting population was sorted using FACS to select $eGFP^+/iCherry^-$ cells. Once sorted, the cloning procedure was done to isolate cells containing strong and amenable to Flp-recombination loci. Noteworthy, the target cassette also contained iFlp coding-gene to avoid its addition in subsequent RMCE steps.

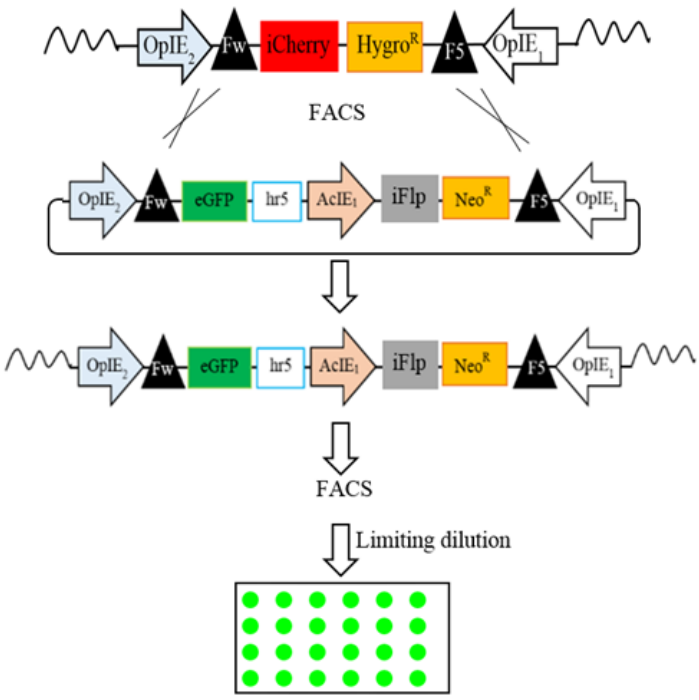

Figure 18 - Cell line development from tagging to cloning

3.3.2 Clones' characterization

Before using the clones to produce the genes of interest, it is important to have them thoroughly characterized. Two isolated Sf9 cell clones (#3 and #4) were analysed for the potential expression of the tagging genes (iCherry and hygromycin). By using specific primers for each gene (products size of 206bp and 245bp) mRNA analysis confirmed that tagging genes were not expressed (Figure 19A) and flow cytometry data also supports these results (Figure 19B). Furthermore, relatively stronger eGFP gene expression in clone #3 was observed compared to clone #4 (Figure 19A). As expected, the resistance gene expression is much lower than the reporter's gene due to the difference in promoter strength ($OpIE_2$ vs $OpIE_1$).

To evaluate if the tagging cassette had been eliminated upon RMCE, genomic DNA of both Sf9 clones was extracted and primers were used in a PCR targeting for $OpIE_2$ and $OpIE_1$. The agarose gel electrophoresis represented in Figure 19C shows the amplification of a 5,6kbp band in clones and a 2,9kbp band for the tagging population and this size difference shows that tagging cassette was eliminated in the RMCE step. In addition, it is confirmed

that there is expression of iFlp in clones (Figure 19A), thus suggesting that addition of iFlp is not needed in further experiments of RMCE. In order to evaluate if this expression is enough to allow efficient cassette exchange, both cell clones were transfected with an iCherry-containing cassette in the presence/absence of an iFlp-containing vector. After 72 hours post transfection, flow cytometry analysis showed 0,3% of iCherry positive cells when adding iFlp and 0% when the enzyme was not supplied (data not shown). Cultures were kept in selection with hygromycin and the same trend was observed over time as confirmed by fluorescence microscopy and flow cytometry. Therefore, although iFlp is being expressed it could not promote RMCE.

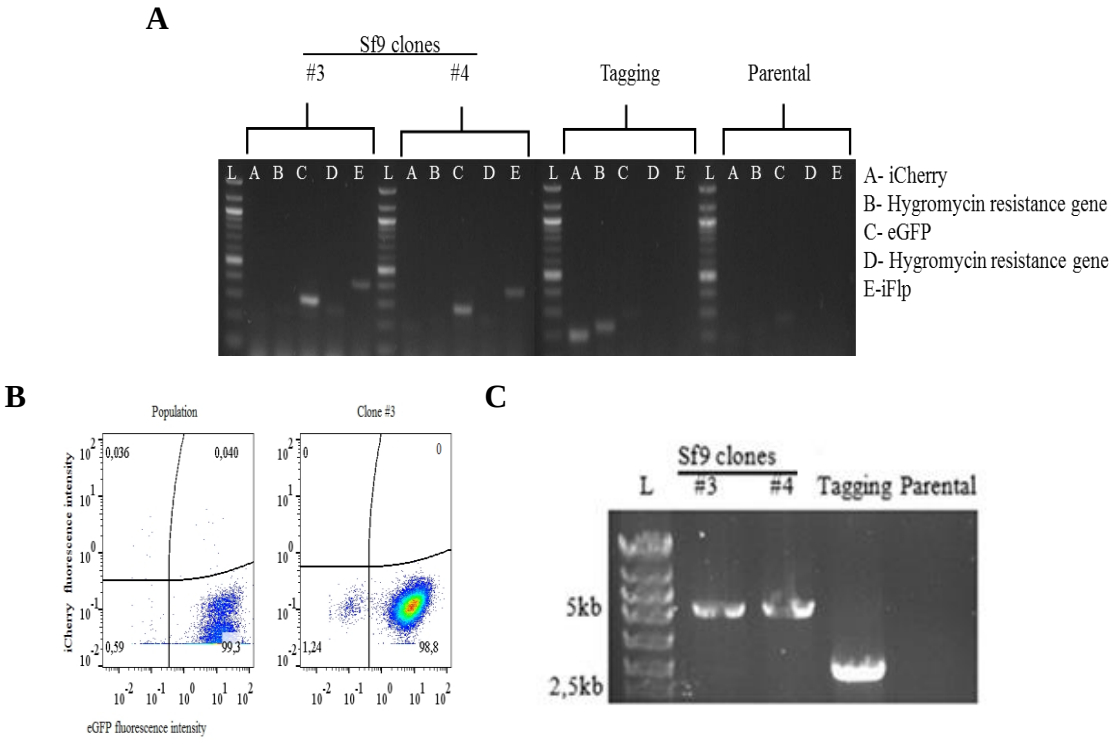

Figure 19 - Characterization of Sf9 clones #3 and #4 according to the presence of (A) expression of tagging and target genes by mRNA analysis and (B) flow cytometry. Primers were constructed to anneal with specific regions of each gene in study; L-ladder (Quick-Load 100bp); C) Identification of tagging cassette in clones. Genomic DNA was extracted and primers were located at OpIE2 and OpIE1 promoters amplifying 5,6kbp fragment in a target population and a 2,9kbp in a tagging population. Ladder used: NZY DNA ladder III.

3.3.3 Production of influenza VLPs in the same locus

The Sf9 clone #3 was selected for the production of influenza VLPs due to the (1) absence of tagging cassette and expression of tagging genes and (2) enhanced eGFP expression when compared to Sf9 clone #4.

This clone was submitted to RMCE with a M1-HA encoding cassette as depicted in Figure 17B. After the selection process, resistant cells were transferred to suspension cultures and purity of this population was assessed by flow cytometry. Results showed that 31% of the cells were still eGFP positive (Figure 20A), also confirmed by fluorescence microscopy (Figure 20B). M1 and HA gene expression was evaluated by mRNA analysis. Although regulated by the same promoter (OpIE2), HA gene expression was considerably stronger than M1 (Figure 20C). The growth curve of this population was followed and the levels of HA assessed for culture supernatants (data not shown) in order to check if HA protein could be released from the cell. HA was detected after 165 h of culture but at a very low concentration (0,04μg/mL increasing to 0,08μg/mL at 187.5 h). Later, the amount of M1 (28kDa) in cellular extracts and supernatants was examined by western blot (data not shown) though its detection was not achieved even at the end of culture (187.5 h). This explains the low concentration of HA in the supernatant as M1 is very important for the secretion of HA. The lack of M1 expression was not expected as gene expression was confirmed by mRNA analysis.

Summing up, the inability to detect M1 production and the low concentration of HA in the supernatant may be the due to weak targeted locus in this Sf9 clone. Even though eGFP fluorescence intensity was acceptable, as shown in Figure 20D, this was not reproducible to a more complex multi-protein product.

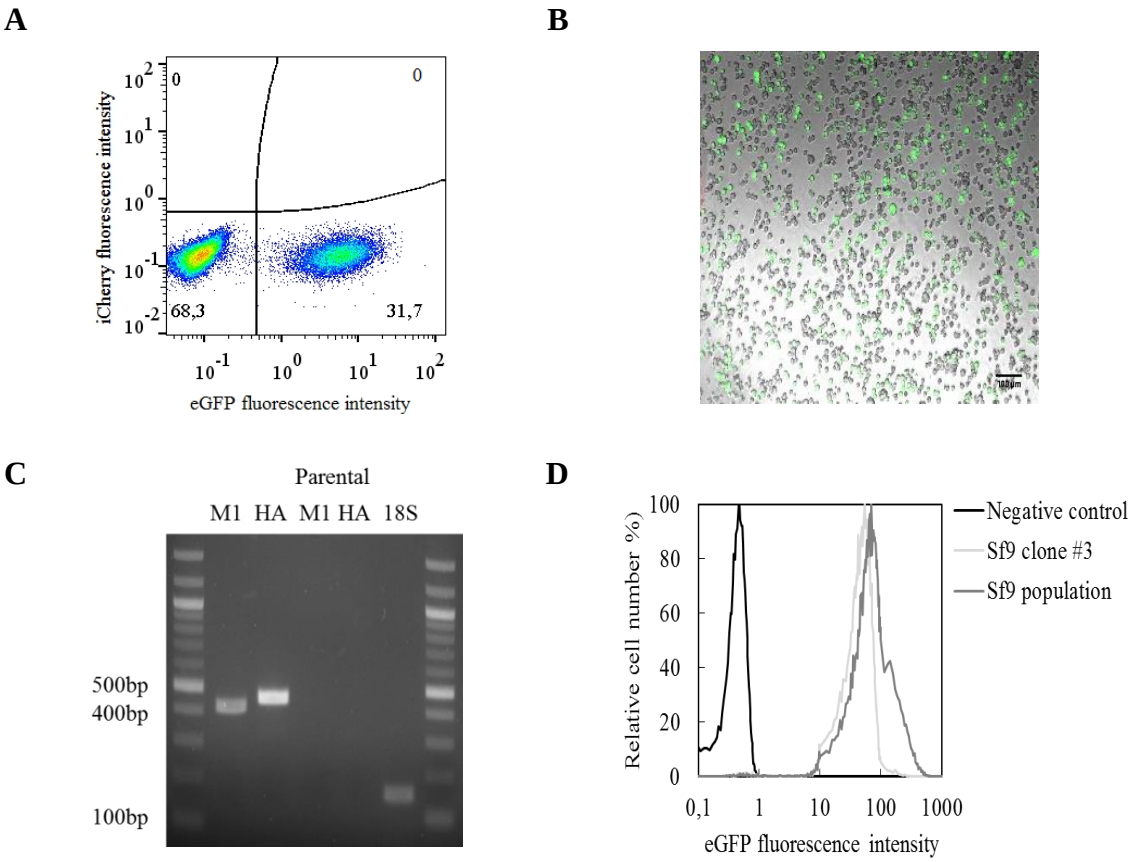

Figure 20 - Detection of eGFP positive cells in population that was submitted to RMCE to M1-HA by (A) flow cytometry and (B) fluorescence microscopy (scale bars are in 100µm). (C) M1 and HA gene expression analysis. Parental cDNA was added as a negative control in a PCR with primers for M1 and HA amplification and 18S cDNA analysis was added as an endogenous control. Ladder used: Quick-load 100bp. (D) Comparison of fluorescence intensity between a population and clone #3 of Sf9.

4 Discussion and conclusions

In this work, optimization of two different insect cells platforms for production of influenza VLPs was approached. A strategy for supporting baculovirus production of multiple proteins was designed and bioprocess optimization was conducted. Three major tasks were performed to achieve such goal: 1) identification of key nutrients during cell growth and their extra addition at a critical time of the culture to increase viable cell concentration with high viabilities; 2) apply this feeding strategy to increase the CCI and consequently protein production; 3) prove the scalability of the process. Then, preliminary work in establishing a double-locus flippase RMCE system was performed and Sf9 clones were generated to produce multimeric products.

4.1 Dual strategy for production of Influenza VLPs

To our knowledge this is the first time stable expression of HA was attempted in insect cells. Cell growth impairment due to genetic instability and/or protein-induced cytotoxicity is a potential bottleneck for stable/continuous cell cultures. Furthermore, product yield and/or quality must be maintained during several cell passages. The results presented in this thesis show that the growth performance of Hi5 cells is dependent on the HA proteins being produced, illustrated by the differences in early passages (below 14) between pool 1 and pool 2. Also, it is shown that it is possible to obtain a similar growth performance over several sub-culture steps in a given population. Likewise, although not constant along passages, the HA expression levels were similar for both populations tested, thus corroborating the observation that insect cells are a competent platform for stable protein production, which has been previously shown by other authors (Fernandes et al., 2014). The PDT of both populations were slightly higher than those observed for non-transformed Hi5 cells which might be explained by the competition between endogenous and heterologous genes for the cellular machinery.

When using the insect cells-baculovirus system, the production of recombinant proteins as well as baculovirus is impaired at high CCIs (above $2x10^6$ cells/mL) - the so called cell density effect (Carinhas et al., 2009; Caron et al., 1990). This phenomenon is not

well understood, but key factors implicated are the lack of nutrients and/or oxygen, and accumulation of toxic by-products (Bernal et al., 2009; Carinhas et al., 2010). In order to investigate this phenomenon, the HA production levels of the two stable Hi5 pools herein generated were assessed at two different CCIs ($2x10^6$ cells/mL and $3x10^6$ cells/mL) using the same baculovirus. Results show that HA expression is independent of CCI for pool 1 but not for pool 2. However, maximum HA production was obtained one day earlier (day 2 post-infection) for pool 1, at the highest CCI. From a bioprocess perspective, this encloses a major advantage: due to higher cell viabilities, less contaminants such as viral and host DNA are present in the culture supernatant, thus potentially reducing downstream processing time and cost.

To cope with the cell density effect problem, a number of strategies ranging from improving medium composition, total or partial replenishing of medium at infection, nutrient supplementation schemes (Caron et al., 1990; Nguyen et al., 1993; Wang et al., 1993), fed-batch processes and perfusion cultures have been used to improve protein production (Ikonomou et al., 2003). In this work, the consumption and production of metabolites in the supernatant was followed along culture time and identified the depletion of glucose and crucial amino acids, such as asparagine and glutamine (Mendonça et al., 1999; Monteiro et al., 2014). Then, by replenishing these nutrients together with a cocktail of lipids and vitamins (Fernandes et al., 2014), it was possible to increase the peak of cell density (from $3.6x10^6$ cells/mL to $4.9x10^6$ cells/mL) and keep high viabilities during longer culture time. Performing infections at a CCI of $4x10^6$ cells/mL, with two different baculovirus, using this supplementation scheme, enabled to increase HA titers up to 4-fold. Infection with a rBac-M1 showed that the metabolic boost induced to the cells enabled the enrichment of culture supernatant in HA, showing once again that this re-feed strategy improved stable protein expression.

Noteworthy, significant build-up of lactate in later stages of growth was observed, especially in supplemented cultures (up to 14mM). This issue has been reported by other authors and can be due to 1) limitations of oxygen supply experienced in shake flask cultures (Bédard et al., 1997; Rhiel and Murhammer, 1995) and/or (2) the high glucose concentration in the medium (Drugmand et al., 2005). In addition, Hi5 cells consume a great amount of

glucose during growth which when depleted can be “replaced” by lactate consumption thus explaining the decrease in lactate concentration by the end of the culture (Monteiro et al., 2014).

As a proof-of-concept, the scale-up process was performed from shake-flasks to 2L bioreactor. The high CCI used ($4x10^6$ cells/mL) for influenza VLPs production and the subsequent increase in cells specific O_2 uptake rate after infection require high oxygen supply that only bioreactors can offer (Kioukia et al., 1995). Oxygen levels seem to be very important for product expression and its quality (Cruz and Peixoto, 1998). In addition, lactate concentrations above 5mM impair specific protein productivities (Drugmand et al., 2005). Although a rapid consumption of glucose was observed during infection (data not shown), this was not accompanied by an increase in lactate concentration. These results suggest that careful monitoring and control of oxygen supply in the bioreactor is essential to avoid build-up of toxic compounds. That is probably why the infection process lasted longer in bioreactor than in small scale experiments (3 days instead of 2). Likewise, by allowing the culture to last longer, cells had more room for protein production before cell death occurred. These conditions allowed a 1.5-fold volumetric increase in HA production compared to shake flasks, proving that the scalability of the process could be successfully achieved. An increase was also seen in specific productivity, from 0,69 $\mu g/10^6$ cells to 1,08$\mu g/10^6$ cells.

HA and M1 proteins accumulated during infection as a result of the successive viral replication. In addition, it is known that both proteins interact with each other and are more prone to be released from the cell when together (Enami, 1996; Gómez-puertas et al., 2000). The fact that HA is detected in the supernatant prior to infection and without expression of M1 can be due to changes in membrane permeability induced by influenza hemagglutinin, leading to leakage of contents from cells, a phenomenon reported in influenza virus infected cells (Blumenthal and Morris, 1999; Frolov et al., 2003).

4.2 Suitability of RMCE for production of complex proteins

To further improve the flexibility of a RMCE-based platform, a double-tagged approach was designed to provide two loci for gene expression, hence higher copy numbers and accumulated gene expression, as well as to allow the production of multi-subunit products more easily. However, the cross-interaction between both pairs of target sites chosen was confirmed (Turan et al., 2010) making it unfeasible.

The next step was to rationally develop a strong expresser cell line that could be pure concerning tagging genes/cassette due to RMCE, which was successfully accomplished, and robust in protein production. The inclusion of iFlp gene aimed at eliminating the need of its further addition in subsequent steps, thus shortening cost in plasmid preparation and more unwanted DNA material integration. Despite this effort, iFlp gene expression was not sufficient to allow RMCE. This negative result was not expected and indicates that maybe it is a process dependent on the amount of enzyme provided.

Clones were obtained by limiting dilution which is a technique that leads to a broad expression pattern of the selected clones. In this sense, characterization of two Sf9 clones was conducted to assess their potential usefulness for expression of complex proteins. Results revealed the absence of tagging genes and cassette in both clones, which means that RMCE was successful in eliminating previous tagged genes as already suggested in the literature (Fernandes et al., 2012). However, a more sensitive technique should be performed to confirm the absence of tagging cassette in the genome, such as Southern Blot. Regarding gene expression, a positive relation between tagging and target genes is suggested as a result of RMCE which may enable to predict levels of expression (Coroadinha et al., 2006; Fernandes et al., 2012; Qiao et al., 2009). Intensity profiles of the Sf9 population compared to clone #3 do not show an improvement in eGFP fluorescence intensity which lead to the conclusion that this was not a strong clone. The difference in levels of expression for eGFP and neomycin resistance gene was expected and are related to the strength of their respective promoters where OpIE2 can be, at least, 5-fold stronger than OpIE1 (Pfeifer et al., 1997). However, such a low expression of the resistance marker was not foreseen.

For the production of influenza VLPs, the most important proteins – M1 and HA – were expressed under the control of the OpIE2 promoter. The relatively low abundance of

HA found in the supernatant can be due to the 32% of contamination with eGFP or a problem in M1 production. The fact that M1 identification was not possible by western blot but with gene expression detection leads to the speculation that the problem resides in a poor translation efficiency. Reports have been made on the existence of a threshold of M1 to more efficiently allow the release of HA from the cell (Bourmakina and García-Sastre, 2005). Thus, if M1 is not present in such a level to drive the release of HA, it explains the low amount of this protein in the supernatant. The presence of HA in the supernatant, if M1 is present in small amounts or not at all, can be explained by HA-membrane induced permeability as described for infected cells (Blumenthal and Morris, 1999; Frolov et al., 2003). Comparison between M1 and HA gene expression revealed to be stronger for HA, a difference that was not expected as their expression is driven by the same promoter. Reports have been made on the influence of chromosomal context and the vector construction in promoter strength (Nehlsen et al., 2009; Pfeifer et al., 1997). Thus, maybe the FRT settled between M1 and the promoter had a negative impact in gene expression and/or the transcriptional elements implicated in the targeted locus had different effects in both genes.

4.3 Conclusions and future work

In this work, two different insect cells based platforms for production of influenza VLPs were designed and implemented. The first one consisted in combining stable expression of multiple HAs in Hi5 cells, based on random integration of the GOIs, with baculovirus-mediated expression to produce multi-HA influenza VLPs. Bioprocess optimization was conducted in order to enhance HA protein expression. It was shown that identification of key nutrients being exhausted during cell growth and their ensuing supplementation to the culture medium had a positive impact on viable cell concentration and, most importantly, on HA production via the increase of CCI. In addition, a proof-of-concept scale-up experiment was performed in order to assess the potential of the strategy herein develop for rapid delivery of substantial amounts of influenza VLPs. Successful scale-up was attained with enhanced HA protein levels observed between 2L bioreactors and shake flasks experiments. As future work, a comprehensive study of Hi5 cells metabolism before and upon baculovirus infection will be essential to design re-feed strategies capable of further

extending cell growth and CCI, thus potentially inducing higher HA expression. Likewise, a perfusion system in bioreactor can be designed to allow continuous feeding and removing spent media as a way to extend culture time. In order to take advantage of RMCE-based cell line development, stable expression can be translated into strong expresser clones, once isolated, to allow expression/production predictability, bypassing the unpredictability associated with random integration.

The second insect cell based platform herein developed for the production of multi-HA influenza VLPs was based in a RMCE approach. Efforts were conducted into developing a double-locus system though not successfully. Also, iFlp integration in the population did not allow RMCE to occur in clones, thus indicating that this is a process dependent on the amount of enzyme provided. In addition, the negligible expression of M1 and HA proteins in clone #3 combined with its low enrichment in fluorescence intensity when compared to a population of cells suggested that clone #3 is extremely weak regarding gene expression. A more robust cloning method should be used (e.g. FACS) to enable the selection of high expressing cells based on their fluorescence intensity. In addition, a thorough evaluation of Sf9 and Hi5 clones and cell populations for HA protein production should be attempted to discriminate the best platform.

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Appendix A

A.1 Table of primers used in the construction of vectors needed in this work.

Amplified gene(s)	Fw primer	Rv primer	Receiving vector	Final vector
HA_1	CGAATTTAAAGCTTGAAACTCGTCAAAGCCACCATGA	GTGGATCCGAGCTCGGACGCCAGAAAGGGGATTAGATAC	pIZT/V5-His	pIZT/HA_1
HA_2	AGCACAGTGGCGGCCAAACTCGTCAAAGCCACCATGA	TAGACTCGAGCGGCCGACGCCAGAAAGGGGATTAGATAC	pIZT/V5-His	pIZT/HA_2
OpIE2 and HA_2	TCGATGCTCACTCAAGATCATGATGATAAACAATGTATGG	ACATGTTCTTTCCTGCCTGATTCTGTGGATAACCGTATTA	pIZT/HA_1	pIZT/$HA_{1,2}$
HA_3	GCTTGGTACCGAGCTCAAACTCGTCAAAGCCACCATG	GGACTAGTGGATCCGTGATCCTTAGACGCCAGAAAGG	pIZT/V5-His	pIZT/HA_3
OpIE2 and HA_2	TCAAGCGCGTGGGATGATCATGATGATAAACAATGTATGG	TTTGAGTGAGCATCGCCTGATTCTGTGGATAACCGTATTA	pIZT/HA_3	pIZT/$HA_{2,3}$
iCherry + hygromycin	CGGCCGCCATGGTTAGCTTCTACCATGGTGTCCAA	TTGCGAATTCGCTAGCATGAAGAAACCTGAACTGAC	pTagg	pTaggF_{13}/F_{14}
eGFP + neomycin	TCCGAAGTTCCTAGCCGATTTAGGTGACACTATAGAACTC	TTGCGAATTCGCTAGTGGATGATTGAACAAGATGG	Promoterless pTaggF_{13}/F_{14}	pTargetF_{13}/F_{14}
OpIE2 + HA	TCAAGCGCGTGGGATGGGGCATGCGGATCATGATG	GTATGGGCTAGCTCACTGGTTCTTTCCGCCTCAGAAG	peGFP/Hygro	pOpIE2 M1/HA+eGFP
M1	TAGGAACTTCGGATCGTTACAGGGGTCAATTCAGAGG	GCTAGCTTTGCGGCCATGAGCGGAAATTGAGGAGAAGC	pOpIE2 M1/HA+eGFP	pOpIE2 M1/HA

A.2 Primers used for cDNA detection.

Amplified cDNA	Fw primer	Rv primer	Fragment size (bp)
iCherry	CCCCCCAGTTCATGTACGGTTC	AGGGGAAGTTGGTACCACGCAG	206
Hygromycin	CTGCAGTGATTCTGCCATTGTCTGT	GATTTGGTCCTTTTGGTCCCCA	245
eGFP	CCCTCGTGACCACCCTGACCTA	GTTCTTCTGCTTGTCGGCCATG	301
Neomycin	CCTTGAGCCTGGCGAACAGTTC	ACTGGCTGCTATTGGGCGAAGT	257
Flippase	GACATCGTGTCCCGTCTGCA	CTCGGAGTTGCGCAGGAACT	408
M1	AGACCAATCCTGTCACCTCTGACT	TCGATCCAGCCATTTGCTCCAT	442
HA	CCAACCACACCGTAACCGGAGT	GGTGTTTGACACTTCGCATCAC	472
18S	AGGGTGTTGGACGCAGATAC	CTTCTGCCTGTTGAGGAACC	163

A.3 Primers used for cDNA detection. Primers used for detection of tagging and target cassettes.

Annealing site	Primer
$OpIE_2$ promoter	Fw: GCCGCGCGTTATCTCATGCGC
$OpIE_1$ promoter	Rv: GCCGTTGGTGGCGTGAGGCATGTAA