Flexible insect cell platforms for fast production of pseudotyped virus-like particles

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Abstract

The incorporation of membrane proteins on the surface of virus-like particles (VLPs) from enveloped viruses, such as Influenza virus and Retrovirus, can be a powerful strategy to providing high concentration of membrane proteins.

This study demonstrates the development of insect cell platforms using targeted gene integration based on recombinase mediated cassette exchange technology for production of Gag-VLPs displaying target membrane proteins.

Sf9-Gag and Hi5- Gag clones with a red reporter protein (iCherry) previous obtained were thoroughly characterized in order to identify those to then co-express from the same locus the model G-Protein coupled receptor (GPCR), Adrb2. A significant increase in the specific Gag secretion rate was obtained from the populations to the isolated clones, demonstrating the value of the cell line development strategy here implemented.

To further improve the stable production of recombinant proteins in insect cells, we tested several bioprocess strategies, such as, the adaptation of cells to hypothermic conditions, addition of NaBu or DMSO, and supplementation with key nutrients. The production of Gag protein using lower culture temperature was successful and we determine that Gag production can be enhanced through adaptation of cells to low culture temperature. In addition, supplementation with DMSO, NaBu or different nutrients also have positive impact in protein production.

Ongoing work focuses on the combination of these strategies to assess their synergistic effect and contribute to further increase the production of Gag VLPs in insect cells.

Keywords: virus-like particles (VLPs), membrane proteins, GPCR, Insect cells, recombinase mediated cassette exchange, cell line development

Resumo

A incorporação de proteínas de membrana na superfície de partículas semelhantes a vírus (VLPs) derivadas de vírus de envelope pode ser uma robusta estratégia para obter elevadas concentrações de proteínas de membrana.

Este estudo demonstra o desenvolvimento de plataformas de células de inseto usando estratégias de integração sitio-específicas baseadas no sistema de troca de cassete mediada por recombinases para produção de proteínas de membrana ancoradas em Gag-VLPs. Previamente foram obtidos clones de células Sf9 e Hi5 integrando o gene da proteína Gag fundida com uma proteína repórter (iCherry) foram caracterizados de modo a identificar os que iriam co-expressar no mesmo local o recetor ligado à proteína G (GPCR), Adrb2. A secreção de Gag das populações para os clones isolados aumentou, demonstrando o valor da estratégia de desenvolvimento da linha celular aqui implementada.

De modo a aumentar a produção de proteínas recombinantes em células de inseto, nós testámos diferentes estratégias de bioprocesso, nomeadamente: adaptação de células a condições hipotérmicas, adição de NaBu ou DMSO e a suplementação com nutrientes chave. A produção de proteína Gag usando baixas temperaturas de cultura foi bem-sucedida, 22ºC foi a temperatura onde houve maior produção de proteína. Comparando células adaptadas e não adaptadas, a adaptação foi crucial para um maior aumento na produção. A suplementação com DMSO, NaBu ou diferentes nutrientes também teve um efeito positivo na produção de proteína. O trabalho futuro irá focar-se na combinação destas estratégias de modo a ter um efeito sinergético e contribuir para aumentar ainda mais a produção de Gag-VLPs.

Palavras-chave: partículas semelhantes a vírus (VLPs), proteínas membranares, recetor ligado à proteína G, células de inseto, troca de cassete mediada por recombinase, desenvolvimento de linha celular

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AcMNPV- Autographa californica multicapsid nucleopolyhedrovirus
Adrb2- beta-2 adrenergic receptor
BCA- bicinchoninic acid assay
BEVS- Baculovirus expression vector system
BHK-21- Baby hamster kidney cell
BV- Baculoviruses
CA – capsid domain
CHO- Chinese hamster ovary
Cys- Cysteine
DMSO- dimethyl sulfoxide
DNA- Deoxyribonucleic acid
***E.coli* -** Escherichia coli
ECL- enhanced chemiluminuscence detection system
FACS- fluorescence-activated cell sorting
Flp- Flipase
G418- Neomycin
GFP- green fluorescence protein
Glc- Glucose
Glu- Glutamine
GOI- Gene-of-interest
GPCRs- G-protein coupled receptors
HA- Hemagglutinin
HBV- Hepatitis B virus
HDAC- histone deacetylase
hESCS- embryonic stem cells
Hi5 - High-Five™ cells
HIV- human immunodeficiency virus
HPV- Human papiloma virus
HR- Homologous recombination
Hygro- Hygromycine
iPSc- induced pluripotent stem cells
IR- Illegitimate recombination
MA- matrix domain
mABs- Monoclonal antibodies
MPs- Multispanning membrane proteins
NaBu- sodium butyrate
NaCl- sodium chloride
NC- nucleocapsid domain

PBS- phosphate buffered saline

PCR- polymerase chain reaction

PEG- plyethylene glycol

rBV - Recombinant baculovirus

RMCE- recombinase-mediated cassette

RV- rotavirus

Ser- Serine

Sf21- Cell line derived from *Spodoptera frugiperda* ovarian cells

Sf9- Cell line derived from Sf21

SSR- Site-specific recombinase

VLPs- Virus-like particles

Zeo- Zeocine

1. Introduction

1.1 Expression of recombinant proteins

Efficient strategies for the production of recombinant proteins are increasingly needed because several applications require high amounts of high-quality protein at lower production costs. To study the function and potential of most proteins as therapeutics or drug targets they have to be produced with the aid of genetic and protein engineering [1].

In order to produce a recombinant protein, the first step is to assemble the corresponding gene sequence into a vector that will delivery it into the chosen biological system. There is an extensive variety of protein expression systems available [1], including many unicellular (prokaryotic and eukaryotic) organisms and immortalized cell lines derived from a variety of organs and types eukaryotic multicellular organisms (Table 1) [2]. Depending on the cell host different molecular biology techniques can be used, as well as different production modes and product recovery strategies.

Despite the complexity, expression in eukaryotic systems has been increasing in popularity within the scientific community over the last two decades [3]. This is mainly because these cells have the capacity to carry out post-translational modifications, such as glycosylation or phosphorylation [3–5]. Non-glycosylated proteins are usually produced in *Escherichia coli* (*E. coli*) in contrast with glycosylated ones where more complex systems, as insect and mammalian cells, are needed.

Table 1 Comparison of expression systems in terms of advantages and yield regarding recombinant pharmaceuticals (adapted from Beljelarskaya, 2002 and Demain et al, 2009)

Expression System	Advantages	Protein yield % dry weight
Bacterial cells	• Rapid expression • High yields • Mass production fast and cost effective • Ease of culture and genome modifications	1-5%
Yeast	• High yield • Stable production strains • Durability • Cost effective • High density growth and productivity • Product processing similar to mammalian cells	1%
Insect cells	• Post translational modification similar to mammalian systems • High expression levels • Easy scale up • Safety • Multiple genes expressed simultaneously	25%
Mammalian cells	• Improved levels of correct posttranslational modifications • Increased probability of obtaining fully functional human proteins	<1%

1.2. Membrane proteins

In all cellular organisms, the plasma membrane is the furthest layer of the cell and has the function to separate the cell from the external environment. Membranes contain several types of interacting membrane proteins [6,7]. These proteins represent approximately 20-30% of the open-reading frames of an organism's genome and play crucial roles in basic cell functions including signal transduction, energy production, nutrient uptake, and cell-cell communication [8]. Despite their prevalence in the genomes, less than 2% of the listed 3D structures in the protein data bank are membrane proteins, due to the technical challenges associated with membrane protein solubilisation and purification in sufficient quantities for crystallisation [8]. It is important to

develop strategies to increase the production yields of these proteins to be studied and used in clinical applications.

1.2.1 G-protein coupled receptors

Multispanning membrane proteins (MPs), such as ion channels and G-protein coupled receptors (GPCRs) comprise the largest family of membrane receptors in the mammalian genome with approximately 850 members [9,10]. These proteins interact with extracellular ligands to produce multiple biological responses, including cell adhesion, signalling and regulatory events.

GPCR dysfunction has been implicated in a variety of pathological conditions including cancer, cardiovascular, metabolic, neurodegenerative and psychiatric disorders, making them important targets for therapeutic intervention. [9,10]. Approximately 50 % of all marketed small drugs act on these receptors[11]. Furthermore, the sequencing of the human genome uncovered many novel orphan GPCRs with unknown function, demonstrating that much remains to be explored in this protein family for drug discovery [12]. Recently, the potential of GPCRs also as antibody targets has started to be explored [13]. Raising antibodies to GPCRs has been difficult due to problems in obtaining suitable antigen because GPCRs are often expressed at low levels in native cells and are very unstable when purified. Interpretations of cell-based assays studies using traditional techniques with fluorescently labelled or radiolabelled ligands may in some cases be complicated by other cell surface components present at much higher concentrations that the GPCR of interest [14]. Due to the importance of these proteins is important to find new methods of purification/enrichment to solve some of the problems associated.

1.3 Virus-Like particles

Virus-like particles (VLPs) are viral proteins that self-assemble into complex structures mimicking the conformation of the native virus, but devoid of viral genetic material [15]. They are therefore non-infectious and non-replicative with a similar morphology to the natural virus [16,17]. VLPs can be divided into two major types based on the structure of their parental viruses: non-enveloped VLPs and enveloped VLPs.

Non-enveloped VLPs are typically composed of one or more components of a pathogen with the ability to self-assemble into particles and do not include any host components[17]. On the other hand, enveloped VLPs are relatively complex structures consisting of the host cell membrane, the envelope, with integrated target antigens displayed on the outer surface [17].

It has been demonstrated that VLPs can be useful in several applications such as serological testing, vaccination and gene therapy with delivery of immunogens or nucleotide sequences [16]. These structures represent sophisticated subunit vaccines with enhanced immunogenicity over inactivated virions because during the inactivation process native epitopes lose their folding thus reducing their capacity to stimulate a strong immune response [18]. Currently, there are four

recombinant vaccines on the market that are based on highly purified VLPs: GlaxoSmithKline (GSK)'s Engerix® (hepatitis B virus [HBV]) and Cervarix® (human papillomavirus [HPV]) and Merck and Co., Inc.'s Recombivax HB® (HBV) and Gardasil® (HPV) [17]. There are also a number of VLP-based vaccine candidates in pre-clinical or clinical development, targeting pathogens such influenza virus, rotavirus (RV) and human immunodeficiency virus (HIV) [17]. The ability of VLPs to cause strong immune responses makes them attractive antigen-presenting vehicles to be administered against many diseases [15]. Chimeric VLPs have also been developed by genetic fusion or chemical conjugation of foreign epitopes to viral structural proteins capable of self-assembly into VLPs (normally viral proteins with limited or no surface exposure) [15].
More recently, new VLPs that can be used as carrier in a variety of applications have been explored, including drug delivery, vaccines, imaging and developing diagnostics kits.

1.3.1 Enveloped VPLs pseudo-typed with membrane proteins

Enveloped VLPs provide a higher degree of flexibility for integration of more antigens from the same or heterologous pathogens and their production requires co-expression of several structural viral proteins and their assembly into particles (budding) from the cell membrane [17].
Enveloped VLPs when used as scaffolds for membrane proteins enable an enrichment of this specific protein concentration in their native structural conformation. For this the target membrane proteins needs to be co-expressed with capsid proteins from enveloped virus leading to the release of virus like particles displaying the membrane proteins correctly folded on their lipidic surface, the viral core protein will trigger VLP budding and release from lipid raft regions of the plasma membrane taking along the anchored target protein. Examples of enveloped viruses that be used as scaffold for pseudotyping are *Influenza* virus or Retrovirus [19].
The main structural protein of the Retroviridae virus family, Gag, has been the mostly used to produce VLPs as shown in Figure 1 [20]**.** In a VLP context, it has been shown that Gag assembles even in the absence of any other viral factor in the lipid raft regions of transduced/transfected cell and leads to the budding of VLPs into the culture supernatant [21]. The use of Gag as a carrier has been mainly explored to produce viral antigen-displaying VLPs that have been proposed as alternatives to conventional vaccines [22–24].
Furthermore, pseudo-typed VLPs could be an ideal platform for high-throughput screening of large libraries of compounds when searching for inhibitors of highly pathogenic viruses (e.g. H5N1 influenza virus), substituting the use of live viruses for obvious biosafety reasons.

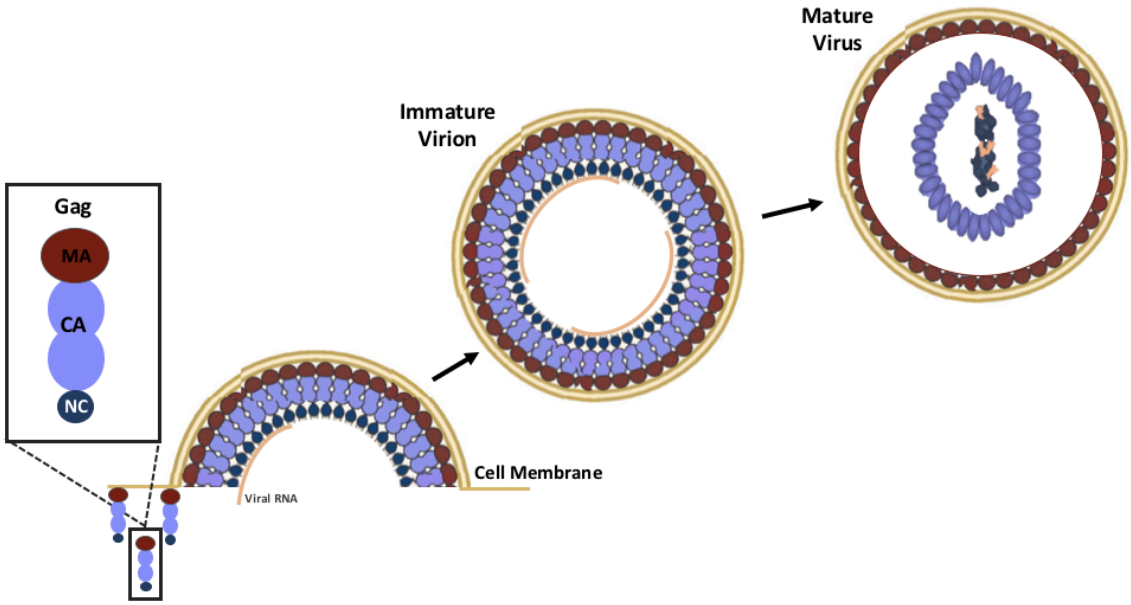


Figure 1 Gag and retrovirus particle assembly. Cross section of the assembly of a prototypic retrovirus particle, emphasizing the oligomeration of Gag (the main structural protein of retroviruses) along the inner leaflet of the plasma membrane. Two copies of viral rna are incorporared and occurs the budding of immature virus particles and the conversion of the immature virus particle to mature infectious virus particle that is catalysed by the viral-encoded protease. Gag is shown as being composed of the matrix domain (MA), the capsid domain (CA), and the nucleocapsid domain (NC). Two copies of the viral RNA (two orange lines inside the viral particle) are shown packaged into the virus particles [19,20]. Adapted from Maldonado JO et al, 2014.

1.3.2 VLPs displaying membrane proteins for antibodies screening

Monoclonal antibodies (mABs) have attracted considerable interest in the treatment of cancer and autoimmune disorders. One of the important issues for the next generation of therapeutic antibodies is to obtain a higher affinity for the purpose of targeting less abundant surface molecules [25]. It is difficult to raise high affinity antibodies against membrane proteins. Considering for instance GPCRs, which possess seven transmembrane regions with both intracellular and extracellular loops, an effective antibody must recognize the receptor in its native conformation by binding to critical epitopes that are accessible in the extracellular domain and elicit a biological response. Current immunization approaches using peptides, purified membrane proteins, membrane preparations, or whole cells had limited success in generating conformational antibodies against many membrane proteins [13]. There are some advantages of targeting GPCRs with antibody therapeutics related with drugability, selectivity and distribution. The costs of antibody development and manufacture are higher but they have in general a higher approval success rates when compared with new chemical entities and have a much longer duration of action [13].

Membrane proteins can be incorporated in enveloped VLPs to be further used to generate antibodies. For the successful generation of VLPs as modular antigen-presenting platforms, it is

required the co-expression of a viral core protein with the target membrane proteins in a host cell [17,25].

1.4 Platform to produce virus-like particles

There are many expression systems for the production of VLPs, such as mammalian cells transiently or stably transfected or transduced with viral expression vector, the baculovirus/insect cell system, various species of yeast including *Saccharamoyces cerevisiae* and *Pichia pastoris* and *E. coli* and other bacteria [26]. Oral vaccine initiative has also produced HBV and Norwalk virus VLPs from various plants, including tobacco and lettuce leaves as well potato [26].

The baculovirus/insect cell system has been extensively used for VLP production, both non-enveloped and enveloped VLPs. In clinical development the enveloped VLP vaccines produced in insect cells are among the most advanced [17].

1.4.1 Insect cells - Baculovirus expression vector system

Insect cell culture is a mature technology, which has been applied in the production of recombinant proteins for the past 30 years [27]. Insect cell lines are able i) to grow in suspension in chemically defined, serum- and protein-free culture media reaching high cell densities [27], ii) to carry out complex post-translational modifications (including glycosylation, phosphorylation, fatty acid acylation, and amidation), and iii) properly fold mammalian proteins [1].

An advantage of insect cells in comparison with mammalian cells is that they are relatively cheap to maintain in culture, can be scaled up relatively easily and have high expression levels [28]. The optimal growth temperature of insect cells is 27ºC and the pH is lower (6,2-6,6) which is commonly maintained by a phosphate buffer without the need of carbon dioxide in the culture [27]. In comparison to mammalian cells, insect cells are characteristically more resistant to temperature and osmolarity fluctuations which is another advantage for their biotechnological applications [29,30]. Insect cells can be cultivated in static (e.g. T-flasks) and in suspension (e.g. Shake flasks and bioreactors) systems.

The increasing interest in insect cells led to the generation of different cell lines. The most used are Sf9, Sf21 and Hi5 cell lines: the first two were derived from the pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda* in 1977 [31], and the last one from the ovarium tissues of the cabbage lopper-*Trichiplusia* ni [32] in 1994 [33] . All cell lines grow rapidly with a doubling time of approximately 24 hours (Table 2), enabling fast expansion and short overall processes [28]. The Sf9 cell line is normally used to produce intracellular or membrane proteins, while Hi5 cell line was reported to increase the specific and volumetric yield of secreted proteins. The Sf21 cell line is preferred for propagation of baculoviruses [27].

Table 2 General characteristics of most used insect cell lines (adapted from thermofisher.com)

Cells	Doubling time	Cell Appearance	Characteristics
Sf9	24-30 hours	Spherical with some granular appearance Firm attachment to surfaces	Grow well in monolayer and suspension culture Adaptable to serum-free medium
Sf21	24-30 hours		
Hi5	18-24 hours	Spherical with some granular appearance Loose attachment to surfaces	Double in less than 24 hours Grow well in adherent cultures, but forms irregular monolayers Adaptable to suspension culture and serum-free medium Provides 5-10 fold higher secreted expression that Sf9 cells

Concerning the metabolism, glucose is the most important carbohydrate for insect cell growth and sucrose is not consumed by either Sf9 or Hi5 cells[34]. Regarding amino acid consumption, these two cell lines have different performances. Asparagine is rapidly consumed in Hi5 cell culture and its depletion coincides with the beginning of the stationary phase. Hi5 cells additionally require significant amounts of glutamine, cysteine and tyrosine [34]. As opposed to most mammalian cells in culture, insect cells do not produce much lactate as by-product, even in media with high initial glucose content. However, in oxygen-limiting conditions Hi5 cells accumulate lactate at levels varying from 7 to 16 mM in shake flask suspension cultures [34]. In the case of ammonia, insect cells are not as sensitive as mammalian cells. Sf9 cells do not usually accumulate ammonia during the growth. On the other hand, Hi5 cells do it in a concentration that depends on the initial concentrations of glutamine and asparagine in the culture medium [34].

Baculovirus expression vector system (BEVS)

The baculovirus expression vector system (BEVS) as 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 production yields that can be achieved [35]. In BEVS the insect cells are infected by recombinant baculovirus that were genetically modified to carry genes of interest. The Baculovirus is rod-shaped with double-stranded DNA genome and is capable of infecting insects and other arthropods. The wild type baculovirus replication cycle is biphasic giving rise to two types of virions: occlusion-derived virions and budded virions. There are three phases concerning gene expression in the virus life cycle: immediate early/early, late and very late[36].

In the very late phase of infection, polyhedrin is expressed by a very strong promoter [37]. Foreign proteins placed under control of the polyhedrin promoter are also produced in large quantities by recombinant baculovirus (rBac). This allows high productivities that can reach more than 25% of total cell proteins in the very late stage of infection[38]. Other option is the use of p10 protein gene expression that is also driven by a very late strong promoter and this gene can also be replaced by a GOI in the rBac without affecting the replication cycle. The most commonly used baculoviruses are Autographa californica multicapsid nucleopolyhedrovirus (AcMNPV), which has a genome of approximately 134kbp[39].

The BEVS has been used for expression of a variety of recombinant proteins, including cytosolic, nuclear, mitochondrial, membrane bound and secreted proteins. This expression system has become one of the most widely used due to its eukaryotic proteins processing capabilities and relatively short process development timelines. The three insect cell lines mentioned above (Table 2) are the mostly applied as hosts of the BEVS [40].

There are two approaches developed for expression in insect cells based on the cellular localization of a given protein in the native state. Proteins with extracellular function are expressed as secreted proteins and are collected from the medium. In the case of proteins that function in the nucleus or in the cytoplasm are expressed intracellularly [3].

The baculovirus-insect cell technology is used in numerous companies to produce custom recombinant proteins for research and commercial applications. Also this system is an accepted technology for the production of viral antigens with vaccine potential; several biopharmaceutical companies have different vaccine candidate products in pre-clinical testing for animal or human purposes [41].

Furthermore, the baculovirus-insect cell system has proven particularly valuable for the expression of GPCRs and co-expression with G proteins has proved valuable for studying receptor-G proteins interactions [42]. Insect cells are the most common expression system used in crystallographic studies of GPCRs giving milligram amounts of pure protein per liter of cell culture. The majority of those receptors were expressed in Sf9 cells [43].

Nevertheless, some disadvantages on the use of this system are related to the lytic nature of virus infection which makes inevitable the presence of proteases in the reaction bulk at the time of harvest, promoting protein degradation and requiring additional efforts in the purification step.

While BVs are considered safe because they cannot replicate in mammalian cells, its genome is able to integrate in the mammalian genome [44] and the consequences of it still remains unclear. Therefore, BVs and host's cell DNA contamination are a concern when the goal is to generate a product for human use. Additionally, proteins requiring complex processing are often produced with low quality as the cellular proteins processing machinery is less efficient in late stages of infections [45]. With BV infection exist a damage of the protein folding and secretion capacity of the cell [28], there are some deviations of the posttranslational modification pattern, which could act immunogenically and the major disadvantage of this system rely on the impossibility of continuous protein production due to the lytic nature of the viral infection process [46].

1.4.2 Cell line development

The inefficiencies of Baculovirus-insect cell expression system have motived the development of stably transformed lepidopteran insect cell lines for heterologous proteins expression [45]. The cell line development process consists on engineering cells to stably express the GOI [45]. High transcriptional rates of the GOI are dependent on the use of strong promoters, enhancers elements and *cis* and *trans*-acting elements [47,48]. The most common promoters used for stable expression in insect cells include the baculovirus immediate early (*IE1* and *IE2*) promoters, the constitutive promoters *Actin* and HSP70, and the inducible metallothionein promoter from *Drosophila* [28,45]. To transport the foreign DNA into the cells different methods can be used, such as electroporation or cationic lipid mediated transfection [45]. The integration of foreign DNA into the host chromosomes will occur in a small portion of the cells that have captured the DNA, a process mediated by cellular DNA repair enzymes that occurs at random sites of the host genome.

The establishment of stable cell lines have many advantages but still have a major drawback concerning the long and laborious timeline necessary to screen and identify stable and high expressing clones [45]. This is required because the expression of the GOI greatly depends on the chromosomal elements close the integration site[47]. Furthermore, in a random integration of the GOI there is the possibility of inducing mutagenic effects by inhibiting or activating host genes at the integration site [45,47]. To overtake these issues, it has been developed targeted integration strategies to express relevant therapeutic proteins.

1.4.2.1 Locus-specific integration

In locus-specific integration, if good loci have been previously identified/tagged they can be re-used for expression of different GOIs without the need of screening. Characterized genomic site can be exploited and reused by homologous recombination (HR) leading to a precise, predictable and reproducible process. Nevertheless, HR has an inefficient rate due to dominance of illegitimate recombination (IR) with a ratio of HR/IR of 1:1000 hindering the wider applicability in transformed cell lines [49]. To overcome this, methods to achieve higher site-specific integration frequencies have been developed.

Recombinase-mediated cassette exchange

Recombinase-mediated cassette (RMCE) exchange, firstly introduced by Schalke and Bode (1994), is a process in which a tagging cassette, flanked by a pair of heterologous recombinase recognition target sites, can be exchange by a target cassette after being integrated into de genome [50]. RMCE systems make use of site-specific recombinases, such as flipase (Flp) from Sacharomyces cerevisiae and Cre from P1 bacteriophage, to mediate the integration of a gene of interest in a pre-characterized chromosome locus flanked by recombinase recognition sites.

The anchored cassette (tagging) encodes a reporter protein and a given selective marker (antibiotic resistance gene, for example), and it is then exchanged for a GOI by means of a site-specific recombinase (SSR), as represented in Figure 2.

After the tagging step an intensive screening of the best locus is necessary with the RMCE system. The advantage is that then it is possible to reuse the same locus to express any target protein, decreasing the time spent in further screening process [48]. With this method it is possible to have stable and high levels of gene expression[51].

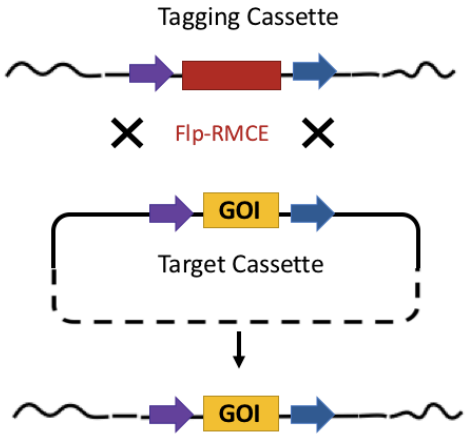


Figure 2 RMCE principle. Tagging a locus with a cassette flanked with heterospecific target sites into the genome and then exchanging it for the GOI.

The RMCE technology has been successfully used in mammalian and insect cells for different purposes [51]. For instance, in human embryonic and induced pluripotent stem cells, which have a great interest in regenerative medicine, RMCE is of crucial importance to by-pass random integration, preventing the possibility of mutagenesis and subsequent tumour formation [52].

Other application of RMCE in on cell line development for production of retroviral vectors for gene therapy, in which the target integration is used for eventual exchange of the therapeutic gene [51]. This strategy has been adopted to express relevant therapeutic proteins, such monoclonal antibodies, in different host cell lines[52–55].

In the insect cell field, our group has recently developed an Sf9 and Hi5 master cell line making use of Flipase-RMCE with the objective of taking advantage of the insect cell expression capabilities but by-passing the use of baculovirus vectors. This cell line produces similar levels of enhanced green fluorescent protein (eGFP), as well as more complex proteins such as rotavirus-like particles, when compared to the BEVS system [45,56]. Despite the recommended single copy integration of the gene of interest in RMCE systems, the identification of a potent integration site can enable competitive productivity levels.

1.5 Strategies for improved recombinant protein production

In the biotechnological field, highly productive cells are important to the technical and economic viability of the process when it is scaled up for industrial production [57]. Over the last twenty years, besides improvements in expression vector systems and cell engineering, optimization of culture conditions and media formulations have resulted in significant increases in the yield of recombinant proteins [57,58].

When compared to microbes, insect cells have significantly slower growth rates and are much more complex in their nutritional requirements. To meet the demands of commercial production and regulatory requirements, bio-manufacturers are faced with the challenge of maximizing productivity whilst controlling product quality. In order to meet these challenges and improving protein yield and quality several strategies for improved recombinant protein production have been followed.

Some of the strategies to improve protein production take advantage of hypothermal growth conditions (i.e. culturing cells at temperatures lower than the optimal for growth), others the addition of substances that promote the expression of the recombinant protein, such as dimethyl sulfoxide (DMSO) or sodium butyrate (NaBu) [57,59]. Another factor that has significant impact on recombinant protein yield and quality is the culture media. Media supplementation is usually implemented to increase cell proliferation, the maximum cell density reached and the culture longevity, that in the end results in an increase in final products [59,60] .

1.6 Previous Work

This master thesis work was developed in the scope of an FCT project, already ongoing when this thesis started. To fulfill the gap of expression systems for fast production of high quantities of membrane proteins of interest in their native lipidic environment, we set up a strategy to develop novel insect cell platforms combining i) Flp/FRT site specific integration and ii) the capability of HIV Gag to bud and drag along target proteins expressed at the plasma membrane of producer cells (Figure 3). The RMCE strategy pursued consists of a unique set of cassettes and two key successive recombination steps combined with FACS technology for screening cells tagged in loci supporting high expression and high recombination efficiency. In the tagging cassette, the FRT sites (Fw and F5) are flanking a green reporter gene (eGFP) and a hygromycin resistance gene, while the OpIE2 and OpIE1 promoters controlling these genes are placed outside the FRTs. One of the FRT sites (Fw) is in-between a fusion gene composed by a core protein from enveloped viruses (HIV-1 Gag) and eGPF (Gag-eGFP). This fusion enables the screening of genomic “hot-spots” supporting high expression of enveloped VLPs, as well as the possibility to eliminate the fluorescent protein from the VLPs once the producer cell line is established.

The exchange target cassettes have two promoterless genes (reporter and antibiotic resistance), which will be only expressed if they replace the FRT flanking region in the cell genome, and this will improve the selection of cells which have exchanged cassettes. In addition, a Flp-recombinase gene (iFlp, codon optimized for insect cells) was included in the intermediate target cassette to avoid the need of co-transfecting the cells with two plasmids. In a first RMCE, the tagging population is submitted to cassette exchange and G418 selection to enrich the population with cells tagged in *loci* amenable to Flp-recombination. Also, given the low efficiency inherent to Flp-recombination, the resulting population is expected to be mainly comprised of single tagged cells. The final target cassette will remove the fluorescent protein fusion from the VLP and express the membrane protein of choice (Figure 3).

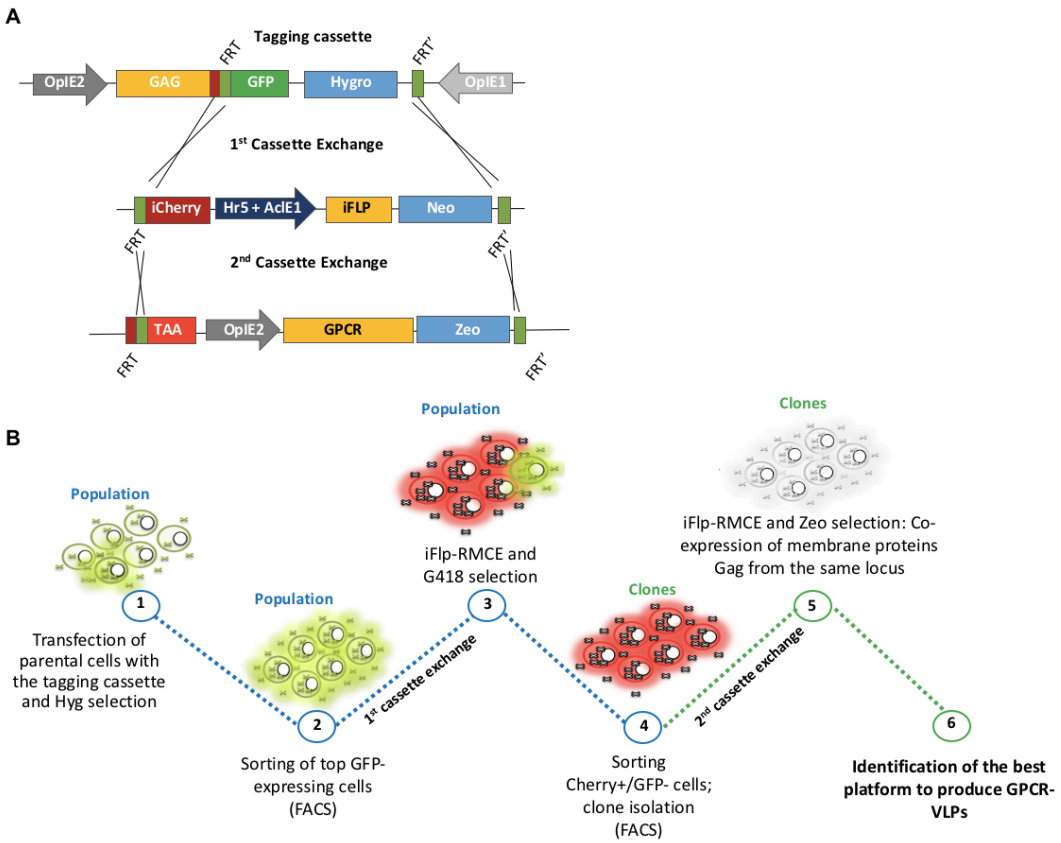


Figure 3 Cell line development strategy based on Flp/FRT site specific integration technology for co-expression of target membrane proteins and enveloped VLPs from the same locus. (A) Design of the cassettes: the tagging cassette is composed by the promoters OpiE2 and OpiE1 driving expression of the Gag scaffold protein fused to GFP and the resistance Hygromycin (Hygro) gene, respectively; one of the FRT sites (Fw) is part of the linker between Gag and GFP and the second FRT site (F5) is immediately upstream of the OpiE1 promoter; the 1st target cassette encodes the red reporter gene iCherry and the Neomycin resistance gene (Neo), without promoters to guarantee that upon transfection only cells which exchanged cassettes will have red fluorescence and will be resistant to G418; this target cassette also includes the promoter Hr5+AcIE1 driving expression of the Flipase codon optimized for insect cells (iFlp); the 2st target has a stop codon (TAA) downstream to the FRT site, the promoter OpiE2 driving expression of the target membrane protein (GPCR), and the Zeocin resistance gene (Zeo). (B) Sequence of steps from tagging parental cells until isolating cell clones co-expressing the scaffold protein and the target membrane protein. Firstly, the tagging cassette is randomly integrated into parental cells, which are then selected with hygromycin. The resulting cell population is enriched with top producer cells by cell sorting (FACS), enhancing the average productivity of the population. The next step is to perform RMCE by a iCherry-expressing cassette, followed by G418 selection to select the cells most prone to the recombination process. The cells which exchanged cassette (iCherry^{+}/GFP^{-}) will be recovered by FACS. Finally, a second RMCE process will remove the fluorescent protein fusion from the VLP and express the membrane protein of choice in the final master cell line.

This master thesis work started when Hi5 and Sf9 cell clones encoding the iCherry cassette (Figure 3B) had been isolated. Briefly, parental Hi5 and Sf9 cells were transfected with Gag tagging cassette (Figure 3A), and selected during 2 weeks with hygromycin. The resulting

populations expressing Gag-GFP were analysed by confocal and fluorescence microscopy (Figure 4); we can see that Gag mainly localizes at the cell membrane for both cell hosts (Figure 4 C). It is known that the Gag protein does not require any other viral protein to form VLPs and budding out of the cell [21,23]. The fusion with GFP allowed to select the cells expressing more Gag by sorting for the best GFP expressing cells.

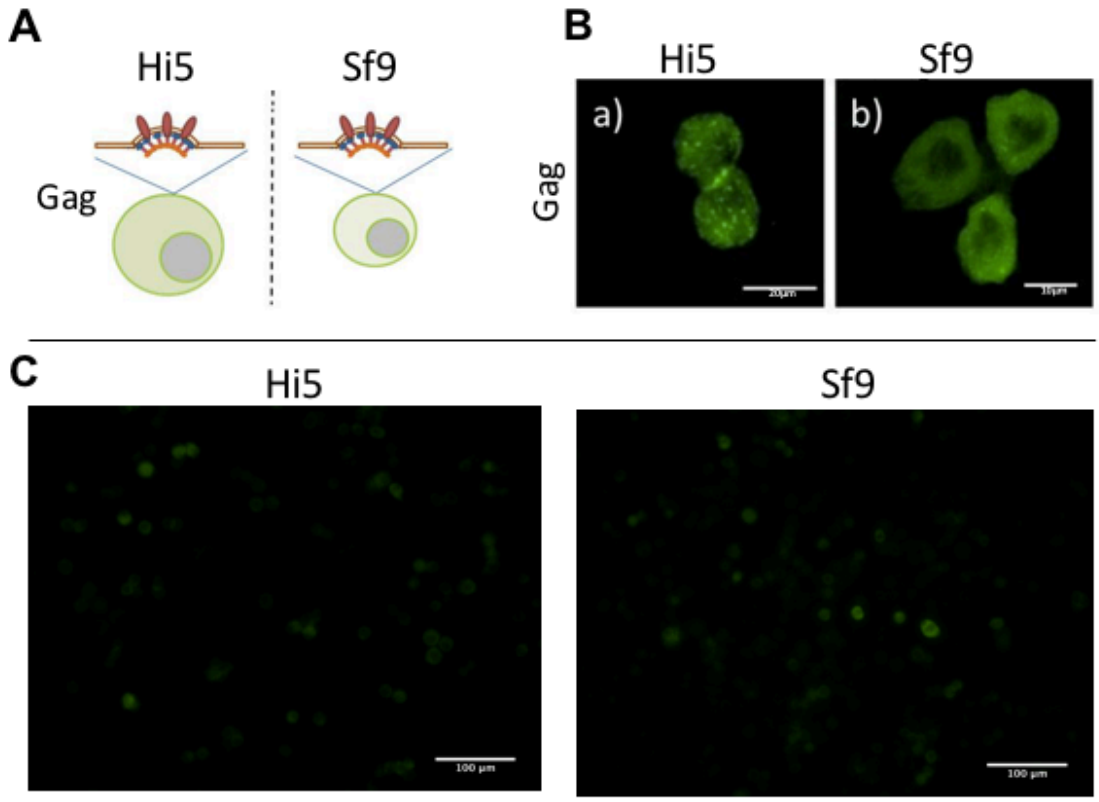


Figure 4 Establishment of the two populations expressing naked Gag VLPs. (A) Scheme of the populations, two insect cell hosts (Sf9 and Hi5) expressing a VLP scaffold, HIV-Gag (B) Confocal fluorescence microscopy a) Hi5 cells (scale bar is in 20 µm) and b) Sf9 cells (scale bar is in 10 µm) (C) Fluorescence microscopy analysis of GFP expression 96 hours post inoculation of populations Hi5 Gag and Sf9 Gag (scale bars are in 100µm; all images were obtained with the same parameters).

To eliminate the cells tagged in loci not amenable to Flp recombination, the tagging populations were transfected with an intermediate cassette encoding a promoterless red reporter gene (iCherry) downstream to the first FRT site, and by G418 selection we recovered the cells which have undergone cassette exchange. Approximately 24h post-transfection a few red cells started to appear (not observed in parental cells transfected with the same cassette), demonstrating that cassette exchange was well-succeeded. The resulting populations were submitted to limiting dilution with FACS, gating to high and pure Cherry-expressing cells. The isolated clones from the Sf9 (#8, #10, #11, #12, #13, #14 and #17) and Hi5 (#1, #2, #3m, #4, #4m, #5m, #6, #9, #10, #12, #13, #14, #15, #16) cell populations were amplified and stored in cell banks for further characterization.

2. Aim of the thesis

This thesis aims at developing insect cell lines specialized in the production of VLPs displaying membrane proteins of interest, using RMCE technology. Clones tagged with the core protein of an enveloped virus (Gag from retrovirus) fused to iCherry were previously developed. Then the clones will be herein thoroughly characterized to identify those potentially better to produce Gag protein to then co-express the target membrane protein. The selected clones will be transfected with a target cassette encoding the model GPCR β-Adrenergic receptor, and upon selection will express Gag and the receptor from the same locus.

In the second part of the thesis, bioprocess engineering strategies will be used to optimize the production of Gag-VLPs, including the adaptation of cells to hypothermic conditions, addition of sodium butyrate (NaBu) or DMSO, and supplementation with key nutrients.

3. Materials and Methods

Molecular Biology

pTarget ADRB2 Fusion vector design and construction

pTarget ADRB2 Fusion was derived from an in house vector, pTarget. The backbone of this vector with a Zeocin resistance gene was amplified from the original pTarget with an inverted PCR with the primers TargetADRfu Fw and TargetADRfu Rv (Table 3).

Three different melting temperatures (54°C; 59°C and 64°C) and two different polymerase buffers were tested to obtained the best amplification reaction. The insert, OpIE2 promoter with the B2AR-i-pep gene, was amplified by polymerase chain reaction (PCR) with the primers ADRfu Fw and ADRfu Rv (Table 1) from another in house plasmid modified from pCDNA_B2AR-i-pep [61]. Also three different temperatures (58,5°C ; 63,5°C and 68,5°C) and two different polymerase buffers were tested for the best condition. Finally, the ligation of the two DNA sequences was done by In-Fusion® HD Cloning kit.

Vector design

In order to have the final cassette exchange process to express the target membrane protein along with the Gag scaffold from the same locus, it was necessary to first construct the desired plasmid containing the Adrb2 gene of interest, the OpiE2 promoter and zeocin resistance marker. Adrb2 gene encodes the beta-2 adrenergic receptor (β_2 adrenoreceptor) which is a member of the G protein-coupled receptor (GPCR) superfamily.

PCR amplifications were performed to obtain the insert (4277 bp) and the vector (2762 bp) with the best conditions for each PCR.

To identify bacteria colonies positive for the plasmid construction, a colony PCR was performed. The plasmid DNA of the positive colonies was purified by miniprep, digested with Pst I HF enzyme and run by agarose gel electrophoresis analysis. The construct with positive result was sequence verified.

General PCR-protocol

The oligonucleotides used for PCR were custom-made by Sigma Aldrich. 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 98°C, followed by 30 cycles of 10sec denaturation at 98°C, primer annealing for 30s performed with the already stablished melting temperature condition, and extension at 72°C according to the fragment size. The next step in the cycle was final extension at 72°C for 10 min.

Table 3 Sequence of primers used in PCR analyses

Primer names	Sequence
ADRfu Fw	5'-TATAGGAACTTCGGATGACCGACGCCGACCAACAC-3'
ADRfu Rv	5'-CTCGCCGATCCCACGATTACCGCCTTTGAGTGAGC-3'
Target ADRfu Fw	5'-CGTGGGATCGGCGAGTCAGT-3'
Target ADRfu Rv	5'-TCCGAAGTTCCTATACTTTC-3'

Agarose gel electrophoresis

Agarose gel electrophoresis was performed to separate DNA-fragments. The concentration of each gel varied according to 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. When needed, Illustra GFX kit (GE Healthcare) was used to purify the bands. Gels were photographed using GelDoc^{TM} system (Bio-Rad) and DNA quantification was done using Nanodrop ND-2000c (Thermo Scientific).

Ligation with in-Fusion

For the ligation of DNA-fragments the In-Fusion® HD Cloning kit was used following the instructions of the manufacturer, proportion of 1:2 (insert/vector ratio) (Clontech; ref. 638910). The ligated vector- DNA mix was used to transform bacterial cells, as previously described.

Transformation and vector isolation

Competent *E.coli* cells (DH5α stellar-NZYTech) were transformed with the DNA obtained from the insert and vector ligation according to the manufacturer's protocol (Clontech, ref. 636763). Transformed cultures were spread on LB-agar plates containing ampicillin 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 37^{o}C and 190rpm. After 12-16h, 1mL 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, and then the DNA was sent to sequencing.

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). The reaction included 2 µl the appropriate buffer, 4 µl of DNA, 0.2 µl of enzyme. RNAse-free water (Sigma) was also

added to the final volume of 20 µl. When digestion of a vector was desired, further excision and purification from agarose gel was performed with Illustra GFX purification kit (GE Healthcare).

Colony PCR-screening

PCR was used to screen transformed bacterial colonies and evaluate if they were positive for the ligation vector. Single transformed bacterial colonies were selected from the LB-agar-plate and transferred into a PCR-tube containing 20 µl of the pre-pipetted PCR-reaction mixture. PCR was performed immediately and checked by agarose gel electrophoresis. Gene specific 5'- and 3'-primers were used.

Insect cell culture

Sf9 and Hi5 Cell Culture Maintenance

For suspension cultures, cells were routinely cultured either in 125 mL or 500 shake flasks (10% working volume) at 27^{o}C in orbital shakers at 100rpm. Sf-900^{TM} II serum-free medium (Gibco) and Insect-Xpress^{TM} (Lonza) were used for Sf9 and Hi5 cultures, respectively. The cell inoculum was 0.5x10^{6}cells/mL and 0.3×10^{6} cells/mL for Sf9 and Hi5 cells, respectively. Cells were sub-cultured every 3-4 days when cell density reached 2-3×10^{6} cells/mL.

Cell concentration and viability were assessed by haemocytometer counting (Brand) using trypan blue exclusion dye (Merck). For adherent cultures, cells were maintained in T-flasks (75 cm^{2}) with the respective medium supplemented with 10% (v/v) of serum (Gibco) and sub-cultured when confluency was reached.

Freezing and Thawing cells

Exponentially growing cells (2-3×10^{6} cells/mL) were centrifuged at 200g, 4^{o}C for 10 min, and cell pellets were ressuspended in cryopreservation media (CryoStor®, Sigma) to obtain a concentration of 1-2x10^{7} cells/mL. Aliquots were frozen using a freezing container (Mr. Frosty) (Thermo Fisher Scientific) and stored at -80^{o}C until further use. Thawing was performed by centrifuging cells in 12 mL of medium at 200g for 10 min. Cell pellet was re-suspended in medium, according the volume to achieve the cell density desired.

Transfection and Cassette exchange

Foreign DNA was inserted into cells using lipotransfection based on Cellfectin® II reagent (Invitrogen). 40 µL of Cellfectin® II reagent (Invitrogen) and 500µL of Grace's Insect medium (Gibco), were used 5x10^{6} cells (5 units of transfection UT).

Cell clones were co-transfected with 0.1 µg of pTarget ADRB2 fusion and 0.3 µg iFlp-expressing vector Transfections were conducted in 125 mL shake flasks (10 mL working volume). Selection was performed with antibiotic zeocine (0.1 mg/mL; Invivogen). When viabilities dropped below 50%, cells were transferred to T-flasks (75cm^{2}). The medium with antibiotic was replaced every four 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 thereof as mentioned above.

Culture supplementation schemes

Chemical supplementation

Sodium butyrate (NaBu; VWR) and DMSO (Sigma) were added at different concentrations to shake flask cultures with cell densities of 2×10^6 cells/mL and 5×10^6 cells/mL. NaBu was added at 0.75 mM; 1 mM; 5 mM and 10 mM whereas DMSO was added at 0.5%, 1% and 2% (v/v).

Nutrients supplementation

A mixture containing lipids (Chemically Defined Lipid Concentrate; ref. 11905-031) (Gibco) was added to shake flask cultures at inoculation and 96 hours post-inoculation. A set of different nutrients were also added along culture time, the first being 10 mM Serine (Ser) (Sigma) and 1 mM of Cysteine (Cys) (Fluka) at 96 hours post-inoculation, the second 20 mM Glucose (Glc) (Merk) and 2mM Glutamine (Gln) (Sigma) at 144 hours post-inoculation in Sf9-Gag. In Hi5-Gag cells at 72h post-inoculation was added 5mM Gln, 10mM Asparagine (Asn) (Sigma), 20 mM Glc. The nutrients were added to the culture in 1mL of the respective insect growth medium.

Adaptation of cells to growth at lower temperatures

The populations Sf9-Gag and Hi5-Gag were subjected to an adaptation process of three months. The culture temperature was decreased from 28/27ºC to 26ºC, 24ºC and finally to 22ºC. The adaptation to 26 ºC was achieved in 3 cell passages (about 1 week); to further decrease the culture temperature to 24ºC, 9 cell passages were performed in about 4 weeks. The adaptation to 22ºC was achieved in 9 cell passages for Hi5-Gag cells (6 weeks) and 12 additional passages for Sf9-Gag cells (10 weeks).

For the adaptation to low temperature, Sf9-Gag and Hi5-Gag cells were cultivated in 125 mL shake flasks (10% working volume) at 26ºC, 24ºC or 22ºC in orbital shakers at 100 rpm. For the experiments carried out at 24ºC and 22ºC, two different inoculums were used: for Sf9 cells $0.5\text{x}10^6$ cells/mL and $1\text{x}10^6$ cells/mL, and for Hi5 cells $0.3\text{x}10^6$ cells/mL and $0.6\text{x}10^6$ cells/mL. Cells were sub-cultured when cell density reached $2\text{-}3\text{x}10^6$cells/mL. The cells are adapted to the new conditions when the viability reached more than 90% and the duplication time stabilized along the passages.

Analytical methods

Flow Cytometry

CyFlow® space (Partec GmbH) was used to evaluate the recombination efficiency. To characterize the stability of the Gag clones in terms of iCherry fluorescence intensity and

percentage, CyFlow® space (Partec GmbH) and BD LSR Fortessa™ (BD Biosciences) were used. Samples were collected and diluted in PBS (Gibco). Analysis from 30 000 events per sample was done using FlowJo software.
To characterize the Gag-Adrb2 clones the laser used in fluorescence microscopy was that used for GFP (509 nm) as mCitrine emits fluorescence at a close wavelength (527 nm).

Western Blot analysis

Samples were denatured with a reducing agent (Novex® NuPAGE®), heated to 95°C 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 MES 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) with Tween® 20 (Merck) (TTBS). The membrane was incubated overnight at room temperature with primary antibody mouse anti-HIV1 p24 (1:1000 dilution) and then 1h with secondary antibody anti-mouse IgC (1:5000 dilution). Detection was performed with the enhanced chemiluminescence detection system (ECL) (Amersham Biosciences).

MicroBCA quantification

Total protein quantification in cell extracts was performed by the bicinchoninic acid assay (BCA) with Micro BCATM Protein Assay Kit (Pierce Biotechnology), according to the manufacturer's instructions.

Quantification of Gag-VLPs by Lenti-X p24 Assay –ELISA

Quantification of Gag-VLPs was performed using the Lenti-X p24 Rapid Titer Kit (Clontech) that allows the determination of the titer of any HIV-1-based lentiviral supernatant using an ELISA method. The wells of the microtiter plate (12 x 8-well strips) are coated with an anti- HIV-1 p24 capture antibody, which quantitatively binds the HIV-1 p24 in the test samples. Specially-bound p24 is detected in a typical "sandwich" ELISA format using a biotinylated anti-p24 secondary antibody, a streptavidin-HRP conjugate, and a colour producing substrate. Colour intensity of the samples is measured spectrophotometrically at 450 nm, which is then quantified against a p24 standard curve.

Purification of VLPs

For evaluation of Gag-VLPs concentration methods, protein recovery was calculated according to equation 1:

$$\textbf{Protein recovery}\ (\%) = \frac{\textbf{final volume} \times \textbf{final concentration}}{\textbf{initial volume} \times \textbf{initial concentration}} \textbf{x100} \qquad \textbf{(Equation 1)}$$

Ultracentrifugation

Upon reaching maximum cell density, cell culture was harvest and centrifuged at 200xg for 10 min. Clarified supernatant was layered over 20% sucrose (VWR) in PBS and then centrifuged for 90 min at 28 000 rpm. Supernatant is removed and the pellet ressuspended in 1 mL PBS overnight. VLPs are then re-pelleted through 20% sucrose in PBS and centrifuged for 45 min at 40 000 rpm. The pellet is ressuspended overnight in PBS.

Polyethylene glycol precipitation

Upon reaching maximum cell density, cell culture was harvest and centrifuged at 200xg for 10 min. Clarified supernatant was collected and PEG (8.5%) and NaCl (0.3 M) were added. This mixture was agitated for 1h30 min at 4ºC, and then centrifuged at 4500xg for 30 min at 4ºC. The pellet obtained was ressuspended in PBS.

Centrifugation with Vivaspin® centrifugal concentrator

VLPs can be concentrated with appropriate device size and membrane cut-off. We have used Vivaspin 20 300kDa (Sartorius). To improve the recovery of low-concentrated protein samples the following treatment should be done. A passivation procedure by washing the concentrators filled with mili-Q water and spinning the liquid through by centrifugation. The residual water is removed thoroughly by pipetting carefully without damage the membrane with the pipette tip. After the washing procedure, the concentrators are filled with the blocking solution (Triton X-100 5%) (Sigma) and incubated at room temperature for 2 hours. Then the device is washed again with mili-Q water 3-4x very thoroughly, and finally the VLP samples are concentrated.

4. Results

4.1. Cell line development

The first part of this thesis focuses on the generation of a flexible insect cell platform for production of enveloped VLPs displaying membrane proteins of interest using RMCE technology. The work performed can be divided in two tasks: 1) characterization of Sf9-Gag and Hi5-Gag clones, and 2) cassette exchange for production of GAG-Adrb2 VLPs.

4.1.1. Characterization of Sf9- and Hi5-Gag clones

The Gag-iCherry expressing Sf9 and Hi5 clones isolated previously (see Previous Work section) were compared based on several characteristics: (1) cell growth performance, (2) iCherry fluorescence intensity, (3) stability of iCherry expression along passages, and (4) secretion of Gag protein.

Cell growth performance

To assess the potential of the cell line, the growth performance is an important feature; a cell line that can reach high cell densities can contribute to higher volumetric productivities, allowing to obtain higher VLP titers at the end of the culture.
In order to compare cell growth performance, isolated clones were cultured in shake flasks and sampled daily to assess cell concentration until viability dropped below 90% (Figure 5).

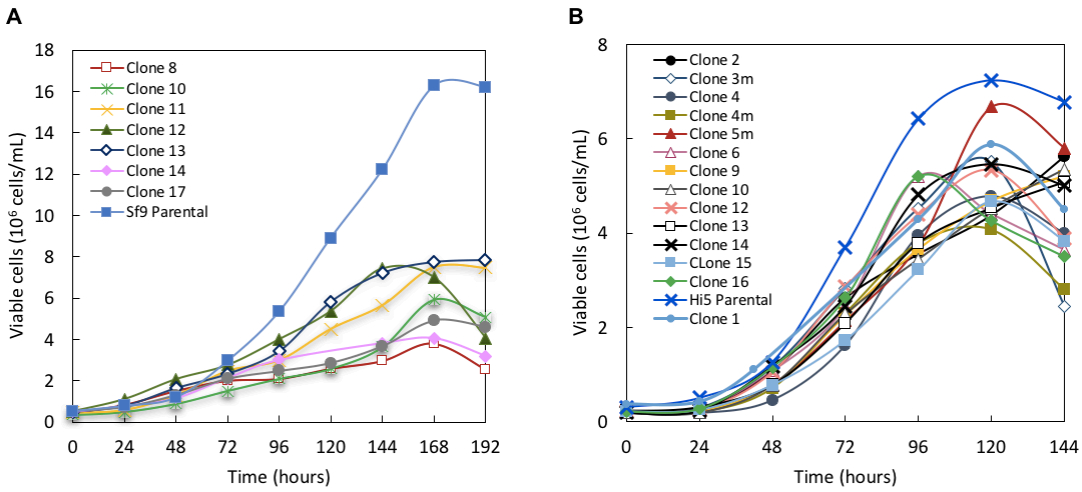


Figure 5 Cell growth kinetics of (A) parental Sf9 cells and Sf9-Gag clones #8, #10,#11,#12,#13,#14 and #17 and (B) parental Hi5 cells and Hi5-Gag clones #1, #2 and #3m, #4, #4m, #5m, #6, #9, #10, #12, #13, #14, #15 and #16.

Compared with parental Sf9 cells, the Sf9-Gag clones show lower growth rates and peak cell

densities (8×10^6 cells/mL vs 16×10^6 cells/mL of parental cell line). The Sf9 clones with higher cell growth rate and peak cell density are clones #11, #12 and #13 (Figure 5A). In contrast, Hi5-Gag clones show growth performances similar to the parental cell line, reaching up to 6.7×10^6cells/mL versus the 7×10^6cells/mL reached by the parental cells (Figure 5B). The Hi5 clones with higher cell growth rate and peak cell density are clones #5m and #1.

iCherry fluorescence intensity

We took advantage of the Gag-iCherry fusion to compare the clones in terms of protein expression. The cell clones were analysed by flow cytometry and fluorescence microscopy. In principle, the clones expressing higher amounts of iCherry will be those producing higher amounts of the Gag protein. The Figure 6 shows the mean iCherry fluorescence intensity of the clones from the two cell hosts analysed by flow cytometry. The Sf9-Gag clones with highest fluorescence intensity are clones #14, #10, #13 and #11, reaching intensities between 500-900. The Hi5-Gag clones with highest fluorescence intensity are clones #4m, #10, #15 and #5m, reaching intensities between 3000-6000. Noteworthy, Hi5-Gag clones reach higher mean fluorescence intensity as compared to Sf9-Gag clones.

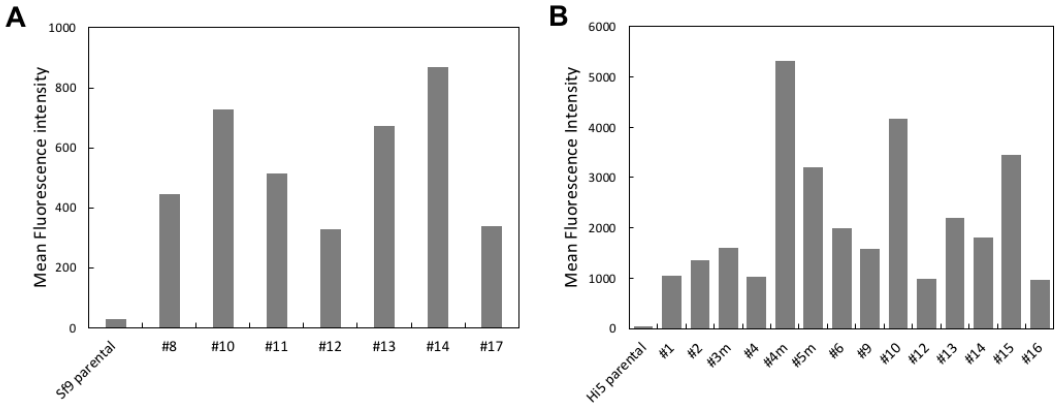


Figure 6 Flow cytometry analysis. Mean iCherry fluorescence intensity of the clones at 96 h post inoculation for (A) Sf9-Gag clones #8, #10 #11, #12, #13, #14 and #17 and (B) of Hi5-Gag clones #1, #2, #3m, #4, #4m, #5m, #6, #9, #19, #12, #13, #14, #15 and #16.

Sf9-Gag clone with highest fluorescence intensity (#10, #13, #11) have a single population (Figure 7) with exception of Sf9-Gag clone 14. Although showing the highest fluorescence intensity, the flow cytometry profiles of the Hi5-Gag clones 4m and 10 show bimodal distributions, suggesting that they were not derived from single cells (Figure 8). Therefore, we discarded these clones as they are not a good option to pursue with the final cassette exchange. On the other hand, the profiles from clones 5m and 15 reveal clearly defined single populations.

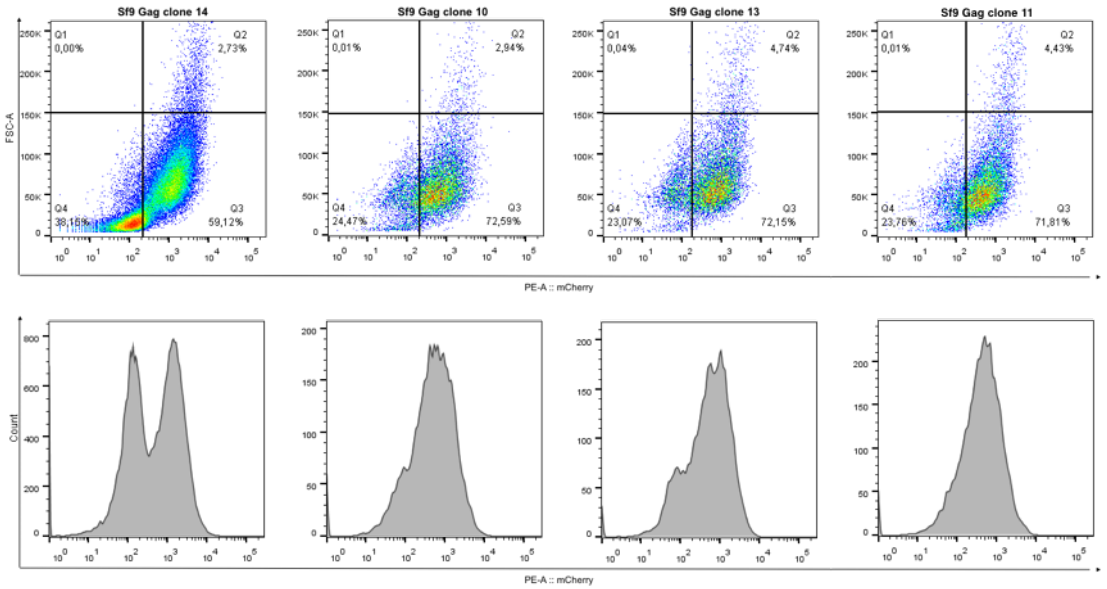


Figure 7 Flow cytometry analysis of the higher Gag-iCherry expressing Sf9 Clones (#14, #10, #13 and #11).

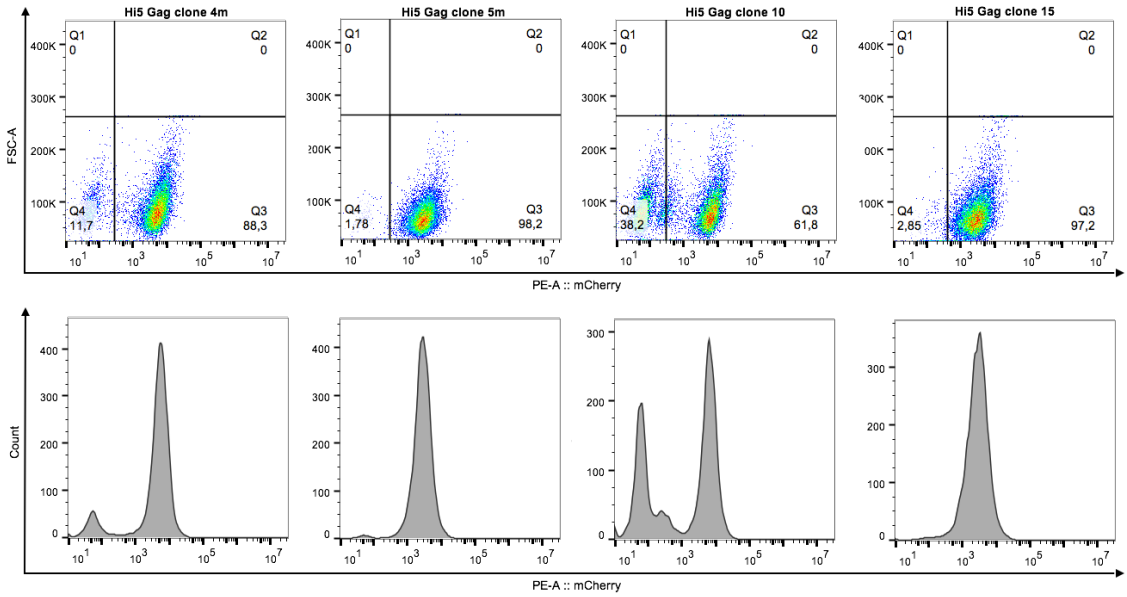


Figure 8 Flow cytometry analysis of the higher Gag-iCherry expressing Hi5 Clones (#4m, #5m, #10 and #15).

In parallel, the clones were analysed by fluorescence microscopy (Figures 9 and 10). Although the Sf9-Gag clone 12 was not within those with higher mean fluorescence by flow cytometry, it seems to be one of strongest by fluorescence microscopy. The red fluorescence of Sf9-Gag clone 14 seems dispersed within the cells, as opposed to its localization at the plasma membrane observed for the remaining clones. Therefore, Sf9-Gag clone 14 was not a good option for further studies. In agreement with the flow cytometry analysis, the Hi5-Gag clones 4m, 5m and 15 seem to have higher iCherry expression.

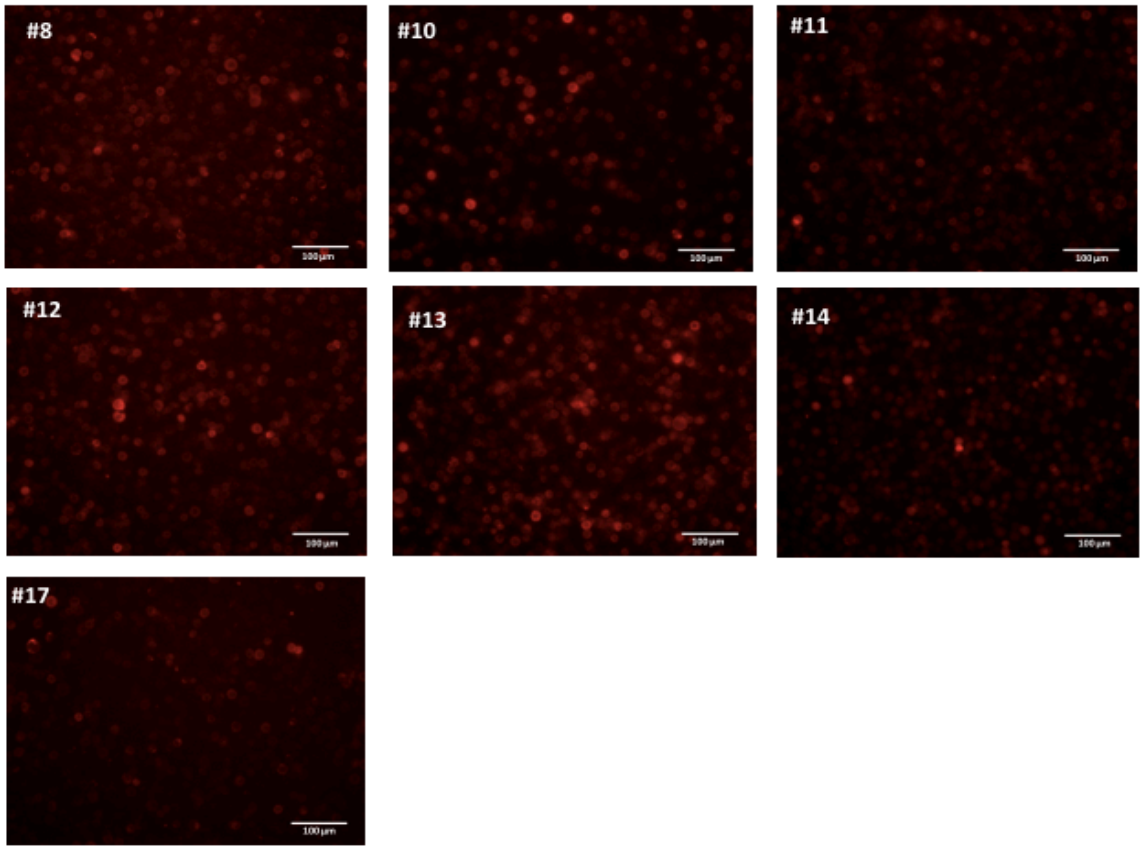


Figure 9 Fluorescence microscopy images of Sf9-Gag clones #8, #10, #11, #12, #13, #14 and #17 at 96 hours post inoculation (scale bars are in 100µm; all images were obtained with the same parameters).

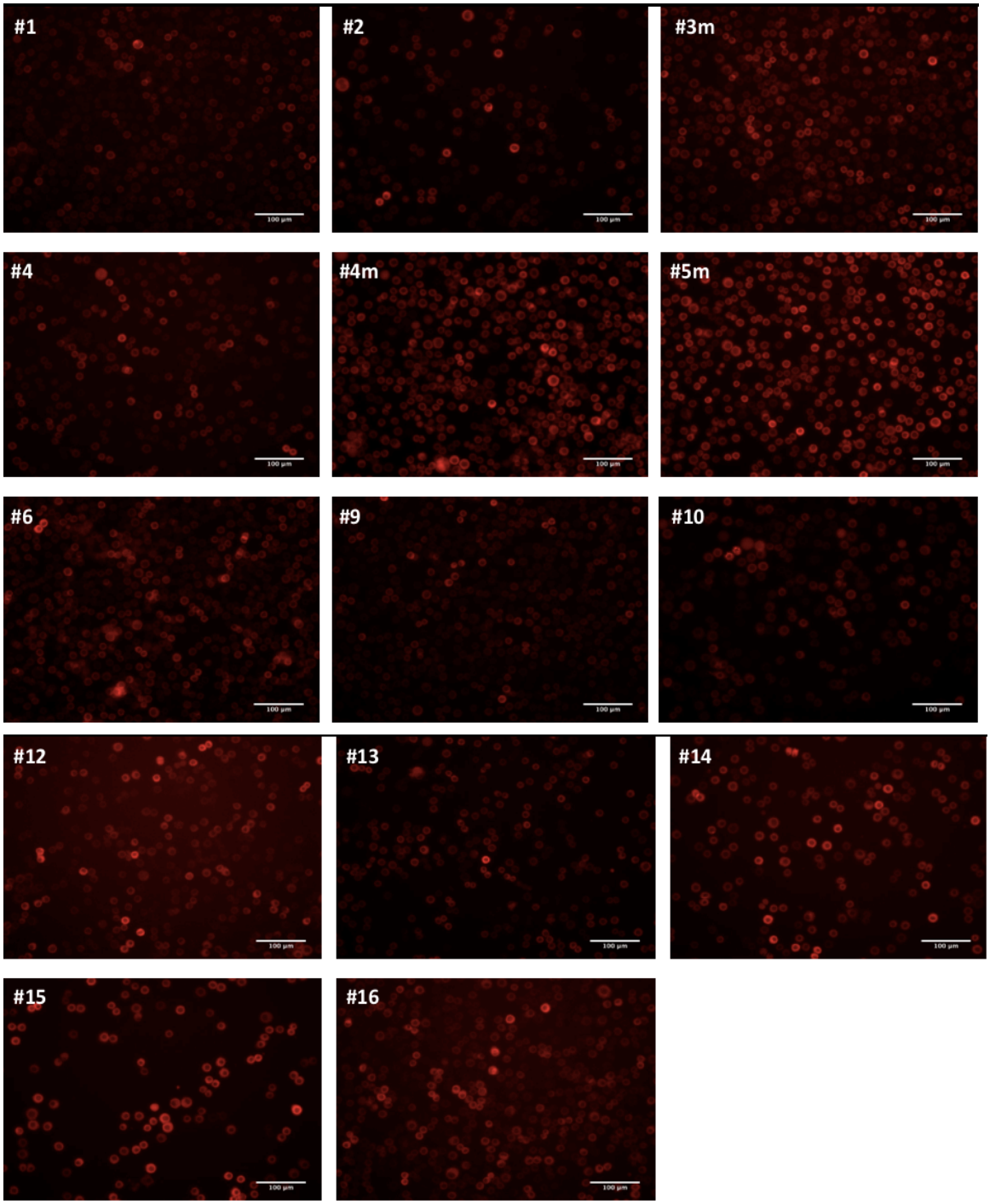


Figure 10 Fluorescence microscopy images of Hi5-Gag clones #1, #2, #3m, #4, #4m, #5m, #6, #9, #19, #12, #13, #14, #15 and #16 at 96 hours post inoculation (scale bars are in 100µm; all images were obtained with the same parameters).

Stability of iCherry expression along passages

It is important that the expression of the recombinant protein is stable along passages even after the selective pressure was removed. The clones were kept under G418 selection to eliminate the cells which did not exchange cassettes, i.e. cells that did not turn into red-expressing cells. Once the selection is finished we can test the effect of removing the antibiotic.

The stability of iCherry expression along twelve passages (with and without antibiotic) was assessed by flow cytometry for Sf9-Gag clones #11, #12, #13 and Hi5-Gag #1, #5m, #10,#15 clones (Figure 11, 12 and 13)

After twelve passages with and without antibiotic the Sf9-Gag clones and Hi5-Gag clones the fluorescence intensity doesn't change along passages with exception of Hi5-Gag clone 10. In Figure 11 A Hi5-Gag clone 5m is used as an example of fluorescence stability with the exception of Hi5-Gag clone 10 (Figure 11B) which is really unstable. Hi5-Gag clone 10 had already shown instability of iCherry (Figure 8) but along passages get worse. As a result, Hi5-Gag clone 10 was not considered a candidate for the final cassette exchange. In addition, there seems to be no impact of antibiotic in stability of the clones (Figure 12 and 13).

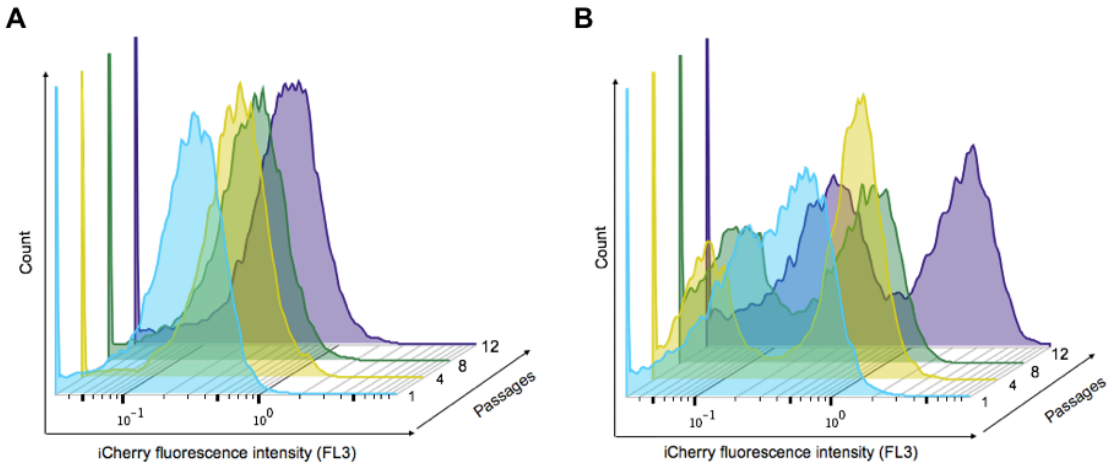


Figure 11 Representative graphic of cherry fluorescence intensity stability of (A) Hi5 Gag clone 5m and (B) Hi5 Gag clone 10 during twelve passages with antibiotic (neomycin).

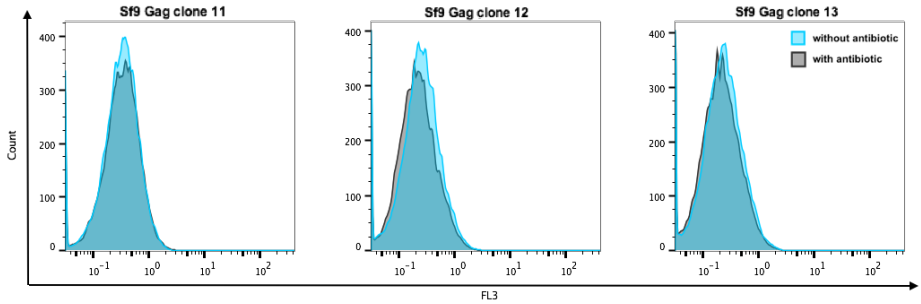


Figure 12 Flow cytometry analysis. iCherry expression of Sf9-Gag clones #11, #12 and #13 at 96h post inoculation, after twelve passages with and without antibiotic.

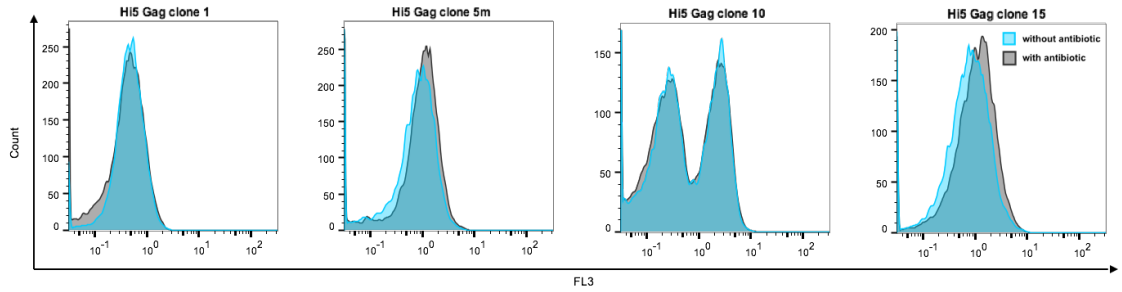


Figure 13 Flow cytometry analysis. iCherry expression of Hi5-Gag clones #1, #5m, #10 and #15 at 96h post inoculation, after twelve passages with and without antibiotic.

Secretion of Gag protein

Besides the iCherry fluorescence of the cell clones, we also analysed the Gag secreted to the culture supernatant by Western blot. Figure 14 shows the typical profiles of Gag accumulation along culture time for one clone of each cell host. Supernatant samples from the Sf9 Gag clone 11 were analysed from 24 h (P2) to 216 h (P9) of growth (Figure 14A), and it is possible to identify in P6, P7, P8, P9 the Gag protein fused to iCherry in the non-mature form (Pr55+iCherry; ~83 kDa) and for P7, P8 and P9 the mature form (MA+CA; ~40 kDa). Comparing the band intensity of the western blot (using the ImageJ), we can conclude that the maximum concentration of Gag is reached at day 9 (Table 4). In Figure 14B, it is represented the Hi5 Gag clone 5m from 48h (P2) to 144h (P6) of growth. It is possible to identify only in P5 the Gag protein fused with iCherry in the non-mature form.

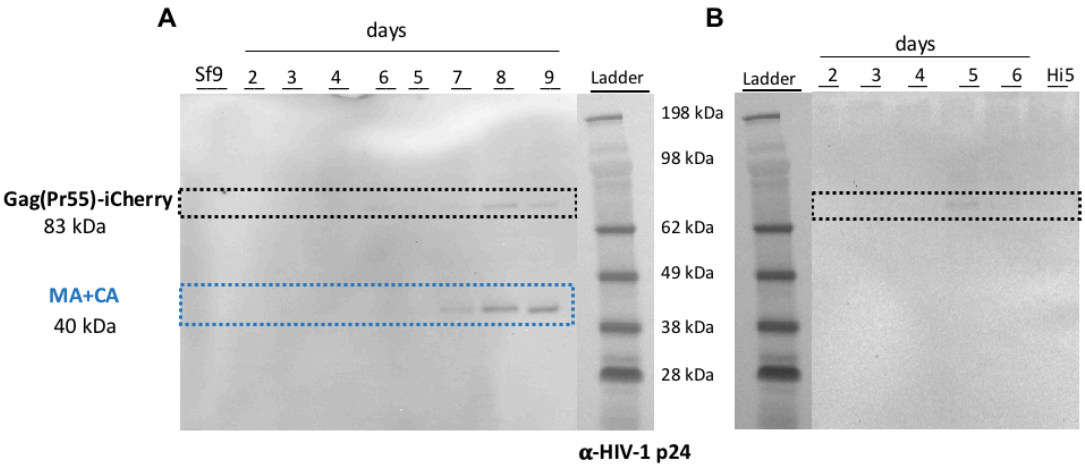


Figure 14 Western blot analysis of (A) Sf9-Gag clone #11 and (B) Hi5-Gag clones #5m along culture days. All samples were clarified and was used the supernatant for the western blot analysis. Gag protein fused with iCherry (Gag-iCherry; 83 kDa) and matrix domain + capsid domain (MA+CA; 40 kDa).

Table 4 Quantitative analysis of band in intensity in western blot of Sf9-Gag clone 11 along 10 days of culture (Figure 14A)

Sample	Fold increase in relation to P6
P7	5
P8	14
P9	15

The expression of Gag protein in Sf9-Gag and Hi5-Gag clones was also assessed by micro BCA assay (total protein quantification) and ELISA (p24 protein quantification) (Table 5). p24 is a protein from Gag capsid. The total protein concentration is similar in all Sf9-Gag and Hi5-Gag clones analysed. On the contrary, p24 concentration varies significantly within Sf9-Gag and Hi5-Gag clones. The Sf9-Gag clone 11 secretes over 2-fold higher Gag protein than clones 12 and 13. These results do not correlate directly with the flow cytometry analysis, from which the strongest of the three clones was the clone 13.

Table 5 Quantification of total protein and p24 protein of Sf9 clones at day 9 of culture and Hi5 clones at day 5 of culture.

ID	Total protein quantification (µg/mL)	Cell concentration (10^6 cells/mL)	[p24] (pg/mL)	Specific productivity (pg p24/(10^6cells.h))
Sf9 Gag Clone 11	5209 ± 42	7.5	6680 ± 964	7.3
Sf9 Gag Clone 12	6534 ± 1170	7.4	3000 ± 500	3.6
Sf9 Gag Clone 13	5931 ± 451	7.2	3360 ±136	3.0
Hi5 Gag Clone 1	3194 ± 527	5.9	160 ±3	0.2
Hi5 Gag Clone 5m	4188 ± 104	6.7	3950 ± 154	4.7
Hi5 Gag Clone 15	3562 ± 98	4.7	7080 ± 727	15.9

Regarding the Hi5 clones, the p24 quantification correlates better with the flow cytometry analysis: the clones 5m and 15 are secreting much more Gag protein than clone 1. In terms of specific productivities, the Hi5-Gag clones surpass the Sf9-Gag clones by up to 2.2-fold, since the former reach lower maximum cell densities and have shorter culture times.

4.1.2 Co-expression of Gag and target membrane protein from the same locus

The last step in the cell line development process is to perform RMCE in the Gag-iCherry expressing clones using a cassette encoding the target membrane protein (the GPCR Adrb2), generating cells that produce Gag VLPs decorated with Adrb2 proteins. In this step, the fluorescent protein fusion (iCherry) is removed from the VLP and the Adrb2 is expressed from the same locus.

To assess the Adrb2 expression and secretion, we first performed a transient expression assay in which parental Sf9 cells and the Sf9-Gag population were transfected with a plasmid encoding the adrb2 gene controlled by the OpIE2 promoter. Five days after transfection, we analysed the supernatant from both cultures by western blot and observed and increased amount of the receptor in the Sf9-Gag population (Figure 15), suggesting that Gag increases the secretion of the receptor. This GPCR has an epitope from hemagglutinin (HA) being possible to identify it by western blot using a HA antibody.

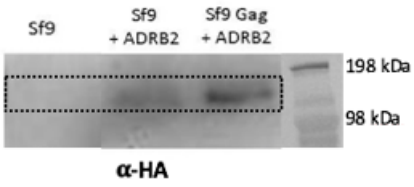


Figure 15 Western blot analysis of Adrb2 in the supernatant of parental Sf9 cells and Gag-expressing Sf9 cells, both transfected with a plasmid encoding the Adrb2 gene. Non-transfected Sf9 cells were used as negative control.

We then co-transfected three clones from each cell host - the Sf9 clones 11, 12 and 13 and the Hi5 clones 5m and 15 - with the target cassette encoding the receptor and a plasmid encoding iFlp. Two days post-transfection, zeocin was added in order to initiate the selection process of the cells that had exchanged cassettes.

In order to evaluate the cassette exchange and selection process, the clones were analysed by flow cytometry and fluorescence microscopy along time. The fluorescence of the clones is related to the fact that this GPCR is fused to two fluorescent proteins, Citrine (mCir) and Cerulean (mCer). Twelve weeks after targeting, it is possible to see that the cells lost the red fluorescence and are all mCir positive (Figure 16).

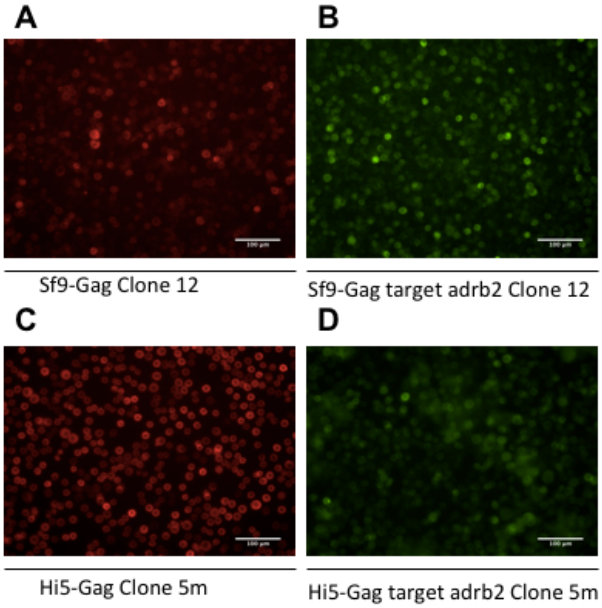


Figure 16 Fluorescence microscopy images of Gag-iCherry clones before and 12 weeks after targeting with the Adrb2-mCir cassette. Sf9 Gag clone 12 (A) before and (B) after targeting. Hi5 Gag clone 5m (C) before and (D) after targeting.

The concentration of p24 protein accumulated in the supernatant at the end of the cultures was also assessed and compared with the amount produced by the Gag-expressing cell pools and by the respective Gag-expressing clones from which they were derived (Figure 17).

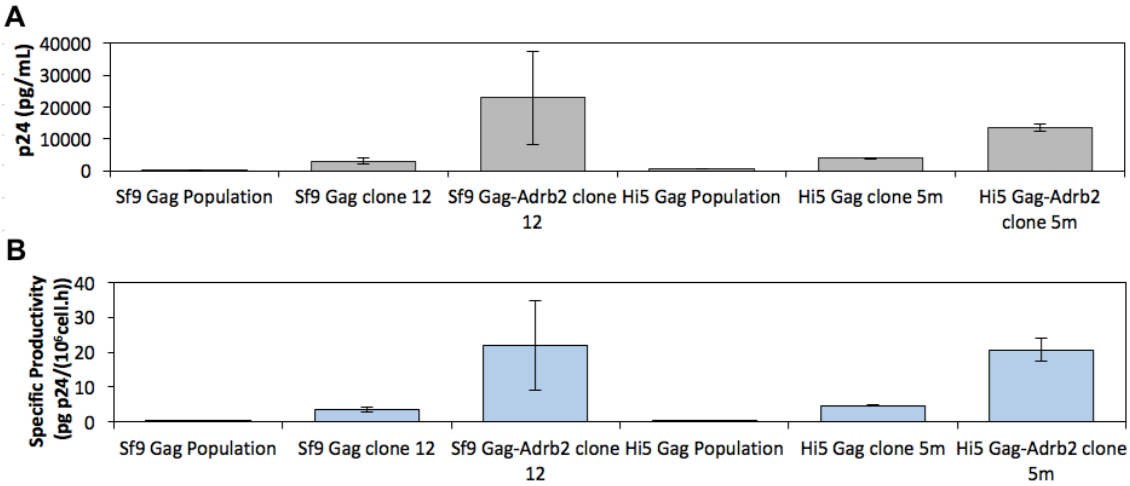


Figure 17 Comparison of secreted p24 protein in Sf9 and Hi5 populations and derived clones before and after Gag co-expression with Adrb2. (A) Concentration of p24 protein at harvest. (B) Specific productivity.

In both Sf9 and Hi5 cell hosts, there was a significant increase in the specific and volumetric productivities from the population to the clone stage, confirming the powerfulness of the RMCE-FACS based screening and selection process we propose. In particular, the specific productivity

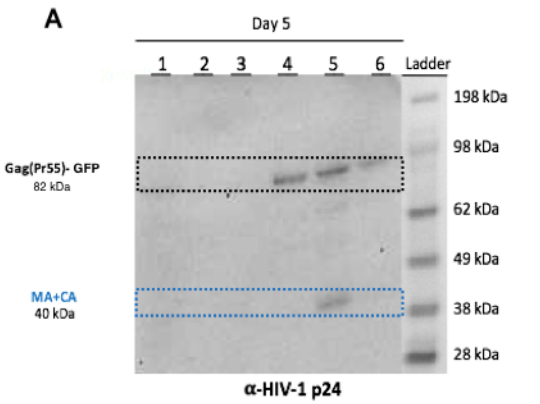
of the Sf9 clone 12 is 36-fold higher than that of the population it was derived, and the specific productivity of the Hi5 clone 5m is 9-fold higher in relation to the Hi5 Gag cell pool. Surprisingly, the subsequent iFlp-mediated cassette exchange at the clone stage, which were transformed into producers of Gag and Adrb2 proteins, further increased the secretion of p24, by 6-fold and 4-fold for Sf9 clone 12 and Hi5 clone 5m, respectively. Similar fold increases were observed for all clones selected for the final target cassette exchange, confirming the platform efficiency.

4.2 Bioprocess Engineering Strategies

The second part of this thesis focuses on the design of bioprocess engineering strategies for optimization of VLPs production. The work performed can be divided in three tasks: 1) evaluation of Gag-VLPs concentration methods, 2) adaptive evolution of cells to non-optimal growth conditions and 3) design of tailor-made nutrients and/or chemical supplements.

4.2.1 Evaluating Gag-VLPs concentration methods

Different methods were evaluated regarding their potential to concentrate Gag-VLPs, namely (1) ultracentrifugation, (2) centrifugation with or without using a Vivaspin® centrifugal concentrator (with and without blocking, see Materials and Methods), and (3) PEG precipitation. Gag-VLPs collected at day 5 from Hi5-Gag cell cultures were concentrated using the different methods and then analysed by western blot (Figure 18). All methods were compared against cell culture supernatant clarified at 200 ×g.



B

Sample	Fold increase in relation to clarification
Centrifugation	0
Ultracentrifugation	0
PEG precipitation	6,1
Centrifugation with Vivaspin® centrifugal concentrator	6,4 (w/ blocking) and 2,9 (w/o blocking)

Figure 18 (A) Western blot analysis of Gag-VLPs harvested at day 5 from Hi5-Gag cultures Lane 1) clarified supernatant; lane 2) clarified supernatant followed by centrifugation at 20 000xg; Lane 3) clarified supernatant followed by ultracentrifugation; Lane 4) clarified supernatant followed by PEG precipitation; Lane 5) clarified supernatant followed by centrifugation with a Vivaspin® centrifugal concentrator blocking with Triton X-100; Lane 6) clarified supernatant followed by centrifugation with a Vivaspin® centrifugal concentrator without blocking; Gag protein fused with GFP (Gag-GFP; 82 kDa) and matrix domain + capsid domain (MA+CA; 40 kDa). (B) Fold increase in band intensity in relation to clarification, integrated with ImageJ.

The band intensities from the various methods were integrated using the ImageJ software. The results show that the Vivaspin® centrifugal concentrator and the PEG precipitation methods are the concentration methods with higher band intensities. In addition, the passivation procedure when using the Vivaspin® centrifugal concentrator improved significantly the recovery of these low-concentrated protein samples.

The different recovery methods were also compared in terms of p24 ELISA assay upon application to both Sf9-Gag and Hi5-Gag cell cultures. Clarified supernatants were concentrated by 150-fold using the different methods before p24 analysis (Table 6).

Table 6 Quantification of p24 protein of Sf9 Gag and Hi5 Gag with different methods of protein concentration by ELISA assay

ID		p24 (pg/mL)	Total protein (ug/mL)	Protein recovery (%)	Purity (10^{-5} %)
Sf9 Gag	**Without concentration**	223 ± 12	3383 ± 146	-	1
	PEG precipitation	3063 ± 182	2872 ±122	9%	11
	Centrifugation with Vivaspin® centrifugal concentrator	8127 ± 671	2604 ±635	24%	31
	Ultracentrifugation	2442 ± 273	823 ± 78	7%	30
Hi5 Gag	**Without concentration**	493 ± 8	1889 ± 279	-	3
	PEG precipitation	3118 ± 487	2689 ± 492	4%	12
	Centrifugation with Vivaspin® centrifugal concentrator	21289 ± 1168	6398 ± 265	29%	33
	Ultracentrifugation	2860 ± 62	1456 ± 72	4%	20

The results obtained suggest that the best concentration method is the centrifugation with Vivaspin® centrifugal concentrator, with p24 concentrations much higher than PEG precipitation and ultracentrifugation methods for both cell hosts. Furthermore, comparing the Gag protein to total protein ratios obtained with the different methods, the ultracentrifugation and centrifugation with Vivaspin® centrifugal concentrator methods allowed higher ratios. This means that these methods are more specific to purify the protein of interest.

4.2.2 Adaptive evolution of cells to non-optimal growth temperatures

Aiming at increasing recombinant Gag production, we tested the effect of growing the cells at hypothermic conditions. Culturing cells under non-physiological temperatures has been widely used in different mammalian expression systems with the objective of increasing the protein production [29,58,59,62–64]. However, this strategy has not been tested for stable expression in the *Lepidopteran* insect cell hosts we are using.

To determine whether the beneficial effect of low culture temperature on Gag production can be enhanced through adaptation, we adapted the Hi5-Gag and Sf9-Gag cell populations to low temperature (24ºC and 22ºC). Shake flask cultures of both populations were sampled daily to

assess cell concentration until viability dropped (Figure 19). The adapted cells at 24ºC and 22ºC were compared with non-adapted cells at this temperature and with cells at normal culture temperature (27ºC). Furthermore, we compared two cell inoculums for each cell host in order to identify which one allows higher protein production when the cell lines growth at lower temperatures.

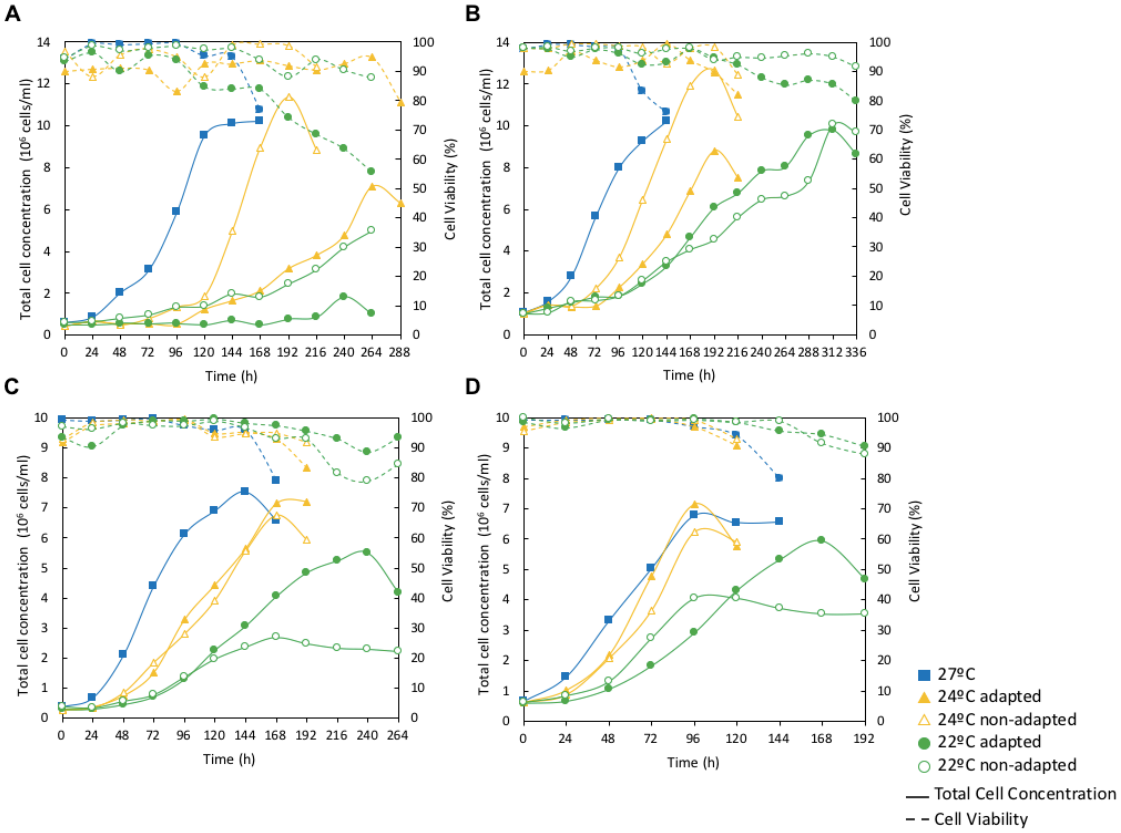


Figure 19 Growth profiles of cells adapted and non-adapted to 24ºC and 22ºC, and control at 27ºC. Sf9 Gag population with inoculum of (A) $0.5x10^6$ cells/mL and (B) $1x10^6$ cells/mL. Hi5 Gag population with inoculum of (C) $0.3x10^6$ cells/mL and (D) $0.6x10^6$ cells/mL

The behaviour of the two cell hosts (adapted and non-adapted) was different at lower culture temperature. In atypical culture conditions, Sf9-Gag cells exhibit more susceptibility than Hi5-Gag cells. When using a low inoculum ($0.5x10^6$ cells/mL), Sf9-Gag cells do not growth and the cell viability was lower than 90%. Despite this, Sf9-Gag cells were able to produce more Gag protein than cells non-adapted or cells growing at 27ºC using the same inoculum, meaning that the specific productivity was significantly higher in this condition (Figure 20). However, with a higher inoculum ($1x10^6$ cells/mL), it was possible to have a better growth performance, and the p24 protein titers reached were much higher than that of cells non-adapted or growing at 27ºC.
In the case of Hi5-Gag cells, the growth performance was less affected by the lower culture temperature; at a lower inoculum ($0.3x10^6$cells/mL) the cells growth but slower than at a higher

inoculum (0.6x10^{6}cells/mL). As this cell growth arrest increases the protein production, there is no need to increase the culture inoculum in Hi5-Gag cells adapted to 22 ºC.

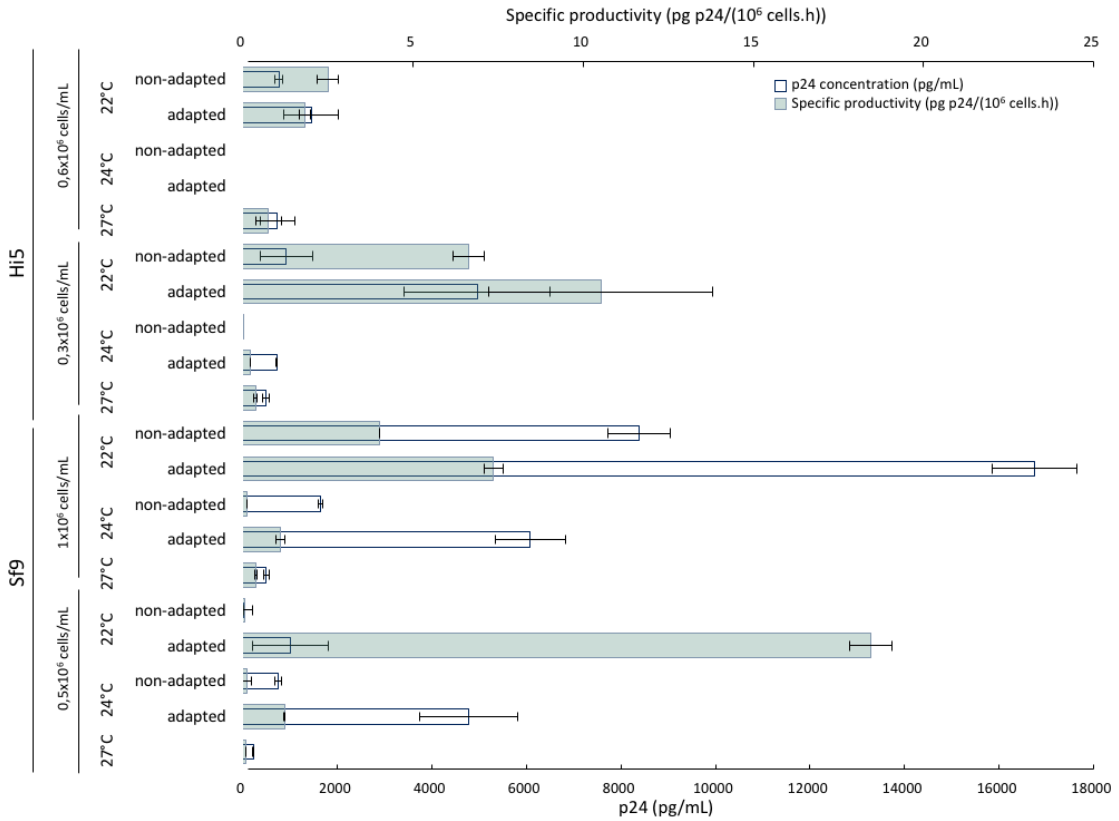


Figure 20 Comparison of Gag protein production in Sf9 and Hi5 cells at different growth temperatures (27ºC, 24ºC, 22ºC); cell inoculums (0.5x10^6 cells/mL and 1x10^6 cells/mL for Sf9-Gag cells; 0.3x10^6 cells/mL and 0.6x10^6 cells/mL for Hi5-Gag cells); and adapted and non-adapted cells. Gag production was evaluated by a p24 ELISA assay when the maximum viable cell concentration was reached in each condition.

Comparing adapted cells to non-adapted cells the protein titers and the specific productivities are in general higher in adapted cells, evidencing the importance of the adaptation process of cells to low temperature.

In agreement with the different susceptibilities of the two cell hosts to atypical culture conditions, the differences between specific productivities in cells adapted and non-adapted are smaller in Hi5 cells. In addition, Hi5-Gag cells with a higher inoculum (0.6x10^{6}cells/mL) demonstrated lower production comparing to a lower inoculum (0.3x10^{6}cells/mL); cultures starting at higher cell densities are much shorter (96h versus 168h), decreasing the Gag-VLPs accumulated at time of harvest.

For both cell hosts, the protein production is increased at 22ºC, comparing with 24ºC and 27ºC. In this atypical culture condition, despite the drop in the specific growth rate, the metabolic activity is reduced, allowing high cell viabilities during longer periods, contributing to increase the accumulation of Gag protein in the supernatant. Still to determine is whether the quality of

the Gag-VLPs is affected or improved in these new culture conditions, as the ELISA assay quantifies all p24 protein (free and assembled).

4.2.3 Effect of productivity enhancers on insect cell growth and Gag expression

There are several chemical compounds (such as NaBu and DMSO) that are known to promote the expression of recombinant proteins in mammalian cells. Their adoption for stable expression in Sf9 and Hi5 cells was hardly tested. To evaluate their effect on growth performance and Gag production, shake flask cultures of Hi5-Gag cells were independently supplemented with different concentrations of DMSO and NaBu at different cell concentrations. The cell concentration was followed daily until the viability started to decrease below 90% and was compared with cultures without supplementation.

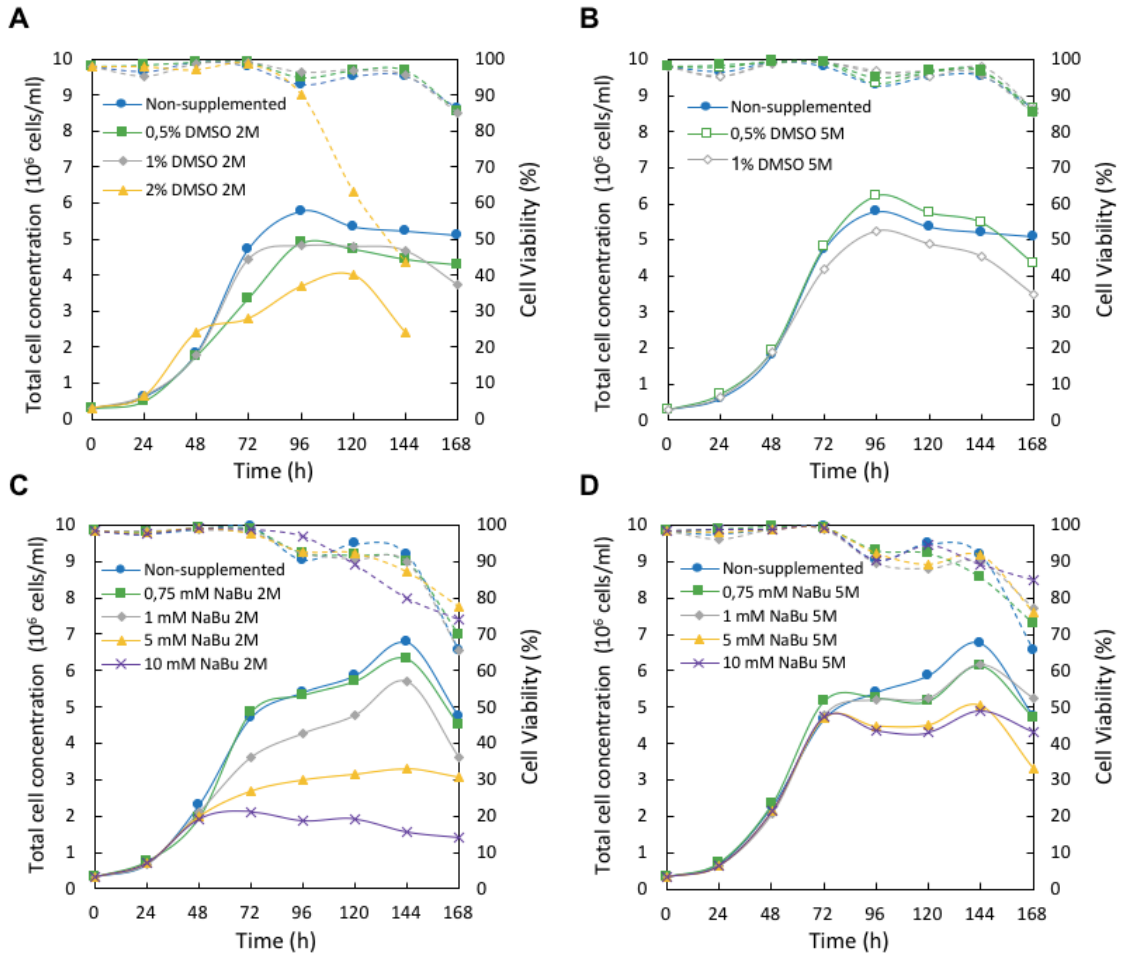


Figure 21 Growth profiles of Hi5-Gag cell cultures under NaBu, DMSO or control conditions (A) Addition of DMSO (0.5%, 1% or 2% of culture volume) when cell densities of $2x10^6$ cells/mL were reached; (B) Addition of DMSO (0.5% and 1% of culture volume) when cell densities of $5x10^6$ cells/mL (5M) were reached. (C) Addition of NaBu (0.75 mM, 1mM, 5mM or 10 mM) when cell densities of $2x10^6$ cells/mL (2M) and (D) $5x10^6$ cells/mL (5M) were reached.

For DMSO, three concentrations (0.5%, 1%, 2%) were added when a cell concentration of $2x10^6$ cells/mL was reached (Figure 21A). There was a small decrease in the cell growth for 0.5% and 1% of DMSO comparing to control conditions, and the viability was maintained above 90%. However, when 2% of DMSO was used, a significant decrease in the growth rate was observed and the viability started to decrease below 90% sooner and much faster. Because of its cytotoxicity, this DMSO quantity was not used in further studies. We also tested the effect of adding DMSO (0.5% and 1%) at higher cell densities ($5x10^6$cells/mL), which impacted less the integral of viable cells (Figure 21B).

To study the impact of NaBu on growth and Gag production, we used 4 different concentrations (0.75, 1, 5 and 10 mM), also at two different growth stages ($2x10^6$ cells/ml and $5x10^6$cells/mL) (Figure 21C-D). When NaBu was added at 5mM and 10mM concentrations (Figures 21C and 21D), the cells could not divide anymore, causing a cell growth arrest. The lowest concentration tested (0.75mM) did not affect the growth performance, while 1mM had a small negative impact when added in the mid-exponential growth phase.

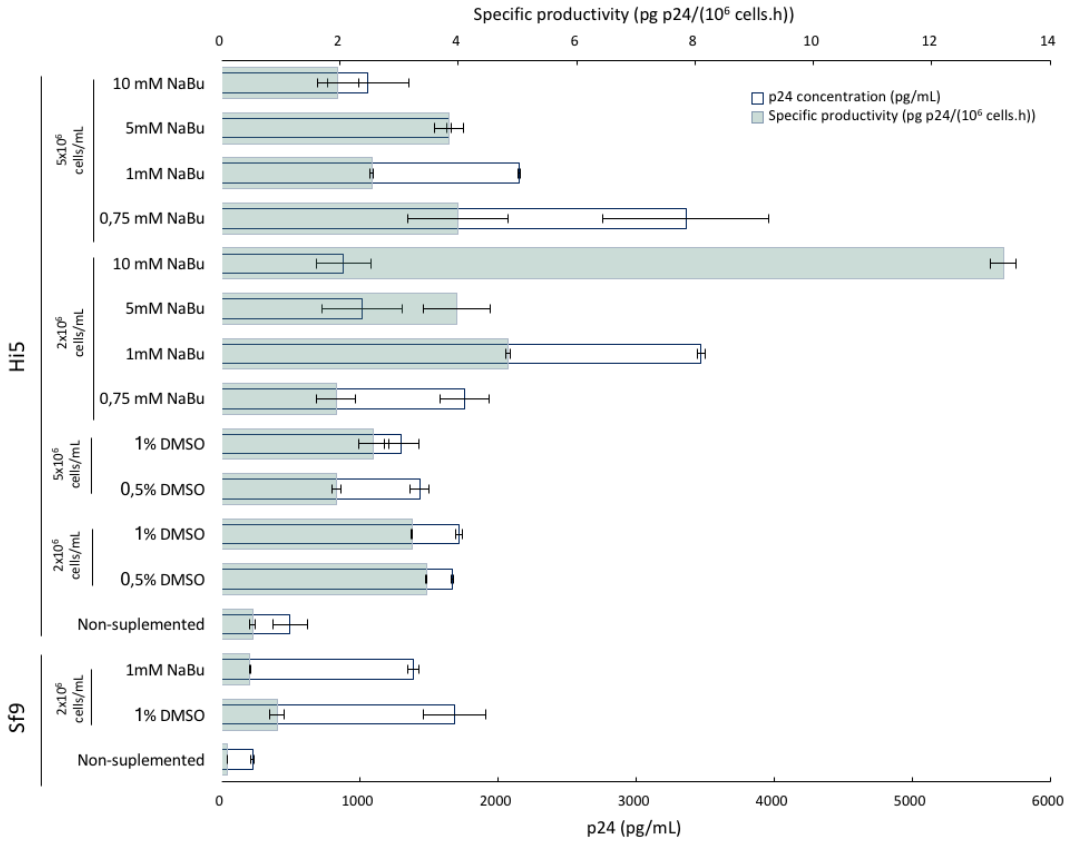


Figure 22 Comparison of Gag protein production in Sf9 Gag and Hi5 Gag supplemented (NaBu, DMSO, nutrients, lipids) and non-supplemented. Supplementation of DMSO (0.5% and 1%), NaBu (0.75 mM; 1mM; 5mM and 10 mM) at different cell densities ($2x10^6$ cells/mL and $5x10^6$ cells/mL). Gag production was evaluated by a p24 ELISA assay.

The impact of the different concentrations of the compounds on Gag production was assessed at 144 hours post inoculation. All conditions tested allowed higher protein titers and higher specific productivities than the control cultures (Figure 22). DMSO allowed up to 3.5-fold increase in protein titer, while the lowest concentrations of NaBu allowed up to 7.0-fold increase in protein titer. In terms of specific productivity, the highest increase was observed for the culture in which 10 mM NaBu was used (25.4-fold increase in relation to untreated cultures).
The DMSO and NaBu conditions allowing higher Gag concentrations in Hi5-Gag cells were applied to Sf9-Gag cell cultures. While the NaBu treatment did not affect the Sf9 growth performance, with the DMSO treatment the maximum cell density reached was lower than non-treated cultures (Figure 23).

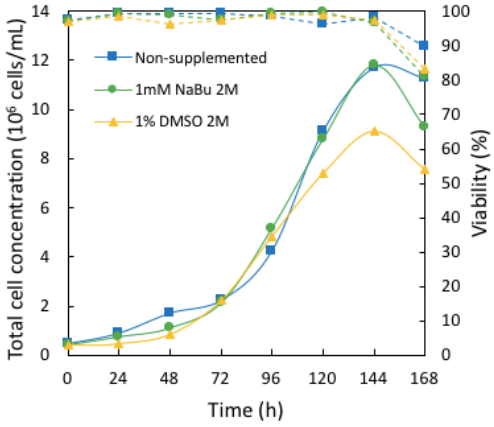


Figure 23 Growth profiles of supplemented (1% DMSO and 1mM NaBu) and non-supplemented cultures of Sf9 Gag. DMSO and NaBu supplementation when the cells reached the cell density of $2x10^6$ cells/mL.

Concerning Gag production, both treatments increased significantly the amount of p24 protein in the supernatant (Figure 22), with a slightly higher fold increase achieved with DMSO (7.5-fold versus 6.2-fold).

4.2.4 Effect of feeding strategies on insect cell proliferation and Gag expression

The supply of limiting nutrients (e.g., sugars, amino acids, lipids, vitamins) can improve the growth rate of cells, increase the culture longevity and consequently increase the protein production[59,65]. We performed cultures of Sf9-Gag and Hi5-Gag cells in which we replenished essential nutrients that are known to be exhausted during typical batch cultures. Furthermore, as Gag-VLPs bud through the plasma membrane taking along lipidic envelope, we tested if supplementing the cultures with extra lipids could improve culture performance (see in Materials and Methods). Regarding the growth performance, both cell hosts supplemented with nutrients achieved higher cell densities comparing to control cultures ($15x10^6$cells/mL in Sf9-Gag cells and $9x10^6$cells/mL in Hi5-Gag cells) (Figures 24A and 24B). On the other hand, the

supplementation with lipids (1× and 5×) didn't increase the total cell concentration, comparing to non-supplemented cells in both cell hosts.

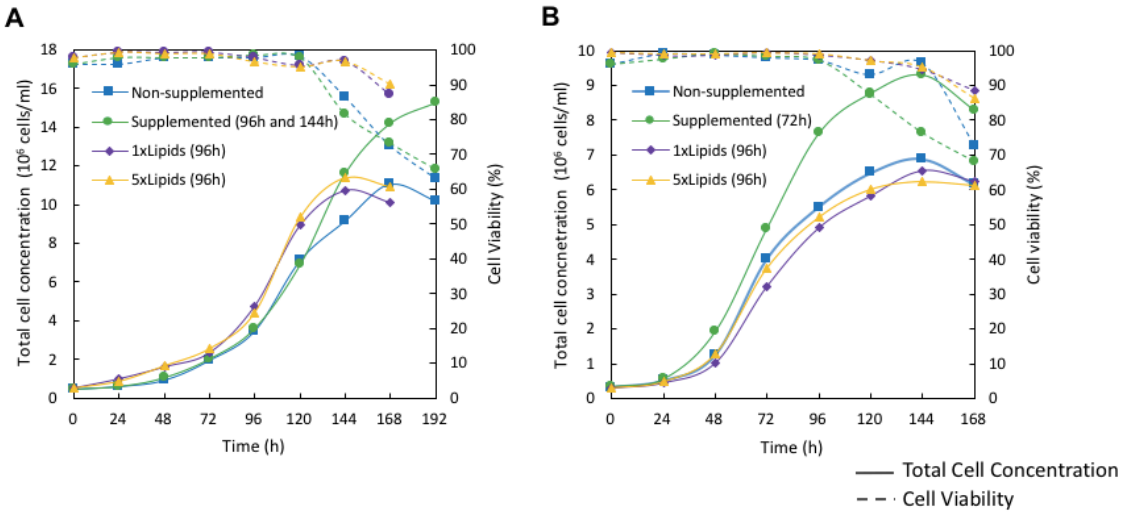


Figure 24 Growth profiles of supplemented and non-supplemented (control) cultures of (A) Sf9 Gag supplemented at 96h (10mM Ser and 1mM Cys) and 144h (20mM Glc and 2mM Gln), 1x Lipids and 5x Lipids (B) Hi5 Gag supplemented at 72h (5mM Gln, 10 mM Asn and 20 mM Glc), 1x Lipids and 5x Lipids.

The protein production was assessed by western blot (Figure 25) and by ELISA (Figure 26).

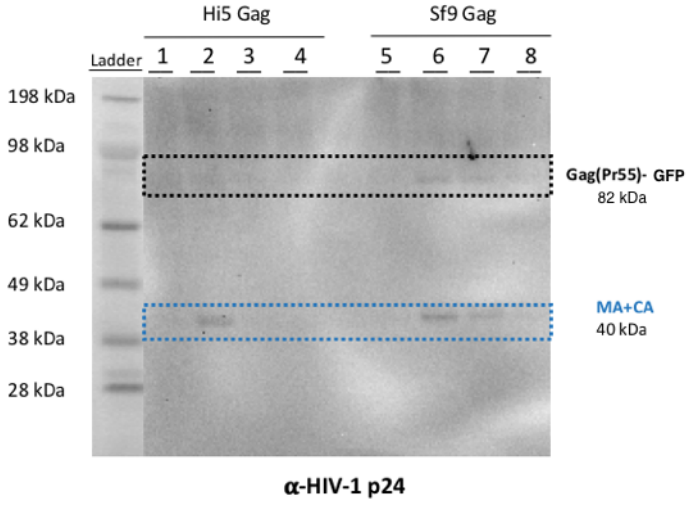


Figure 25 Western blot analysis of Hi5 gag and Sf9 gag cultures supplemented and non-supplemented at maximum cell concentration (1) Hi5 Gag non-supplemented, (2) Hi5 Gag supplemented at 72h (5mM Gln, 10 mM Asn and 20 mM Glc), (3) Hi5 Gag supplemented with 1x Lipids, (4) Hi5 Gag supplemented with 5x Lipids, (5) Sf9 Gag non-supplemented, (6) Sf9 Gag supplemented at 96h (10mM Ser and 1mM Cys) and 144h (20mM Glc and 2mM Gln), (7) Sf9 Gag supplemented with 1x Lipids and (8) Sf9 Gag supplemented with 5x Lipids. Gag protein fused with GFP (Gag-GFP; 82 kDa) and matrix domain + capsid domain (MA+CA; 40 kDa).

The western blot shows an increase of Gag protein when the culture is supplemented with nutrients in both cell lines and in the case of Sf9-Gag population the supplementation with 1xLipids also have an increase in protein concentration (Figure 25).

From the ELISA results (Figure 26), indeed the supplementation of essential nutrients allowed higher Gag concentrations than supplementation with lipids for both cell populations. In the case of Hi5-Gag cells, extra lipids (1× or 5×) have almost no increase in Gag production.

Comparing all the tailor-made nutrients and/or chemical supplements, with 10mM NaBu was obtained higher specific productivity but there was a cell growth arrest and the growth performance of the cells was affected. Regarding the Gag concentration obtained in each conditions the feeding with nutrients was the greatest in the both cell hosts.

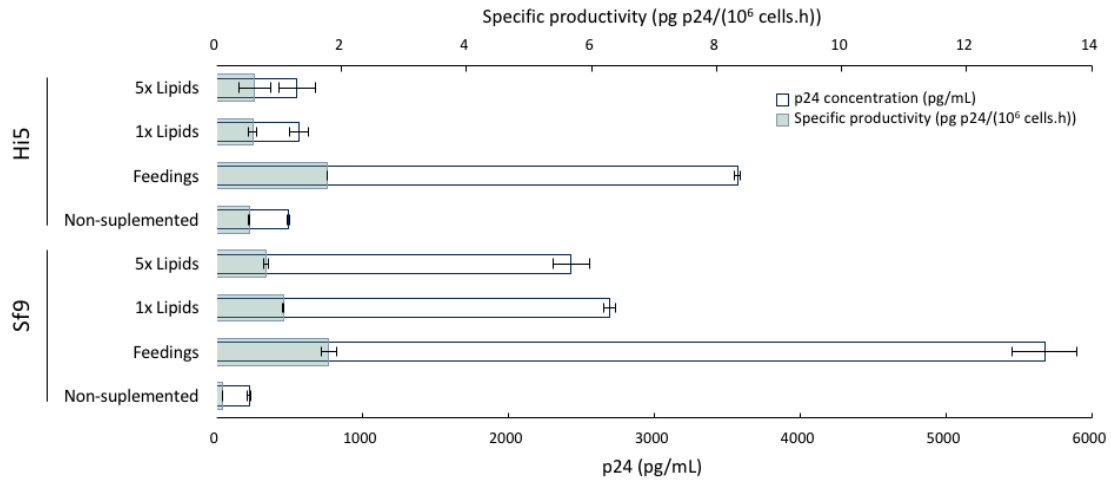


Figure 26 Comparison of Gag protein production in Sf9 Gag and Hi5 Gag supplemented with glucose and specific amino acids, or lipids (see Materials and Methods). Gag production was evaluated by a p24 ELISA assay when the maximum cell concentration was reached in each condition.

5. Discussion

Important steps in the development of flexible and re-usable insect cell factories for continuous production of pseudo typed enveloped VLPs were undertaken in this thesis. The insect cell expression system here developed is expected to be advantageous over the transient baculovirus-insect cell expression system, which is the current gold standard to produce multimeric proteins. Two major tasks were performed during this thesis: 1) characterization of Sf9 and Hi5 clones tagged in different loci with a Flp/FRT cassette encoding the fusion Gag-iCherry protein, and their further use to co-express a model GPCR, obtaining Gag VLPs decorated with the GPCR; 2) Implementation of different bioprocess optimization strategies in order to enhance stable production of Gag-VLPs in insect cells.

5.1 Cell line development

RMCE technology has been widely adopted for production of recombinant proteins in several mammalian cell lines[51,66] and more recently also in insect cells, including contributions from our lab [15,45,56]. The focus of this work was to establish re-usable high producer insect cell lines, relying on the flipase site-specific recombination system, bypassing the need to perform extensive clone screening for every new target membrane protein to be produced at the surface of Gag-VLPs. Indeed, the incorporation of membrane proteins on the surface of VLPs from enveloped viruses, such Retrovirus, can be a powerful strategy for applications such as drug screening or manufacture of vaccines. Our new platform can combine the production of Gag-VLPs with any membrane protein of interest by means of RMCE. The Gag core protein will trigger VLP budding and release from lipid raft regions of the plasma membrane, taking along the anchored target protein, thus providing a native conformation for downstream assays. The use of RMCE with a Gag fused to fluorescent reporter proteins enabled the use of FACS to screen higher producer clones. It has been reported that such fusion proteins result in VLPs which are morphologically indistinguishable from VLPs formed by Gag only [67]. The linker between the fused moieties includes one FRT site enabling the removal of the reporter gene by cassette exchange. Cells tagged in loci supporting high transcription rates and high Flp recombination efficiencies can be pre-selected at population level, a remarkable advantage of this tagging/targeting strategy. In this way, all clones tested supported cassette exchange, which did not happen when using the conventional RMCE cell line development process [45,68.] Furthermore, the fact that we have an FRT site composing the linker fusing the two genes does not seem to impact the recombination efficiency, as the percentage of cells which exchanged cassettes is similar to those achieved with standard FRT cassettes (unpublished results).

After the first cassette exchange at population level, several clones from Gag-iCherry expressing Sf9 and Hi5 cell populations were isolated by cell sorting, which were then subjected to a comprehensive characterization. In both cells hosts, the clones with best growth performance were not necessarily those with high fluorescence intensity or high Gag protein

production. Furthermore, some clones show high intracellular fluorescence intensity but low Gag production; this could be because Gag-iCherry may be staying within the cell instead of coming out to the supernatant. In addition, it is important that the clones have stable expression along passages (fluorescence stability), such that the antibiotic can be removed after the selection process.

Improved growth performance is normally associated to higher amounts of recombinant protein which accumulate into the supernatant. However, the production of Gag VLPs could have influence in the growth of the cells because the stress related with the disruption of the membrane when they are released [21]. Clones with different morphogenetic characteristic found during the selection and cloning process were found to release VLPs inefficiently [69]. This phenomenon indicates that maintaining similar morphology and growth characteristics with the parental cells may be important in the engineering cell lines with high efficiency and stable exogenous gene expression.

To produce Gag-VLPs displaying the Adrb2 receptor, several clones were submitted to RMCE and selected with zeocin to isolate the cells which exchanged cassettes. All clones lost red fluorescence and gained the fluorescence associated to the receptor, demonstrating successful cassette exchange, but the selection process was very long. Consequently, we are still optimizing the protocol in order to have a faster and efficient recombination and selection process.

Of note, a significant increase in Gag production was achieved from both Sf9- and Hi5-Gag tagging populations to the isolated Gag-iCherry clones, demonstrating the efficiency of the RMCE strategy here proposed. When these clones were further submitted to cassette exchange to co-express Gag (without iCherry fusion) and the receptor, an additional increase in Gag production was observed for all clones tested. Although an increase was expected due to the removal of the reporter protein fused to Gag, we were surprised by the observed improvements (up to 6-fold depending on the clone), These results deserve further investigation; for instance, assessing by real time PCR the amount of Gag transcripts before and after the co-expression with adrb2.

5.2 Bioprocess Engineering Strategies

In the biotechnological field is important to have highly productive bioprocesses [57]. In the second part of this work, we explored different bioprocess strategies to further improve the recombinant protein titers produced by the developed RMCE insect cell lines, and make them strong alternative platforms to the baculovirus-insect cell system.

In addition to protein production improvement, it is also important to establish the optimal concentration method for Gag-VLPs, bearing in mind scalability, costs, protein recovery and purity. Indeed, centrifugation with Vivaspin® centrifugal concentrator yields more protein and enables higher purification levels, although being dependent on costly material. Despite the lower protein recovery obtained with ultracentrifugation as compared to what has been reported

[71–73], it enabled reasonable VLP purification yields. This method is dependent on the ressuspension of VLPs, which can be problematic given that those are particularly sensitive particles [74]. However, both methods described above are challenging when higher volumes of culture are processed. Consequently, protein concentration with PEG precipitation would be more suitable for large scale protein production, although the low efficiency.

Hypothermal growth conditions (i.e. culturing cells at temperatures lower than the optimal for growth) or the addition of small chemical compounds have been applied to different expression systems to enhance recombinant protein production [57,59,75]. Furthermore, the supplementation of essential nutrients is normally employed to improve cell proliferation and culture longevity as a means to also increase final product concentration [59,60] .

For both cell hosts used in this thesis, the hypothermal growth increased the production titers of the Gag protein, with the minimum temperature tested, 22ºC, yielding the largest improvements. In addition, cells adapted to this culture temperature showed a higher fold increase in Gag production than cells non-adapted. The culture temperature at 22ºC allowed up to 34-fold increase in specific productivity in Sf9-Gag cells, while in Hi5-Gag cells allowed up to 10-fold increase in specific productivity, demonstrating that the adaptation process is important to have optimal yields. Culturing *Drosophila melanogaster* derived S2 cells at 22ºC was also described to enhance production of recombinant rabies virus glycoprotein (rRVGP) by almost 10-fold [76]. Reducing the temperature of suspension cultures of CHO cells from 37ºC to 31ºC during late exponential growth phase results in an immediate cessation of proliferation, and in an increase in the cell specific productivity, resulting in a 6-fold increase of the product titter [64]. Indeed, the growth capabilities of the cells decrease at low temperatures. Also the metabolites are consumed are lower rates, contributing to increase the culture longevity, and consequently, allowing higher protein titers to be accumulated. Nevertheless, in this work we were assessing the total p24 protein accumulated in the supernatant. As future work, it is important to evaluate the Gag-VLPs stability at 22ºC and confirm that hypothermic growth conditions do not have influence in the assembling of the VLPs. This can be performed by using a Vivaspin® centrifugal concentrator to concentrate only Gag-VLPs based on molecular weight, and then analyse p24 protein by ELISA. Also still to be performed is the observation of the produced Gag-VLPs in the different conditions by electron microscopy.

The treatment with the chemicals DMSO and NaBu allowed to obtain higher protein titers when compared to non-treated cultures. It has been shown that DMSO act as a stabilizing agent during protein folding and also have an extensive impact on the transcription rate [59,77]. For example, the production of endostatin or rRVGP in S2 cells increased 17% [78] or 3.6-fold[76], respectively, in the presence of DMSO in relation to non-treated cultures. It has also been demonstrated that DMSO induces an efficient and reversible G1 phase arrest in CHO cells [59,77]. In our study, the concentrations of DMSO used did not cause cell growth arrest but allowed a 10 and 6-fold increase in specific productivity of p24 in Sf9 and Hi5 Gag cells, respectively.

However, the higher concentrations of NaBu used caused growth arrest in Hi5 cells, condition which also allowed the highest increase in the specific productivity (25-fold increase). NaBu is

known to cause cell blockage at the G1-phase of the cell cycle, inhibit histone deacetylases, induce cell differentiation and apoptosis [59]. Hyperacetylation of histones through the inhibition of histone deacetylases has been found to up-regulate transcription by opening up nucleosome structures. These modifications would make a recombinant gene more accessible to the transcription machinery, thus increasing its transcription rate [58]. For example, in hybridoma cell cultures the addition of NaBu was reported to increase production of a monoclonal antibody by 2.3-fold [77]. With other expression system like *Drosophila melanogaster* S2 cells it was also reported higher production comparing with cells without NaBu [79,80].

Fed-batch is a common strategy used for industrial manufacture of recombinant therapeutics in animal cell cultures [60]. The objective of fed-batch strategy is to increase final product concentration which is possible by extending culture duration and/or by increasing peak viable cell concentration by using feeding strategies based on the consumption rates of nutrients [59,60]. Supplementing Sf9-Gag and Hi5-Gag cell culture with glucose and amino acids enabled to reach higher cell concentrations and extend culture duration, which positively impacted the production titer of p24 leading to 20 and 3-fold increase in specific productivity.

6. Conclusion and future work

In this study, we developed insect cell platforms for production of enveloped VLPs displaying membrane proteins of interest. The baculovirus-insect cell expression system is commonly used for recombinant protein production, especially for difficult-to-express proteins such as receptors and other membrane proteins. Nevertheless, stable cell lines are advantageous over transient expression, due to potential to scale-up and improvement of the production by process optimization. Also, stable insect cell lines don't have the negative effect on protein processing pathways caused by the lytic baculovirus infection cycle, sometimes critical for the protein quality.

Our system is particularly beneficial for proteins expressed in lower quantities as membrane proteins, because of the ability to secrete them in their own native environment without the need to used chemical or physical methodologies for extraction that in the end could interfere with their own conformation and functionality.

Combining RMCE and FACS we could identify cell clones which produce much more Gag than the populations from which they were derived, confirming the potential of our approach. In parallel, by using three independent strategies we could improve the Gag titers produced by the populations. The next step would be to combine these strategies to evaluate the synergistic effect of supplementing cells adapted to low culture temperature with key nutrients, and in the presence of DMSO/NaBu. Furthermore, it is important to assess the impact of such strategies on the protein titers produced by the final clones. As proof-of-concept, it is important to perform lab scale bioreactors with the purpose of assess the scalability of the strategy.

7. References

1. Demain, A. L. & Vaishnav, P. Production of recombinant proteins by microbes and higher organisms. *Biotechnol. Adv.* **27,** 297–306 (2009).
2. Balbás, P. & Lorence, A. *Recombinant gene expression: reviews and protocols*. (Humana Press, 2004).
3. Unger, T. & Peleg, Y. in *Chemical Genomics and Proteomics* (ed. Zanders, E. D.) **800,** 187–199 (Humana Press, 2012).
4. Beljelarskaya, S. N. A Baculovirus Expression System for Insect Cells. *Mol. Biol.* **36,** 281–292 (2002).
5. Geisse, S., Gram, H., Kleuser, B. & Kocher, H. P. Eukaryotic Expression Systems: A Comparison. *Protein Expr. Purif.* **8,** 271–282 (1996).
6. *Molecular cell biology*. (Freeman, 2002).
7. Willis, S. *et al.* Use of virus-like particles as quantitative probes of membrane protein interactions. *Biochemistry (Mosc.)* **47,** 6988–6990 (2008).
8. Smith, S. M. in *Protein Chromatography* (eds. Walls, D. & Loughran, S. T.) **681,** 485–496 (Humana Press, 2011).
9. Webb, D. R., Handel, T. M., Kretz-Rommel, A. & Stevens, R. C. Opportunities for functional selectivity in GPCR antibodies. *Biochem. Pharmacol.* **85,** 147–152 (2013).
10. Lappano, R. & Maggiolini, M. G protein-coupled receptors: novel targets for drug discovery in cancer. *Nat. Rev. Drug Discov.* **10,** 47–60 (2011).
11. Chen, L., Jin, L. & Zhou, N. An update of novel screening methods for GPCR in drug discovery. *Expert Opin. Drug Discov.* **7,** 791–806 (2012).
12. Tang, X., Wang, Y., Li, D., Luo, J. & Liu, M. Orphan G protein-coupled receptors (GPCRs): biological functions and potential drug targets. *Acta Pharmacol. Sin.* **33,** 363–371 (2012).
13. Hutchings, C. J., Koglin, M. & Marshall, F. H. Therapeutic antibodies directed at G protein-coupled receptors. *mAbs* **2,** 594–606 (2010).
14. Stenlund, P., Babcock, G. J., Sodroski, J. & Myszka, D. G. Capture and reconstitution of G protein-coupled receptors on a biosensor surface. *Anal. Biochem.* **316,** 243–250 (2003).
15. Fernandes, F., Teixeira, A. P., Carinhas, N., Carrondo, M. J. & Alves, P. M. Insect cells as a production platform of complex virus-like particles. *Expert Rev. Vaccines* **12,** 225–236 (2013).
16. Buonaguro, F. M., Tornesello, M. L. & Buonaguro, L. Virus-like particle vaccines and adjuvants: the HPV paradigm. *Expert Rev. Vaccines* **8,** 1379–1398 (2009).
17. Kushnir, N., Streatfield, S. J. & Yusibov, V. Virus-like particles as a highly efficient vaccine platform: Diversity of targets and production systems and advances in clinical development. *Vaccine* **31,** 58–83 (2012).
18. Bright, R. A. *et al.* Influenza virus-like particles elicit broader immune responses than whole virion inactivated influenza virus or recombinant hemagglutinin. *Vaccine* **25,** 3871–3878

(2007).

19. Balliet, J. W. & Bates, P. Efficient infection mediated by viral receptors incorporated into retroviral particles. *J. Virol.* **72,** 671–676 (1998).

20. Maldonado, J. O., Martin, J. L., Mueller, J. D., Zhang, W. & Mansky, L. M. New insights into retroviral Gag-Gag and Gag-membrane interactions. *Front. Microbiol.* **5,** (2014).

21. Weldon, RA and Erdie, CR and Oliver, MG and Wills, JW. Incorporation of chimeric gag protein into retroviral particles. *J. Virol.* **64,** 4169–4179 (1990).

22. Szécsi, J. *et al.* Induction of neutralising antibodies by virus-like particles harbouring surface proteins from highly pathogenic H5N1 and H7N1 influenza viruses. *Virol. J.* **3,** 1–7 (2006).

23. Haynes, J. R. *et al.* Influenza-pseudotyped Gag virus-like particle vaccines provide broad protection against highly pathogenic avian influenza challenge. *Vaccine* **27,** 530–541 (2009).

24. Akahata, W. *et al.* A virus-like particle vaccine for epidemic Chikungunya virus protects nonhuman primates against infection. *Nat. Med.* **16,** 334–338 (2010).

25. Hamakubo, T., Kusano-Arai, O. & Iwanari, H. Generation of antibodies against membrane proteins. *Biochim. Biophys. Acta BBA - Proteins Proteomics* **1844,** 1920–1924 (2014).

26. Grgacic, E. V. L. & Anderson, D. A. Virus-like particles: Passport to immune recognition. *Methods* **40,** 60–65 (2006).

27. *Cell and tissue reaction engineering*. (Springer, 2009).

28. McCarroll, L. & King, L. A. Stable insect cell cultures for recombinant protein production. *Curr. Opin. Biotechnol.* **8,** 590–594 (1997).

29. Gerbal, M. *et al.* Adaptation of an insect cell line of spodoptera frugiperda to grow at 37° c: characterization of an endodiploid clone. *Vitro Cell. Dev. Biol.--Anim.* **36,** 117–124 (2000).

30. Yang, J.-D. *et al.* Rational scale-up of a baculovirus-insect cell batch process based on medium nutritional depth. *Biotechnol. Bioeng.* **52,** 696–706 (2000).

31. Vaughn, J. L., Goodwin, R. H., Tompkins, G. J. & McCawley, P. The establishment of two cell lines from the insectspodoptera frugiperda (lepidoptera; noctuidae). *In Vitro* **13,** 213–217 (1977).

32. Hink, W. F. Established Insect Cell Line from the Cabbage Looper, Trichoplusia ni. *Nature* **226,** 466–467 (1970).

33. Granados, R. R., Guoxun, L., Derksen, A. C. G. & McKenna, K. A. A new insect cell line from Trichoplusia ni (BTI-Tn-5B1-4) susceptible to Trichoplusia ni single enveloped nuclear polyhedrosis virus. *J. Invertebr. Pathol.* **64,** 260–266 (1994).

34. Ikonomou, L., Schneider, Y.-J. & Agathos, S. N. Insect cell culture for industrial production of recombinant proteins. *Appl. Microbiol. Biotechnol.* **62,** 1–20 (2003).

35. Harrison, R. L. & Jarvis, D. L. in *Baculovirus and Insect Cell Expression Protocols* (ed. Murhammer, D. W.) **388,** 299–315 (Humana Press, 2007).

36. Passarelli, A. L. & Guarino, L. A. Baculovirus late and very late gene regulation. *Curr.*

Drug Targets **8,** 1103–1115 (2007).

37. Volkman, L. E. Nucleopolyhedrovirus interactions with their insect hosts. *Adv. Virus Res.* **48,** 313–348 (1997).

38. Caron, A. W., Archambault, J. & Massie, B. High-level recombinant protein production in bioreactors using the baculovirus-insect cell expression system. *Biotechnol. Bioeng.* **36,** 1133–1140 (1990).

39. Ayres, M. D., Howard, S. C., Kuzio, J., Lopez-Ferber, M. & Possee, R. D. The Complete DNA Sequence of Autographa californica Nuclear Polyhedrosis Virus. *Virology* **202,** 586–605 (1994).

40. Rhiel, M., Mitchell-Logean, C. M. & Murhammer, D. W. Comparison of Trichoplusia ni BTI-Tn-5B1-4 (high five™) and Spodoptera frugiperda Sf-9 insect cell line metabolism in suspension cultures. *Biotechnol. Bioeng.* **55,** 909–920 (1997).

41. Granados, R. R., Li, G. & Blissard, G. W. Insect cell culture and biotechnology. *Virol. Sin.* **22,** 83–93 (2007).

42. Kost, T. A., Condreay, J. P. & Jarvis, D. L. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. *Nat. Biotechnol.* **23,** 567–575 (2005).

43. MiliÄ‡, D. & Veprintsev, D. B. Large-scale production and protein engineering of G protein-coupled receptors for structural studies. *Front. Pharmacol.* **6,** (2015).

44. Merrihew, R. V. *et al.* Chromosomal Integration of Transduced Recombinant Baculovirus DNA in Mammalian Cells. *J. Virol.* **75,** 903–909 (2001).

45. Fernandes, F. *et al.* Flipase-mediated cassette exchange in *Sf9* insect cells for stable gene expression. *Biotechnol. Bioeng.* **109,** 2836–2844 (2012).

46. Schmidt, F. R. Recombinant expression systems in the pharmaceutical industry. *Appl. Microbiol. Biotechnol.* **65,** 363–372 (2004).

47. Nehlsen, K., da Gama-Norton, L., Schucht, R., Hauser, H. & Wirth, D. Towards rational engineering of cells: Recombinant gene expression in defined chromosomal loci. *BMC Proc.* **5,** O6 (2011).

48. Nehlsen, K. *et al.* Recombinant protein expression by targeting pre-selected chromosomal loci. *BMC Biotechnol.* **9,** 100 (2009).

49. Turan, S., Zehe, C., Kuehle, J., Qiao, J. & Bode, J. Recombinase-mediated cassette exchange (RMCE) — A rapidly-expanding toolbox for targeted genomic modifications. *Gene* **515,** 1–27 (2013).

50. Oumard, A., Qiao, J., Jostock, T., Li, J. & Bode, J. Recommended Method for Chromosome Exploitation: RMCE-based Cassette-exchange Systems in Animal Cell Biotechnology. *Cytotechnology* **50,** 93–108 (2006).

51. Coroadinha, A. S. *et al.* The use of recombinase mediated cassette exchange in retroviral vector producer cell lines: Predictability and efficiency by transgene exchange. *J. Biotechnol.* **124,** 457–468 (2006).

52. Ramachandra, C. J. A. *et al.* Efficient recombinase-mediated cassette exchange at the AAVS1 locus in human embryonic stem cells using baculoviral vectors. *Nucleic Acids Res.* **39,**

e107–e107 (2011).

53. Thyagarajan, B., Olivares, E. C., Hollis, R. P., Ginsburg, D. S. & Calos, M. P. Site-Specific Genomic Integration in Mammalian Cells Mediated by Phage C31 Integrase. *Mol. Cell. Biol.* **21,** 3926–3934 (2001).

54. M., K., S., I., Y., F., K., Y. & T., S. Construction of engineered CHO strains for high-level production of recombinant proteins. *Appl. Microbiol. Biotechnol.* **60,** 442–448 (2002).

55. Wiberg, F. C. *et al.* Production of target-specific recombinant human polyclonal antibodies in mammalian cells. *Biotechnol. Bioeng.* **94,** 396–405 (2006).

56. Fernandes, F. *et al.* Production of rotavirus core-like particles in Sf9 cells using recombinase-mediated cassette exchange. *J. Biotechnol.* **171,** 34–38 (2014).

57. Rossi, N. *et al.* Effect of hypothermic temperatures on production of rabies virus glycoprotein by recombinant Drosophila melanogaster S2 cells cultured in suspension. *J. Biotechnol.* **161,** 328–335 (2012).

58. Sunley, K. & Butler, M. Strategies for the enhancement of recombinant protein production from mammalian cells by growth arrest. *Biotechnol. Adv.* **28,** 385–394 (2010).

59. Kumar, N., Gammell, P. & Clynes, M. Proliferation control strategies to improve productivity and survival during CHO based production culture: A summary of recent methods employed and the effects of proliferation control in product secreting CHO cell lines. *Cytotechnology* **53,** 33–46 (2007).

60. Sauer, P. W., Burky, J. E., Wesson, M. C., Sternard, H. D. & Qu, L. A high-yielding, generic fed-batch cell culture process for production of recombinant antibodies. *Biotechnol. Bioeng.* **67,** 585–597 (2000).

61. Malik, R. U. *et al.* Detection of G Protein-selective G Protein-coupled Receptor (GPCR) Conformations in Live Cells. *J. Biol. Chem.* **288,** 17167–17178 (2013).

62. Fox, S. R., Yap, M. G. & Wang, D. I. The Effect of Culture Temperature on Recombinant IFN-γ Production and Quality. (2004).

63. Al-Fageeh, M. B., Marchant, R. J., Carden, M. J. & Smales, C. M. The cold-shock response in cultured mammalian cells: Harnessing the response for the improvement of recombinant protein production. *Biotechnol. Bioeng.* **93,** 829–835 (2006).

64. Bollati-Fogolín, M. *et al.* Temperature Reduction in Cultures of hGM-CSF-expressing CHO Cells: Effect on Productivity and Product Quality. *Biotechnol. Prog.* **21,** 17–21 (2008).

65. Kishishita, S. *et al.* Optimization of chemically defined feed media for monoclonal antibody production in Chinese hamster ovary cells. *J. Biosci. Bioeng.* **120,** 78–84 (2015).

66. Schucht, R. *et al.* A New Generation of Retroviral Producer Cells: Predictable and Stable Virus Production by Flp-Mediated Site-Specific Integration of Retroviral Vectors. *Mol. Ther.* **14,** 285–292 (2006).

67. Jouvenet, N., Bieniasz, P. D. & Simon, S. M. Imaging the biogenesis of individual HIV-1 virions in live cells. *Nature* **454,** 236–240 (2008).

68. Qiao, J., Oumard, A., Wegloehner, W. & Bode, J. Novel Tag-and-Exchange (RMCE) Strategies Generate Master Cell Clones with Predictable and Stable Transgene Expression

Properties. *J. Mol. Biol.* **390,** 579–594 (2009).

69. Hua, R.-H. *et al.* Generation and characterization of a new mammalian cell line continuously expressing virus-like particles of Japanese encephalitis virus for a subunit vaccine candidate. *BMC Biotechnol.* **14,** 62 (2014).

70. Vicente, T., Roldão, A., Peixoto, C., Carrondo, M. J. T. & Alves, P. M. Large-scale production and purification of VLP-based vaccines. *J. Invertebr. Pathol.* **107,** S42–S48 (2011).

71. Peixoto, C., Sousa, M. F. Q., Silva, A. C., Carrondo, M. J. T. & Alves, P. M. Downstream processing of triple layered rotavirus like particles. *J. Biotechnol.* **127,** 452–461 (2007).

72. Gutiérrez-Granados, S., Cervera, L., Gòdia, F. & Segura, M. Characterization and quantitation of fluorescent Gag virus-like particles. *BMC Proc.* **7,** P62 (2013).

73. Rucker, J. in *Membrane Protein Protocols* **228,** 317–328 (Humana Press, 2003).

74. Yoon, S. K. *et al.* Adaptation of Chinese hamster ovary cells to low culture temperature: Cell growth and recombinant protein production. *J. Biotechnol.* **122,** 463–472 (2006).

75. Swiech, K., Rossi, N., Astray, R. M. & Suazo, C. A. T. Enhanced production of recombinant rabies virus glycoprotein (rRVGP) by Drosophila melanogaster S2 cells through control of culture conditions. *Cytotechnology* **57,** 67–72 (2008).

76. Mimura, Y. *et al.* Butyrate increases production of human chimeric IgG in CHO-K1 cells whilst maintaining function and glycoform profile. *J. Immunol. Methods* **247,** 205–216 (2001).

77. Park, J. H., Chang, K. H., Lee, J. M., Lee, Y. H. & Chung, I. S. Optimal production and in vitro activity of recombinant endostatin from stably transformed Drosophila melanogaster S2 cells. *In Vitro Cell. Dev. Biol. Anim.* **37,** 5–9 (2001).

78. Hwa Chang, K. *et al.* Dimethylsulfoxide and sodium butyrate enhance the production of recombinant cyclooxygenase 2 in stably transformed Drosophila melanogaster S2 cells. *Biotechnol. Lett.* **24,** 1353–1359 (2002).

79. Lamotte, D. *et al.* Na-butyrate increases the production and alpha2,6-sialylation of recombinant interferon-gamma expressed by alpha2,6- sialyltransferase engineered CHO cells. *Cytotechnology* **29,** 55–64 (1999).