NOVA SCHOOL OF SCIENCE & TECHNOLOGY

CHEMISTRY DEPARTMENT

MIGUEL ÂNGELO MATOS GRAÇA

BSc in Biochemistry

RATIONAL GENETIC ENGINEERING OF INSECT CELLS TO IMPROVE RAAVS AND INFLUENZA VLPS PRODUCTION YIELD UTILIZING THE BACULOVIRUS EXPRESSION VECTOR SYSTEM

MASTER DEGREE IN BIOTECHNOLOGY

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“The true sign of intelligence is not knowledge, but imagination.”
(Albert Einstein)

ABSTRACT

The insect cells-baculovirus expression vector system (IC-BEVS) is one of the uprising expression systems in the biopharmaceutical industry to produce vaccines and gene therapy vectors, allowing for high expression levels in short time frames. Despite its benefits, IC-BEVS is a transient system due to the baculovirus lytic nature, which causes the productivity and/or quality of products generated to be impacted. Improvements to the expression platform, and in particular to the cell line, is thus essential in order to bring IC-BEVS products faster to market. However, this has been limited by the lack of well-established tools for genetic engineering in insect cells.

This thesis aimed at overcoming these bottlenecks by establishing a gene editing pipeline based on the CRISPR-Cas9 system for simple and fast gene(s) knockout in Sf9 cells towards improved phenotypes. To establish the pipeline, different transfection methods were explored as to their potential to deliver the ribonucleoprotein complex comprising the Cas9 enzyme and a guide RNA that targeted the fused lobes (*fdl*) gene expressing the β-*N*-acetylglucosaminidase enzyme. Lipofection was identified as the best method with up to 85 % of gene editing efficiency achieved in a single transfection step. To validate the system, *Sf-caspase-1*, a gene associated to apoptosis (pathway demonstrated by in-house data to be up-regulated upon infection and linked to increased product titers), was used as a model knockout target. Editing efficiency of *Sf-caspase-1* was optimized, obtaining a highly edited population. Single-cell cloning by limited dilution was implemented and *Sf-caspase-1* knockout clones identified. Isolated clones were evaluated for cell growth, showing similar kinetics to non-edited Sf9 cells. In addition, clones showed improved resistance to apoptosis and generated slightly improved titers of recombinant adeno-associated viruses and influenza virus-like particles when compared to non-edited Sf9 cells.

Overall, an efficient CRISPR-Cas9 gene editing pipeline in insect cells was successfully established, which can assist rational engineering of insect cell lines towards improved production titers.

Keywords: IC-BEVS, Cell Line Development, CRISPR/Cas9, Ribonucleoprotein complex

RESUMO

O sistema vetorial de expressão de baculovírus em células de insecto (IC-BEVS) é um sistema de expressão em ascensão na indústria biofarmacêutica para produzir vacinas e vetores de terapia genética, permitindo altos níveis de expressão em prazos curtos. Apesar destes benefícios, o IC-BEVS é um sistema transiente devido à natureza lítica dos baculovírus, o que faz com que a produtividade e/ou qualidade dos produtos seja impactada. Melhorias na plataforma de expressão, e em particular à linha celular, são portanto essenciais de forma a levar produtos de IC-BEVS rapidamente para o mercado. No entanto, isto tem sido limitado por falta de ferramentas bem estabelecidas para engenharia genética de células de insetos.

Esta tese visa superar estes limites através do estabelecimento de uma *pipeline* de engenharia genética baseada no sistema *CRISPR-Cas9*, para *knockout* simples e rápido de genes em células de Sf9 para melhorar fenótipos. Para estabelecer o método, diferentes métodos de transfeção foram explorados quanto ao seu potencial para entregar um complexo ribonucleoproteico composto pela a enzima *Cas9* e o guia *RNA* com alvo no gene *fused lobes* (*fdl*) que expressa a enzyma β-*N*-acetilglucosaminidase. Lipofeção foi identificado como o melhor método com até 85 % de eficiência de edição genética alcançada em uma ronda de transfecção. Para validar o sistema, *Sf-caspase-1*, um gene associado à apoptose (uma via mostrada em dados obtidos *in-house* a ser sobreexpressa durante infeção e associada a títulos de produtos melhorados), foi usado como modelo de alvo de *knockout*. A edição de *Sf-caspase-1* foi otimizada, e uma população altamente editada foi obtida. *Single-cell cloning* por diluição limitada foi implementada e clones com *knockout* de *Sf-caspase-1* foram identificados. Clones isolados foram avaliados quanto ao crescimento celular, mostrando comportamento semelhante a células de Sf9 não-editadas. Adicionalmente, os clones mostraram uma melhor resistência à apoptose e geraram títulos ligeiramente melhorados de vírus adeno-associados recombinantes (rAAVs) e partículas *virus-like* (VLPs) quando comparados com células de Sf9 não-editadas.

Em geral, um protocolo eficiente baseado no sistema CRISPR-Cas9 foi estabelecido com sucesso em células de inseto, o que pode assistir engenharia racional de linhas celulares de insecto tendo como alvo títulos de produção melhorados.

Palavras-chave: IC-BEVS, Desenvolvimento de Linhas Celular, CRISPR/Cas9, Complexo Ribonucleoproteico

ÍNDEX

ACKNOWLEDGEMENTS ix

ABSTRACT xiii

RESUMO xv

ÍNDEX xvii

LIST OF FIGURES xxi

LIST OF TABLES xxiii

ABBREVIATIONS xxv

1. INTRODUCTION 1

1.1. Biopharmaceuticals 1

1.2. Insect Cells 2

1.2.1. The Baculovirus Expression Vector System (BEVS) 4

1.2.2. Stable Insect Cells 5

1.3. Cell Line Development 5

1.3.1. Cell Engineering Methods 5

1.3.1.1. RNA Interference 6

1.3.1.2. Ribozymes 7

1.3.1.3. Antisense Oligonucleotides (ASOs) 7

1.3.1.4. Zinc Finger Nucleases (ZFNs) 8

1.3.1.5. Transcription Activator-like Effector Nucleases (TALENs) 9

1.3.1.6. CRISPR-Cas9 9

1.3.2. Engineered Pathways 10

1.3.2.1. Apoptosis 10

1.3.2.2. Metabolism 10

1.3.2.3. Post-translational Modifications 11

1.3.2.4. Cell Cycle Regulation 11

1.4. CRISPR-Cas9 12

1.4.1. Delivery Methods of RNP Complexes 13

1.5. Aim of Thesis 15

2. MATERIALS AND METHODS ... 17

2.1. Insect Cell Culture ... 17

2.1.1. Routine Cell Culturing ... 17

2.1.2. Freezing and Thawing of Cells ... 17

2.2. Establishing a CRISPR-Cas9 Knockout Strategy ... 17

2.2.1. Design of Single Guide RNAs ... 17

2.2.2. Ribonucleoprotein Complexes Formation and Delivery ... 17

2.2.2.1. Lipofection ... 18

2.2.2.2. Electroporation ... 18

2.3.1. Evaluation of Gene Editing Efficiency ... 18

2.3.1.1. DNA Extraction ... 18

2.3.1.2. Target Gene Amplification ... 19

2.3.1.3. Fragmentation by T7 Endonuclease Digestion ... 19

2.4. Single-cell Cloning ... 20

2.4.2. Amplification of Clones ... 20

2.5. Clone Characterization ... 20

2.5.1. Growth Assessment ... 20

2.5.2. Colony PCR ... 20

2.5.3. Toxicity Resistance ... 21

2.6. Production of Biopharmaceuticals ... 21

2.6.1. Baculovirus Stocks ... 21

2.6.1.1. Production of rAAV and VLP ... 21

2.7. Process Analytics ... 22

2.7.1. Cell Concentration and Viability ... 22

2.7.2. Baculovirus Titration by MTT Assay ... 22

2.7.3. Full rAAV Capsid Determination by qPCR ... 23

2.7.4. VLP Titer Estimation by Hemagglutination Assay ... 23

3. RESULTS AND DISCUSSION ... 25

3.1. CRISPR-Cas9 Implementation ... 25

3.1.1. Ribonucleoprotein Complexes Formation and Delivery 25
3.1.2. Establishing Single-cell Clones 29
3.2. Establishing *Sf-caspase-1* Knockout Clones 32
3.2.1. Knockout Efficiency 33
3.2.2. Single-cell Cloning by Limiting Dilution 35
3.2.2.1. Initial Screening 35
3.3. Clone Characterization 36
3.3.1. Growth Profile 36
3.3.2. Evaluating Stress Response in *Sf-caspase-1* Knockout Clones 37
3.4. Biopharmaceutical Production 38
3.4.1. AAV Production 39
3.4.1.1. Infection Kinetics 39
3.4.1.2. Intracellular AAV Titers 40
3.4.2. Influenza HA-VLP Production 41
3.4.2.1. Infection Kinetics 41
3.4.2.2. Extracellular VLP Titers 42
4. Conclusions and Future Perspectives 43
5. Bibliography 45
6. Annex I 53

LIST OF FIGURES

Figure 1.1 - Baculovirus replication cycle in insect cells 4

Figure 1.2 - Mechanism of processing dsRNA into siRNA.. 6

Figure 1.3 - Schematic representation of some ribozyme functions 7

Figure 1.4 - Different methods for gene expression regulation with ASO 8

Figure 1.5 - Recognition of target sequence by Zinc Fingers 9

Figure 1.6 - Recognition of target sequence by TAL effectors...................................... 9

Figure 1.7 - Forms of cell death experienced by cells in culture 10

Figure 1.8 - Mode of action of CRISPR-Cas9 12

Figure 3.1 - Repair mechanisms to Cas9 editing... 26

Figure 3.2 - Successful amplification of fdl gene from Sf9... 27

Figure 3.3 – General pipeline towards evaluation of gene editing efficiency................ 27

Figure 3.4 – Fdl gene with identification of target for gRNA, primers for amplification and expected fragments from T7 digestion 28

Figure 3.5 - Gel analysis of fragments resulting from T7 digestion 28

Figure 3.6 - Limited dilution for single cell cloning 29

Figure 3.7 - Number of colonies observed per condition (first test)............................. 31

Figure 3.8 - Number of colonies observed per condition (second test)........................ 32

Figure 3.9 - Sf-caspase-1 gene with identification of target for gRNA, primers for amplification and expected fragments from T7 digestion.. 33

Figure 3.10 - Initial tests for Sf-caspase-1 targeting efficiency of gRNAs 33

Figure 3.11 - Successful amplification of Sf-caspase-1 gene from Sf9........................ 34

Figure 3.12 - Gel analysis of fragments resulting from T7 digestion 34

Figure 3.13 – Single-cell growth of selected clones for further study........................... 35

Figure 3.14 - Initial screening of clones derived from single-cell cloning 36

Figure 3.15 - Viable cell concentration and viability profiles for growth assessment of Sf-caspase-1 37

Figure 3.16 - Normalized absorbance of viability assessment after exposure to zeocin 38

Figure 3.17 - Viable cell concentration and viability kinetics throughout infection for rAAV production for all populations 39

Figure 3.18 - Titer for intracellular genomic particle production throughout infection assessed by qPCR 40

Figure 3.19 - Viable cell concentration and viability kinetics throughout infection for VLP production for all populations 41

Figure 3.20 - Titer for virus-like particle production throughout infection assessed by hemagglutination assay 42

LIST OF TABLES

Table 1.1 - Examples of FDA approved biopharmaceuticals 1

Table 1.2 - List of BEVS derived products... 3

Table 2.1 - List of gRNAs used for transfection 17

Table 2.2 - PCR reaction for target gene amplification 19

Table 2.3 - PCR conditions for gene amplification.. 19

Table 2.4 - Conditions for heteroduplex formation.. 20

Table 2.5 - Example of plate for titration usage, with virus dilution 22

Table 2.6 - qPCR Program for gene expression quantification 23

Table 3.1 – Gene editing efficiency for fdl knockout 29

Table 3.2 - Previously reported methods for generation of monoclonal insect cell line 30

Table 3.3 -Gene editing efficiency for Sf-caspase-1 knockout..................................... 34

Table 6.1 - First set of conditions tested for improved colony growth in single-cell cloning 53

Table 6.2 - Second set of conditions tested for improved colony growth in single-cell cloning 53

ABBREVIATIONS

ASO	Antisense oligonucleotides
AAV	Adeno-associated virus
BEVS	Baculovirus expression vector system
CHO	Chinese hamster ovary
CASP	Caspase
Cas9	CRISPR-associated protein 9
CCI	Cell concentration at point of infection
CMV	Cytomegalovirus
CRISPR	Clustered regularly interspaced short palindromic repeats
CRISPRi	CRISPR interference
crRNA	CRISPR ribonucleic acid
DBS	Double stranded break
DNA	Deoxyribonucleic acid
DPBS	Dulbecco’s phosphate-buffered saline
dsRNA	Double stranded RNA
FACS	Fluorescence-activated cell sorting
GFP	Green fluorescent protein
GOI	Gene of interest
gRNA	Guide ribonucleic acid
HA	Hemagglutinin
HDR	Homology directed repair
HEK	Human embryonic kidney
HeLa	Human cervical cancer
HIV	Human immunodeficiency virus
hpi	Hours post infection
IC	Insect cells
miRNA	Micro ribonucleic acid
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
NHEJ	Non-homologous end joining
PCR	Polymerase chain reaction
PTM	Post translational modification
PYC2	Pyruvate carboxylase 2

qPCR	Quantitative PCR
rAAV	Recombinant adeno-associated virus
rBac	Recombinant baculovirus
RISK	Ribonucleic acid-induced silencing complex
RMCE	Recombinase-mediated cassette exchange
RNA	Ribonucleic acid
RNAi	RNA interference
RNAse	Ribonuclease
RNAseH1	Ribonuclease H1
RNP	Ribonucleoprotein
RT-qPCR	Reverse transcriptase – qPCR
SEAP	Secreted alkaline phosphatase
SFM	Serum-free media
sgRNA	Single guide RNA
shRNA	Short-hairpin RNA
siRNA	Short interfering RNA
Sf9	*Spodoptera frugiperda* 9
TALENs	Transcription activator-like effector nucleases
tracrRNA	Trans activating crRNA
VLP	Virus-like particle
Wt	Wild type
ZFN	Zinc finger nucleases

1. INTRODUCTION

1.1. Biopharmaceuticals

Since their discovery in the 1980s, biopharmaceuticals and the precision with which they treat diseases have completely changed the face of medical practise. They are defined as prophylactic and therapeutic substances produced from biological sources, such as organs, tissue, microorganisms, or animal cells [1] and reached a global market value of 265.4 billion US dollars according to the Precedence Research report of 2020 . This makes the biopharmaceutical industry one of the fastest growing, due the global population growth and the alarming increase of chronic diseases such as cancer and diabetes [1].

In comparison to pharmaceutics, a chemical process product, biopharmaceuticals present a higher degree of complexity, with several advantages and disadvantages in comparison to their counterparts [2]. As benefits, these compounds usually present more complex mechanisms of action, showing high specificity and activity [1]. Drawbacks include immunogenic reactions as well as complex and tedious characterization needs, as small differences in product quality and/or composition can drastically affect its applications in humans [1,2]. Overall, synthetic drugs present production costs which are approximately 22-times lower than those of biopharmaceuticals, they're mostly process independent, have a lower risk of contamination, and have a higher stability. To highlight the wide variability of biopharmaceuticals, currently approved products are shown in Table 1.1.

Table 1.1 - Examples of FDA approved biopharmaceuticals (adapted from O'Flaherty et al, 2020) [1]

Class	Biopharmaceutical Product
Cytokines	Peginterferon beta-1a
Hormones	Insulin glargine and lixisenatide, Parathyroid hormone
Growth Factors	Sargramostim, Antihemophilic Factor Fc Fusion Protein
Antibodies	Lanadelumab, Ravuzilumab
Enzymes	Elapegademase, Calaspargase pegol
Vaccines	Trivalent influenza vaccine, Smallpox
Gene Therapy	Inotersen

Production of biopharmaceuticals relies mostly on the use of live cells, although cell-free expression systems are also being studied and applied to some specific products, namely mRNA [3]. The vast majority of biopharmaceutical production uses either prokaryotic systems, mainly *Escherichia coli* (*E.coli*), or eukaryotic systems such as yeast, mammalian cells (human and non-human origin), and insect cell lines The choice on the expression system thereby heavily relies on the type of product, for each of the systems present advantages and disadvantages [2].

Mammalian expression systems are advantageous for producing highly complex proteins but present several limitations regarding the production process (mainly costs), have a slower

growth rate, present some limitations in adaptation to suspension culture and have associated contaminations [4]. Amongst them, chinese hamster ovary (CHO) and human embryonic kidney (HEK293) cells are the most commonly used cell lines for protein expression, although several others have already been mentioned, such as HeLa (derived from human cervival cancer), Vero (derived from african monkey kidney) and mouse myeloma, NS0 cells [5]. They produce the vast majority of the recombinant proteins currently on the market, with CHO cell line being used the most, achieving around 70 % of the market, as it is the primary choice for monoclonal antibody production. Nevertheless, the growing field of recombinant proteins products and viral vectors could highlight a possible increase in the use of other expression systems than CHO cell line [1,2].

Bacterial expression systems are highly regarded due to their rapid growth, high product yield, cost-effectiveness, ease of scale up and short turnaround time, but suffer from limitations regarding the complexity of the products they can express, lacking complex post translational modification (PTM) machinery. The most prevalent bacteria is *E.coli*, which can express stably folded globular proteins from both prokaryotes and eukaryotes, but has difficulty expressing membrane and high-molecular-weight proteins.[6]. Proteins produced by this system also tend to aggregate due to formation of inclusion bodies. Some products in the market generated in *E.coli* are long lasting insulin-analogue Lantus®, therapeutic enzymes such as glucarpidase VORAXAZE®, and peptide drugs like the parathyroid hormone Preotact® [1].

Yeast expression systems present a more complex PTM machinery compared to prokaryotic systems and can grow to high densities in protein-free media but can present several drawbacks in post-translational modifications and protein yields are lower when the expressed protein is a hetero-oligomer, membrane attached or prone to proteolytic degradation [2]. The most common species used are *Saccharomyces cerevisiae* and *Pichia pastoris*. Despite advantages, due to hyper-mannosylation, expressed proteins can have altered protein binding activity and altered immunogenic response, as well as a faster clearance following therapeutic treatments. Some of the products in the market generated with this system are insulin and human serum albumin, amongst others [1,2].

In the latest decade, insect cells have emerged as a powerful alternative system, showing fast and comparable expression of products of interest [7].

1.2. Insect Cells

Insect cells, initially established as a model for pathology and physiology research, gained popularity for biopharmaceutical production since the discovery of the baculovirus expression vector system (BEVS), discussed later. Compared to other expression systems, insect cell lines such as S2, derived from *Drosophila melanogaster*, Sf9 from *Spodoptera frugiperda*, and High Five^{TM} from *Trichoplusia ni* [8] show several advantageous characteristics, making them a suitable alternative expression system. Compared with bacteria or yeast, they can produce post translational modifications more similar to humans, allowing for the production of products with more complex structures [7,8]. Other advantages compared to mammalian systems include i) their

capability to grow in suspension to high cell densities, ii) no CO2 requirements and iii) low biosafety demands [7].

Despite these advantages, insect cells don't have the same post translational machinery as human cells, and therefore complex products can have structural differences compared with their mammalian counterpart, which can affect the efficacy and safety of products, hindering their application as therapeutics [8].

The most commonly used insect cell lines in the biopharmaceutical industry are Sf9 and High-Five. While Sf9 are better suited for amplification of baculoviruses, High-Five express recombinant protein at higher levels (up to 20-fold more) [7]. High-Five cells are larger, present a higher resistance to shear stress and osmotic shock, have a faster growth rate, and generally higher consumption of nutrients during both growth and infection. As drawbacks, these usually produce higher levels of ammonia and lactate which can accumulate to toxic levels and have a detrimental effect on the production process. Sf9 cells are more resistant to thermal shock and produce little to no lactate and ammonia during both growth and infection [7]. As mentioned, current production in insect cells are used in combination with BEVS, however some efforts in developing virus-free production systems have been shown [8]. Table 1.2 highlights some BEVS derived products already on the market, although a myriad of others are in different stages of preclinical or clinical trials [9].

Table 1.2 - List of BEVS derived products (adapted from Felberbaum et al, 2015 and Kumar et al, 2018) [9,10]

Product	Manufacturer	Product Type
Human Vacines		
Human papillomavirus – Cervarix®	GlaxoSmithKline	VLP
Influenza – Flublok®	Sanofi (previously Protein Sciences Corporation)	Subunit Antigen
Human Therapeutics		
Prostate cancer – Provenge®	Dendreon	Immunotherapy
Lipoprotein lipase deficiency – Glybera®	uniQure	rAAV-based gene therapy
Veterinary Vacines		
Swine fever – Porcilis® Pesti	MSD Animal Health	Subunit Antigen
BAYOVAC CSF E2®	Bayer Biologicals/Pfizer Animal Health	
Porcine circovirus type 2 - Circumvent® PCV	MSD Animal Health	
Ingelvac CircoFLEX®	Ingelvac	
Porcilis® PCV	MSD Animal Health	

1.2.1. The Baculovirus Expression Vector System (BEVS)

First developed in 1983, the baculovirus expression vector system (BEVS) has revolutionized the potential of insect cells in the production of biopharmaceuticals. This system works through the usage of a recombinant baculovirus (rBAC), a DNA virus that solely infects insects, which was modified to express a gene of interest (GOI) upon infection. One of the main characteristics of the system are the strong naturally occurring viral promoters, namely *polh* and *p10*[8,11]. These promoters are activated at later infection stages (peaking after 48 hours post infection in high MOI processes) and account for 30-50 % of the total protein expression at during infection [12]. Despite the emphasis on the use of *polh* and *p10* some early promoters have been tested (e.g. *bbp*), allowing for protein expression as early as 6 hours post infection with usually better post translational modifications, however lower titers [7].

The system presents several advantages, namely high titers of heterologous proteins in a short time frame, easy manipulation and adaptation as well as the ease of recombinant baculovirus amplification and the short period for the development and production [7].

The main disadvantage of BEVS is the fact that it is a lytic system due to baculovirus replication cycle, as shown in figure 1.1, overburdening the cell until lysis, potentially reducing the quality and production of recombinant proteins at later infection stages. Another disadvantage is the complexity of purification processes, as the baculovirus needs to be removed, as well as the increased amount of cell specific contaminations, such as DNA, cellular debris, proteases (like caspases) and proteins [7,8].

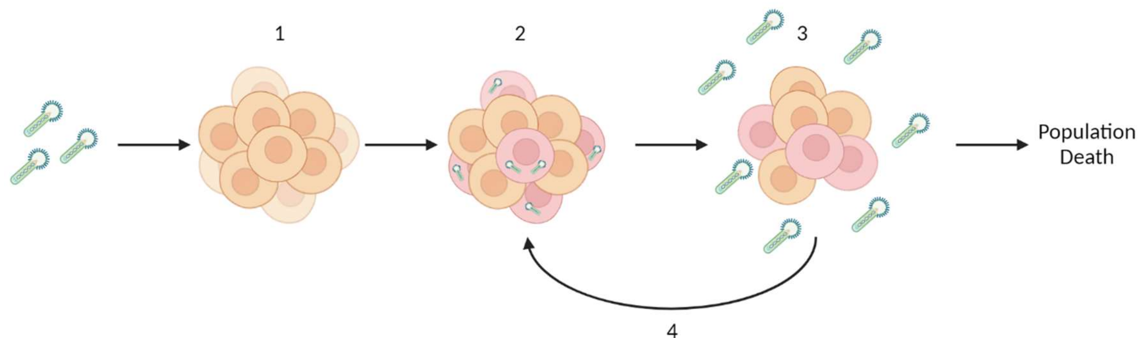


Figure 1.1 - Baculovirus replication cycle in insect cells; 1 – cells in culture are exposed to baculovirus infection, 2 – infected cells (red) start replicating virus and swell, 3 – infected cells lyse and release contents into supernatant, 4 – cycle repeats with new infections due to baculovirus release until complete population death (done using Biorender)

During baculovirus infection, it has been shown that several modifications to the cell occur that drive the intense replication of viruses, or in a biopharmaceutical context, of the GOI. These consist on the rearrangement of the cytoskeleton, cell cycle arrest and cytomegaly (the increase of the cell size), complete shutdown of the hosts protein production and takeover of the metabolism driving it towards catabolic pathways like the glycolysis and the tricarboxylic acid

cycle, with the inhibition of the central metabolism, so the host can support the infection with increased levels of energy production, and the induction of apoptosis [13].

All the above present themselves as avenues for improvement of the expression system (for application in BEVS production processes), but due to the lack of a completely annotated genome and transcriptome, and a low amount of literature on insect cells, the expression system hasn't been the target of many genetic engineering studies, which has emerged as one strategy to the enhancement of productivity in numerous expression systems.

1.2.2. Stable Insect Cells

Aside from the IC-BEVS, efforts into developing virus-free production systems in insect cells have been made. This was achieved by targeted gene integration using recombinase-mediated cassette exchange (RMCE) and selection markers. With this technology cell lines expressing products such as β-galactosidase, or more recently vaccine candidates for influenza based on virus-like particles (VLPs) exhibiting HIV-1 Gag proteins of the surface have been already established [14,15].

The main advantage of stable producing cell lines is the absence of the virus and associated contaminants, as well as higher compatibility to continuous production processes [14,16]. Additionally, virus induced metabolic and secretory pathway limitations, which potentially affect product quality and titer are absent in this system [14,15,17], concluding the possibility of higher product quality compared to BEVS. Nevertheless, significant disadvantages still occur, such as the time needed to establish cell lines, lower adaptability, as well as significantly lower product titers [18].

1.3. Cell Line Development

At the centre of any biopharmaceutical production process there is the cell line expressing the biopharmaceutical. With the advancement of existing tools associated with vector design, cell line screening, genomic and transcriptomic resources and genetic engineering, it has become possible to engineer cell lines with favourable characteristics improving not only productivity but also resistance to different factors throughout production [19,20].

Benefits associated with cell line development are broad, ranging from resistance to different conditions in culture, namely inducement of apoptosis by recombinant protein production, stress associated to conditions, namely physical or biochemical, to improvement of product quality or even engineering of targets associated with hyper productivity traits allowing for direct increase in productivity or growth kinetics [4,19].

1.3.1. Cell Engineering Methods

Early technologies for the study of gene expression were mostly silencing methods. Gene silencing has evolved in a broad range of organisms as a defence mechanism against invasive

nucleic acids by modulating gene expression through transcription and post-transcriptional silencing. The first acts by preventing RNA synthesis, while the latter acts to degrade existing mRNA. Gene silencing technologies are methods that downregulate to a higher or lower degree (depending on efficiency) the expression of specific targets through the use of molecules such as RNA interference (RNAi), ribozymes, and antisense oligonucleotides [4,21].

With the advancement of technology, genome editing has become possible, allowing for both knockout and knock in of genetic information into the target cell genome through techniques like CRISPR-Cas9, TALENs and ZFNs, but also increasing mechanisms through which the previously mentioned methods can be applied [4].

1.3.1.1. RNA Interference

RNA interference or RNAi was first discovered in 1998, when double stranded RNA (dsRNA) was introduced into *Caenorhabditis elegans* and the observed effect was a highly efficient gene silencing, paving the way to the study of small RNA molecules for such purposes. It was discovered that these small RNA molecules could regulate entire pathways by modulating gene expression after transcription, by degrading homologous mRNA. Several pathways for the processing of different precursor RNA molecules into various RNAi forms such as short-interfering RNA (siRNA) and microRNA (miRNA) were discovered in cells [22].

With these pathways, namely through the activity of the cytoplasmic ribonuclease III known as DICER and the multiprotein complex known as RNA-induced silencing complex or RISK, the RNA can be delivered in several formats, namely siRNA, dsRNA, siRNA-based hairpin RNA (or short-hairpin RNA, shRNA) and pre-micro-RNA structures that can be cleaved by DICER into miRNA (with similar effects to siRNA) in order to target a specific mRNA as presented in figure 1.2 [22].

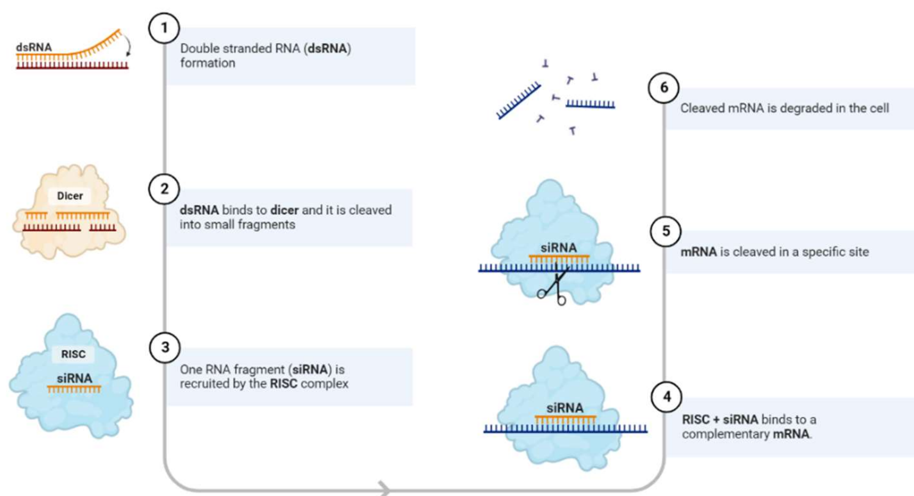


Figure 1.2 - Mechanism of processing dsRNA into siRNA and recognition and degradation of target mRNA (done using Biorender)

More specifically, as an example, manipulation of miRNA levels in CHO cells have shown to improve proliferation and specific productivity, apoptosis resistance and oxidative metabolism,

through both overexpression, stable expression and even inhibition by shRNA of cellular miRNAs [4,23].

1.3.1.2. Ribozymes

Ribozymes are RNA catalysts capable of cleavage or linkage of phosphodiester bonds or both, splicing of introns, and covalent bonding of boundary exons with or without proteins factor(s) amongst many others [24–26]. They are associated with riboswitches, RNA molecules which are capable for instance of sensing changes in several cell physiology parameters, namely temperature, cation sensitivity, and regulatory RNA molecules. By sensing these changes they transduce them into the gene expression machinery, with regulatory proteins only showing later [26]. Moreover they are capable of down- or up-regulating genes through the fine tuning alternative splicing mechanism that they exhibit. Through interaction with mRNA, these can either sequester or expose the ribosomal binding site, effectively shifting the expression of the protein according to the changes in the cell environment [26].

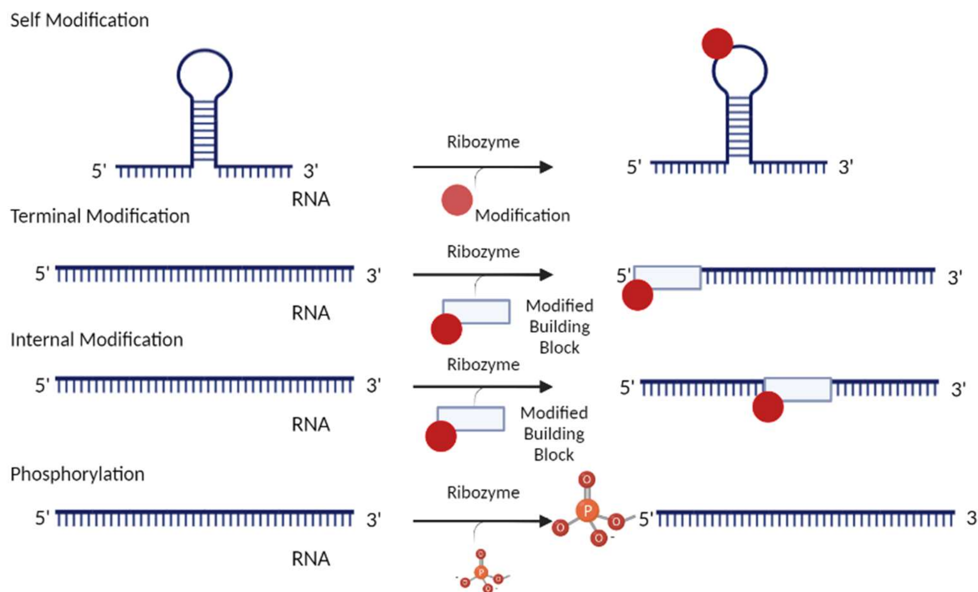


Figure 1.3 - Schematic representation of some ribozyme functions (adapted from Müller et al., 2018) [25] (done using Biorender)

A study found that blocking caspase-3 activity by targeting the mRNA with a ribozyme allowed for the improvement of production of Interferon-β and increased survivability in culture, effectively presenting an anti-apoptotic phenotype [27].

1.3.1.3. Antisense Oligonucleotides (ASOs)

ASOs were identified for the first time in 1978 as a technique for suppressing gene expression by association with mRNA via complementary base pairing to the target, followed by transcript knockdown mediated by endonucleases. The potential for using ASOs in gene expression research has expanded as a result of various breakthroughs. These have not only lowered their turnover inside cells, hence raising intracellular maximum concentrations, but also

varied their mode of action. For instance, by modifying splicing factors through steric hindrance or preventing the recruitment of mRNA translation machinery [28].

These molecules have allowed for not only silencing of genes, but also upregulation of gene expression, through the aforementioned splicing whereby preventing naturally occurring non-productive alternative splicing and generation of non-productive mRNA, full-length protein was produced in higher levels [28,29]. Some of the methods for gene expression regulation using ASOs are present in figure 1.4.

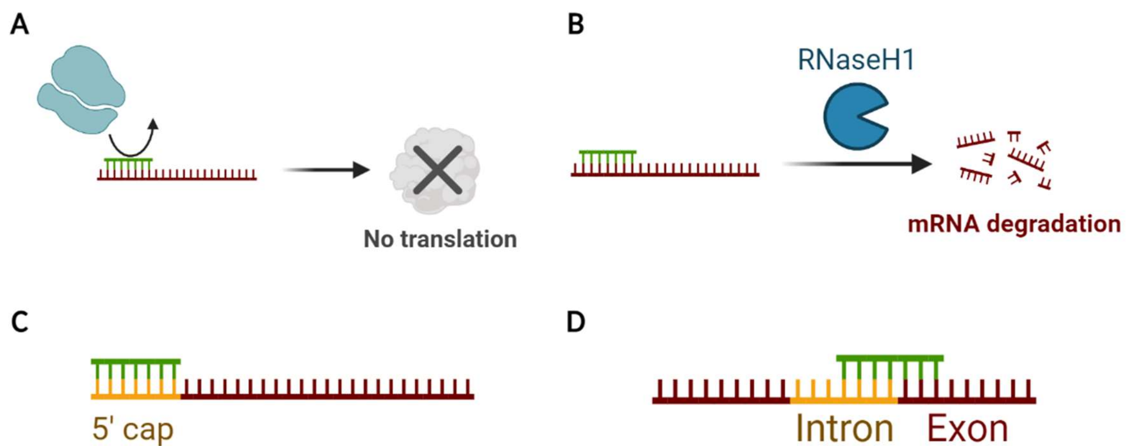


Figure 1.4 - Different methods for gene expression regulation with ASOs: A - blocking the ribosomal binding site, stopping translation, B - marking mRNA for degradation, C – preventing 5' cap formation, allowing for faster degradation of mRNA; D - modulating alternative splicing of introns and exons (done using Biorender)

1.3.1.4. Zinc Finger Nucleases (ZFNs)

Zinc finger nucleases represent the simplest and most common endonucleases. They are artificially designed restriction enzymes derived from eukaryotic transcription factors, with a range of applications, from point mutations to insertions and inversions [4,30].

ZFNs act based on the dimerization of the DNA-binding domain, which can be designed to target any gene theoretically, based on the recognition of a tandem array of Cys_2His_2 zinc fingers of which each can link with a three-nucleotide sequence within the gene sequence. By binding to a series of “fingers”, a pair of ZFNs in inverse orientation dimerize into a *FokI* nuclease which is capable of cleaving the DNA between the binding sites [31].

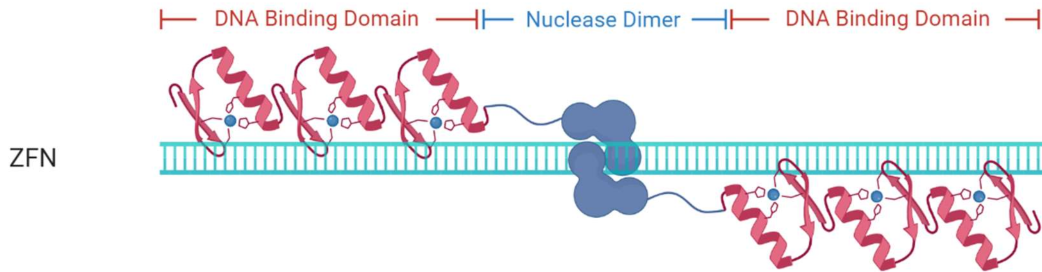


Figure 1.5 - Recognition of target sequence by Zinc Fingers (DNA Binding Domain) and dimerization of FokI nucleases (Nuclease Dimer) (induces cleavage of nucleotides)

ZFNs have been successfully used for both non-homologous end joining (NHEJ) knockout and homology directed repair (HDR) mediated knock-in, but despite the success these present several restrictions. Some of these challenges are linked with imitations in targeting certain genomic sequences, in the surrounding environment of the target that might impair recognition and hence specificity of the sequence, and as a method it is often highly expensive and time-consuming. [4].

1.3.1.5. Transcription Activator-like Effector Nucleases (TALENs)

TALENs are mechanically very similar to ZFNs, with the key difference being the DNA-binding domain, which is derived from transcription activator-like (TAL) proteins isolated from pathogenic bacteria Xanthomonas. Due to this difference, this system has generally higher efficiency in gene editing for both knockout and knock-in events. This is due to the capacity of TAL proteins to recognize single nucleotides, with high specificity and without influence from surrounding domains [4,30].

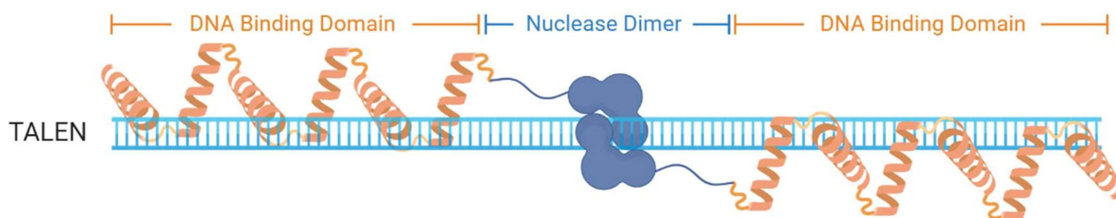


Figure 1.6 - Recognition of target sequence by TAL effectors (DNA Binding Domain) and dimerization of FokI nucleases (Nuclease Dimer) (induces cleavage of nucleotides)

1.3.1.6. CRISPR-Cas9

The clustered regularly interspaced short palindromic repeats system or CRISPR, alongside the endonuclease Cas9 is based on the natural defence mechanism of bacteria. Nowadays it is established as one of the most powerful tools in genetic engineering, mostly due to its simplicity, specificity, and efficiency.

Given its importance in this thesis, a detailed description on the system will follow in Chapter 1.4.

1.3.2. Engineered Pathways

1.3.2.1. Apoptosis

Programmed cell death is one of the major challenges related with cell culture and biopharmaceutical development. This is a significant problem since it reduces productivity (by reducing viable cells) and product quality. To prevent apoptosis, several strategies have been employed in different expression systems. Apoptosis is triggered when a cell is exposed to a mild stress (if the stress is too extreme, cell suffers from necrosis) and allows for the controlled death of the cell, through cellular mechanisms, as shown in figure 1.7 [19]:

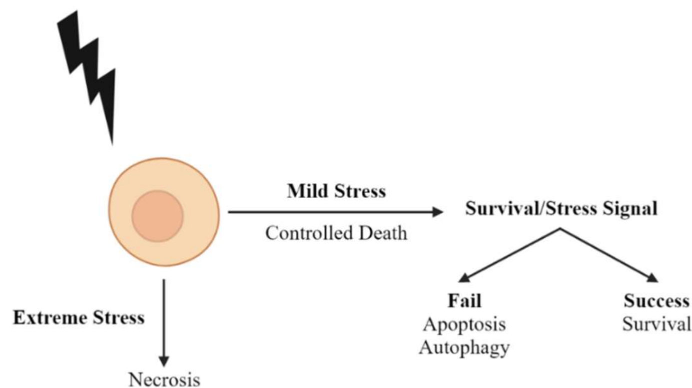


Figure 1.7 - Forms of cell death experienced by cells in culture (done with BioRender)

To address this obstacle, several different approaches have been studied, namely the overexpression of proteins associated with antiapoptotic signalling or knockdown/knockout of genes associated with the effects and signalling of the start of apoptosis. In insect cells, it has been shown that overexpression of a viral ankyrin protein allowed for higher expression and delayed apoptosis triggered by baculovirus expression [32].

Knockdown or knockout of genes associated with the initiation of apoptosis has also been studied. In insect cells, knockdown and knockout of *Sf-caspase-1* has some contradictory results, with some studies showing that knockdown through siRNA or shRNA resulted in improved cell viability and productivity throughout infection [33–35]: However, other manuscripts showed opposite results, using both knockout and knockdown cells. Although these exhibit stronger resistance towards other apoptotic stimuli, during infection the suppression of *Sf-caspase-1* had no effect on cell viability nor productivity. Nevertheless, the possibility that this effect is associated with product-type was theorized in the last case [36].

1.3.2.2. Metabolism

One obstacle that some expression systems, mainly mammalian cell lines but also some insect cell lines, such as High-Five, face during recombinant protein production is the accumulation of by-products of glutamine and glucose metabolism such as lactate and ammonia.

These by-products can reach toxic levels and cause adverse effects in cell physiology and in recombinant product secretion [4,37].

Although insect cells haven't been researched in this area, CHO cells have been the target of some metabolic engineering studies. One approach that has been studied for the amelioration of this problem was the overexpression of an enzyme responsible for the conversion of pyruvate into oxaloacetate, pyruvate carboxylase (PYC2), resulting in higher cell density, extended batch duration and a well-controlled central carbon metabolism [38]. A knockdown study using siRNA investigated the effects of reducing the expression levels of lactate dehydrogenase and pyruvate dehydrogenase, both responsible for accumulation of pyruvate and lactate in the cytosol, and was successful in decreasing lactate levels and increase antibody titers without impacting cell growth and product quality [39].

1.3.2.3. Post-translational Modifications

Post-translational modifications (PTMs) such as N-glycan structures in antibodies, but also several other biopharmaceuticals, have a critical role in their bioactivity, efficacy, and safety. Several expression systems suffer from a lack of cellular machinery or simply different mechanisms for protein PTMs, which then affect their application in human therapy.

To address these differences, several studies attempting to recreate cellular mechanisms that better mimic the human PTM machinery have been reported, with many resorting to cell engineering. In insect cells, the fused lobe gene (*fdl*) has been the target of knockout studies since it represents limits in *N-glycan* elongation. After knockout, an increase in elongated structures was observed for human erythropoietin which are associated with more humanized PTM modifications [40]. By co-expression of heterologous GDP-6-deoxy-d-lyxo-4-hexulose reductase in CHO cells, a study was able to deflect the fucose *de novo* pathway, which creates a core of fucose in antibodies, and produce a therapeutic product with a much stronger effector function in the antibody-dependent cell cytotoxicity, which is the desired effect for this product in specific [4,41].

1.3.2.4. Cell Cycle Regulation

In the eukaryotic cell cycle there are many changes that occur to cells that ensure certain conditions necessary for the integrity and survival of the cells. These changes can often present themselves as being detrimental towards protein synthesis and cell growth. In order to improve biopharmaceutical production, different approaches to regulate cell cycle have been studied [4,42].

In insect cells, "tuning" of cell cycle was done through arrest of the cell cycle at G1 phase by overexpressing and knocking down cyclin E, using a plasmid for overexpression and RNAi for knocking down. With cyclin E overexpression, High-Five cell growth was actively favoured and there was no effect on cell cycle progression. With the silencing of cyclin E using RNAi, the cells tended towards the G1 phase and growth was heavily inhibited. In this study, effects on

baculovirus infection were studied, and intracellular production of GFP was favoured by synchronization [42].

1.4. CRISPR-Cas9

First mentioned in 1987, CRISPR-Cas9 has emerged as a powerful tool for genetic engineering. The main mode of action is through a class of RNA-guided nucleases, with the most common being Cas9, and CRISPR RNA molecules (crRNAs) [43]. The crRNA is responsible for the attachment to the target DNA, through the recognition of a protospacer adjacent motifs (PAMs), a specific DNA sequence unique to different Cas enzymes, existing approximately every 16 nucleotides in a random DNA sequence [43,44].

The Cas9 endonuclease binds to a complex of crRNA and *trans* activating crRNA (tracrRNA) named as guide-RNA (gRNA) and after complementary DNA-RNA base pairing, a double-stranded break (DSB) three nucleotides proximate to the PAM sequence occurs. Next, the cellular DNA repair system recognizes the break and fixes it through either non-homologous end joining (NHEJ) or homology directed repair (HDR). NHEJ is an error-prone mechanism, resulting in either insertion or deletion (indel) mutations, and consequently in a different frameshift and dysfunctional protein product. The resulting mutation caused by the nuclease activity is therefore variable, namely type and size of indel due to variability in Cas9 action but also NHEJ mediated repair [45]. The second possible mechanism of action, namely HDR requires a donor template with homology to the flanking region of the DSB, which allows for the introduction of donor DNA strands, which allow for the knock-in experiments [43].

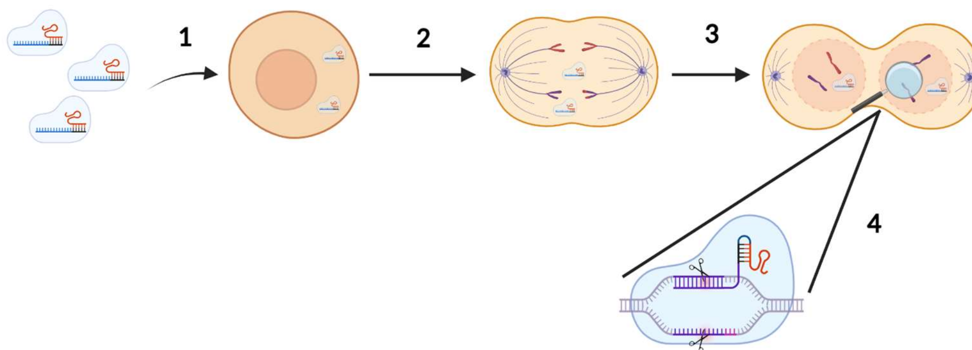


Figure 1.8 - Mode of action of CRISPR-Cas9: 1 - Delivery of gRNA and Cas9 to cells (in a protein format); 2 - Cells go through cell division, lysing the nuclear membrane and exposing the DNA; 3 - CRISPR-Cas9 accesses the DNA; 4 - Homology recognition by the gRNA and double stranded break induction by Cas9

The simplicity of gRNA design and CRISPR-Cas9 application has therefore allowed for efficient and quick targeting of gene sequences, whether the objective is inducing a knockout or a knock-in event. Aside from these, due to engineering surrounding the Cas9 endonuclease, several other applications have arisen to this system [4,43].

Due to the capacity of Cas9 to bind to DNA at specific sites defined by the gRNA, aside from the permanent modification of the target genome, Cas9 with deactivated catalytical activity

has been used as a regulation system, called CRISPR interference (CRISPRi). This strategy has been shown to block transcriptional elongation, RNA polymerase or transcription factor binding depending on the target of the gRNA-Cas9 complex [44].

This has allowed for a reversible system, with no detectable off target effects that allow for the repression of multiple genes simultaneously. Since the CRISPR system is absent in eukaryotic organisms, there is no conflict with endogenous pathways, while RNAi for example can have unpredictable effects when studying for example the RNAi pathway targets, and unlike the CRISPR-Cas9 system, incomplete knockdowns and several off-target effects are still a problem with this system [44].

This system can be delivered into cells not only through different transfection methods, such as electroporation or lipofection, but also in different formats, such as DNA, mRNA, and protein format, each having its advantages and disadvantages [46]. While plasmid DNA offers a cost-effective solution and sustained expression, out of the three options this is the one with the slowest onset for the editing and the one with the highest chance for off-targets due to insertional mutagenesis and longer Cas9 activity. On the other hand, mRNA enables a slightly faster onset of editing because transcription is no longer necessary, but mRNA itself is not only highly unstable but also prone degradation by RNases. This in turn reduces the gene editing efficiency but also the chances of off-target effects [47].

For this thesis, Cas9 was delivered in a protein format, using a ribonucleoprotein (RNP) complex. This method sacrifices any type of sustained expression, and therefore has a really short time frame for gene editing activity, but allows for the immediate editing and reduces the off-target effects to a minimum [47].

1.4.1. Delivery Methods of RNP Complexes

A critical step towards an efficient gene editing experiment using the CRISPR-Cas9 is the delivery of the system to the cell. Methods for intracellular delivery of genetic material and proteins have already been established but taking into consideration the unique properties of RNP complexes, there are specific requirements that need to be considered. Methods already developed include physical approaches, such as microinjection, electroporation, biolistic and microfluidic techniques, and synthetic carriers such as lipid nanoparticles, cell-derived vesicles and nanogels, amongst other [47].

Membrane disruption by electroporation is a commonly used physical method where an electrical pulse produces temporary pores in the cell membrane, allowing for the transport of proteins, nucleic acids and, most importantly, RNPs. This method offers a transient and stable method for transfection, but since a high voltage is necessary to create pores in membrane, this system is usually responsible for high cell death rate [47].

Delivery by synthetic lipid nanoparticles is one of the most used biochemical methods for the delivery of foreign genetic material to cells. Cationic lipids naturally form vesicles due to the

action of the hydrophobic portion, encapsulating RNP complexes inside it. Since the charge for these complexes is negative (in contrast with Cas9, with is cationic), they can be delivered using commercially available cationic lipids used for DNA delivery. Upon endocytosis, which is the main mechanism through which these vesicles enter the cells, the interaction between phospholipids in the membrane and the cationic lipids leads to the release of the RNP into the cytosol, achieving high transfection efficiencies [47].

1.5. Aim of Thesis

Increasing interest has been placed on insect cells and the baculovirus expression vector system (IC-BEVS) as an expression system for biopharmaceutical products. While tremendous effort has been placed to improve IC-BEVS production processes regarding improving viral vectors, development of bioprocess intensification strategies and laboratory evolution [16,48,49]; limited progress has been made on the genetic modification of cell lines such as Sf9 and High-Five for improved and/or prolonged production processes [43]. These results are partly due to limited genomic and transcriptomic reference availability, restricting the identification of engineering targets and the development of engineering tools. Only recently, the first CRISPR-Cas9 study was published, showing the first knockout approach in Sf9 insect cell lines, however in a tedious, labour intensive and inefficient approach.

This thesis therefore aims to establish a faster and more efficient genetic engineering pipeline in Sf9 insect cells to accelerate the application of rational genetic engineering, enhancing the application of insect cell lines towards improved production processes. As a result, two main goals were defined. First, establishing a state-of-the-art CRISPR-Cas9 pipeline for insect cells, based on the delivery of ribonucleoprotein complexes (shown to be the most efficient). For this, several delivery methods were tested, and selected based on the best ratio of efficiency to applicability. Moreover, to improve retrieval of stable knockout cells, currently inefficient single-cell isolation methods were improved. Second, by applying the established pipeline to a target gene of interest, namely *Sf-caspase-1* a gene associated with apoptosis induction we aimed at evaluating knockout clones for improved stress resistant and enhanced production capabilities.

2. MATERIALS AND METHODS

2.1. Insect Cell Culture

2.1.1. Routine Cell Culturing

Sf9 insect cells derived from *Spodoptera frugiperda* (Invitrogen, Cat#: 11496-015) were routinely sub-cultured in serum-free Sf900-II^{TM} SFM medium (Thermo Fischer Scientific) to 0.6 – 1 x 10^6 cell.mL^{-1} when reaching a cell concentration of 2-4 x 10^6 cells.mL^{-1}. Cells were cultured at 27 ºC in an Inova 44R shaking incubator (orbital motion diameter of 2.54 cm – Eppendorf) at 100 RPM using 10 – 12 % working volume (w/v) of 125, 250 and 500 mL shake flasks (Corning).

2.1.2. Freezing and Thawing of Cells

Exponentially growing cells (3-5 x 10^6 cell.mL^{-1}) were frozen by pelleting a defined number of cells by centrifugation (300x g for 5 min at 4°C) and resuspending in cryopreservation media (CryoStor®, Sigma) to a final concentration of 30-50 x 10^6 cell.mL^{-1}. Aliquots of 1 mL were frozen using a freezing container (Mr.Frosty, Thermo Fisher Scientific) guaranteeing a temperature decrease of 1°C/minute. Frozen cells were stored either at -80 ºC (short-term) or liquid nitrogen (long-term).

For thawing, cell aliquots were centrifuged in 10 mL of culture media at 300x g for 5 min. Resulting cell pellets were resuspended in culture media, to achieve the desired cell concentration and culture volume.

2.2. Establishing a CRISPR-Cas9 Knockout Strategy

2.2.1. Design of Single Guide RNAs

Single guide RNAs (sgRNAs) and Cas9 were purchased from IDT (Alt-R® CRISPR-Cas9 sgRNA and Alt-R^{TM} S.p.Cas9 V3 products respectively) using either sequences obtained from literature (fdl gRNA 2 from Mabashi-Asazuma et al., 2017 [40] – 2 bases at the 5'-end were added to comply with the recommended IDT sequencing length) or identified using IDT's sgRNA design software. The sgRNAs used in this thesis can be found in Table 2.1.

Table 2.1 - List of gRNAs used for transfection

RNA	Gene ID	Target gene	Target sequence (5' → 3')
Fdl gRNA1	118265933	*fdl*	GACGATGTGTTCCACTTAGG
Fdl gRNA2	118265933	*fdl*	ACGAAGTGTCGGAACGTTGC
Sf-caspase-1 gRNA1	118267264	*Sf-caspase-1*	GTTGATAGAAATGCCCCTTA
Sf-caspase-1 gRNA2	118267264	*Sf-caspase-1*	TAAGGGGCATTTCTATCAAC

2.2.2. Ribonucleoprotein Complexes Formation and Delivery

Ribonucleoprotein (RNP) complexes were formed by combining sgRNA with Cas9 in conditions specific for the delivery method. Depending on the necessary working volume, RNP

delivery was performed in 6 or 24 well plates (Corning) using different delivery strategies, namely lipofection or electroporation.

2.2.2.1. Lipofection

For lipofection, three different reagents were evaluated, namely RNAiMax™, TransIT™ and Cellfectin™ (all Thermofisher). RNP complexes were formed by a 10-minute incubation period at room temperature using 15.5 pmol Cas9 and 30 pmol sgRNA in 250 µL OptiMEM™ media (Thermofisher). Vesicle formation around formed RNP complexes was achieved by addition of the same volume of OptiMEM™ containing 6 % (v/v) lipofection reagent and an additional 10-minute incubation at room temperature. Finally, 0.6 x 10^6 cell.mL^{-1} were transfected by adding 10 % (v/v) of delivery solution to each well (working volume of 0.5 mL in 24 well-plates, 1.5 mL in 6-well plates).

2.2.2.2. Electroporation

RNP complexes were prepared for electroporation by complexing 19.2 pmol Cas9 and 36 pmol sgRNA in 30 µL Nucleofector™ Solution containing 18.2 % (v/v) Nucleofector™ Supplement (both Lonza) and incubation for 15 minutes at room temperature. For transfection of cells, 0.5 x 10^6 cells were pelleted by centrifugation (300g, 5 minutes at room temperature) and resuspended in 20 µL of RNP containing nucleofection solution before introduction into a Nucleocuvette™ Strip (Lonza). Electroporation was performed using a Lonza 4D Nucleofector, using the CM104 program, recommended for Sf9 insect cells. Immediately after electroporation, 100 µL of fresh Sf900II media was added to cells inside the cuvette, which were then transferred to a 24 well plate containing media for a final volume of 0.5 mL.

2.3.1. Evaluation of Gene Editing Efficiency

After 72-96 hours post-transfection (depending on cell growth), DNA was extracted, amplified for the target gene using polymerase chain reaction (PCR), and analysed for the presence of genome editing using a commercially available mutation detection kit, based on T7 endonuclease fragmentation.

2.3.1.1. DNA Extraction

Extraction of DNA from transfected cultures was performed by using the DNeasy® Blood & Tissue Kit (Qiagen), according to manufacturer's instructions. Briefly, cells were pelleted (300g, 5 minutes) and after removal of supernatant resuspended in 200 µL PBS. Afterwards, 20 µL of Protenaise K (included in kit) and 200 µL Lysis Buffer was added and quickly resuspended, followed by an incubation of 10 minutes at 56 ºC. After incubation, 200 µL of 96 % ethanol was added, solution was vortexed, and contents were transferred into a column with a collection tube. A centrifugation step of 6000g for 1 minute was performed and flow-through discarded. Afterwards, 500 µL wash buffer 1 was added and the previous centrifugation step was repeated. Then, 500 µL of wash buffer 2 was added and a centrifugation step at 20000g for 3 minutes was conducted. For elution, the buffer was pre-warmed to 37 ºC and 200 µL was added to the column.

After incubating for 1 minute at room temperature, a centrifugation step for 1 minute at 6000g was done to elute DNA, into sterile 1.5 mL tube. Samples were then stored at 4 ºC for further analysis.

DNA samples were later quantified using NanoDrop™ (Thermofisher), by measuring 1 µL of DNA and calculating the 280/260 and 280/230 absorbance ratios for determine DNA quality.

2.3.1.2. Target Gene Amplification

Amplification of target genes was performed by PCR reaction, containing 150 ng of DNA and the Platinum™ SuperFi II Green PCR Master Mix (Invitrogen), and primer pairs targeting either the Sf9 *fdl* gene, from Mabashi-Asazuma et al., 2017 [40] (FP: 5'-CGCGGACTTCTCCTTGACACAG-3' , RP: 5'-CGAACCCGCAGTCCAGGTAC-3') or *Sf-caspase-1* (FP: 5'-TGCTGGACGAAAACAAGAC-3', RP: 5'-CTCAGTGCGGCTCAGAGTAA-3'), according to Table 2.2. PCR reaction was performed in a SimpliAmp Thermal Cycler (Thermofisher) according to conditions specified in Table 2.3.

Table 2.2 - PCR reaction for target gene amplification

Component	Volume (µL) per 10 µL reaction
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
Platinum™ SuperFi II Green PCR Master Mix (x2)	5
150 ng DNA	Variable
Nuclease Free Water	Variable

Table 2.3 - PCR conditions for gene amplification

Cycles	Temperature	Time
1	98 ºC	30 sec
	98 ºC	10 sec
35	60 ºC	10 sec
	72 ºC	15 sec
1	72 ºC	5 min

Amplification of the target gene was later evaluated by gel electrophoresis, using 5 µL of PCR product in a 1 % agarose (NZYTech) gel containing 0.005 % RedSafe (iNtRON Biotechnology). NZYDNA Ladder III (NZYTech) was used as reference. Gels were run at 100 V for 1 hour. PCR amplicon bands were revealed by UV, using a Gel Doc™ EZ (BIO-RAD). Images were processed and analysed using ImageJ software.

2.3.1.3. Fragmentation by T7 Endonuclease Digestion

To evaluate mutation efficiency, PCR products (single complementary strands) need to be in a heteroduplex form and are therefore denatured and annealed for genetic recombination. Briefly, heteroduplex formation was performed in 10 µL reactions containing 150 ng PCR product, NEBuffer (x10 concentrated) and nuclease free water using PCR conditions mentioned in Table 2.4.

Table 2.4 - Conditions for heteroduplex formation

Temperature	Ramp Rate	Time
95 ºC		5 min
95 – 85 ºC	- 2 ºC/sec	15 sec
85 – 25 ºC	- 0.1 ºC/sec	15 sec

Heteroduplex mismatching was identified by adding 0.5 µL of T7 Endonuclease I and incubation at 37 ºC for 15 minutes. Afterwards, T7 was inactivated using 0.5 µL Proteinase K for 5 minutes at 37 ºC. Digested/Fragmented amplicon targets were combined with 3 µL 6x concentrated gel loading dye purple (NEB) and 4.5 µL nuclease free water (NFW) and separated by gel electrophoresis for 1 hour at 120 V, using a 4-20 % TBE gel (Invitrogen) in 0.5X TBE buffer (Novex). Quick-Load® Purple 1 kb Plus DNA Ladder (NEB) was used as reference. To reveal fragmented amplicons by UV, gels were stained with a 0.005 % (v/v) RedSafe, MQWater solution and washed twice with deionized water to reduce background signal.

2.4. Single-cell Cloning

2.4.1. Limited Dilution

200 µL of cells at concentrations from 25-50 cells.mL^{-1} were transferred into a 96-well plate (Falcon), so each well could receive around 5-10 cells. We anticipate that a substantial proportion of cells would be lost reducing the number of colonies grown per well. After proliferation, wells where only 1 cell grew were identified, and new cell clones were established. During testing, only 100 µL of cells was added to wells, with the remaining 100 µL being added depending on condition.

2.4.2. Amplification of Clones

After reaching confluency in the 96-well plate, clones were transferred into 48-, 24-, 12- and finally 6- well plates. After having four wells in a 6-well plate reach confluency, cells were transferred to a 125 mL shake flask, and adapted to suspension through sub-culturing.

2.5. Clone Characterization

2.5.1. Growth Assessment

For growth assessment, clones were seeded at 1 x 10 ^{6} cells.mL^{-1} alongside wild type *Sf9* and cultured for six days. Cell concentration and viability were assessed every 24 hours.

2.5.2. Colony PCR

Colony PCR was used for preliminary clone screening. 20 µL of culture were mixed with 60 µL of nuclease-free water and incubated for five minutes at 95 degrees Celsius to perform the lysis stage. This served as the primer pair for a PCR reaction with the same number of cycles discussed before. Following the previous protocol, the reaction was set up using 1 µL of DNA input (or lysis product) and 3 µL of nuclease-free water.

2.5.3. Toxicity Resistance

Resistance to apoptosis induction was done using a MTT (Abcam) assay, after 4 days of exposure to Zeocin® (InvivoGen). For this, 100 µL of cells (clones and wild type *Sf9*) at a concentration of 0.5 x 10^6 cells.mL^{-1} were inoculated and allowed to adhere for around 1 hour in 8 rows of a 96-well plate (Falcon) per cell line. Serial 1:2 dilutions of toxin were carried out in a 96-well plate from columns 1-11 (highest concentration 4 mg/mL) and excess discarded for negative control in column 12. After this, media was aspired from cells and 100 µL of toxin dilution was added to wells and allowed to incubate for 4 days at 27 ºC.

After incubation, cells were then evaluated for viability by MTT assay, where NAD(P)H-dependent cellular oxidoreductase enzymes were revealed by reduction of tetrazolium dye through the addition of 10 µL of a 5 mg/mL MTT solution and an additional incubation of 4 hours at 27 ºC. Supernatant was removed and crystals dissolved with 150 µL of DMSO (Sigma) and incubation for 15 minutes with shaking. Finally, absorbance at 570 and 690 nm (reference) was measured, and the results analysed using GraphPad Prism.

2.6. Production of Biopharmaceuticals

To evaluate the phenotype of Sf9 edited cells, recombinant baculoviruses obtained in-house were used for production of a recombinant adeno-associated virus (rAAV) expressing green fluorescent protein (GFP) (a viral vector for gene therapy purposes) and virus-like particles exhibiting influenza M1 protein (VLP) (a vaccine candidate).

2.6.1. Baculovirus Stocks

Recombinant *Autographa californica nucleopolyhedrovirus (AcMNPV)* including GFP transgene flanked by AAV2 inverted terminal repeats and under control of the cytomegalovirus promoter (hereby named rBac-CMV/GFP) was provided by Généthon; recombinant baculovirus carrying rAAV2 *rep* and *cap* genes (hereby named rBac-REP/CAP) was developed *in-house* [50,51].

Recombinant baculovirus containing influenza M1 (from A/California/06/2009 H1N1 strain) and HA (from A/Brisbane/59/2007 strain) was established in-house [52], to produce virus-like particles (hereby named rBac-VLP).

Baculovirus amplification was performed by infecting Sf9 cells at a CCI of 1 x 10^6 cells.mL^{-1} with a multiplicity of infection (MOI) of 0.1 plaque forming units per viable cell (pfu.cell^{-1}). Upon reaching a viability of 80 %, around 3 days after infection, supernatant was harvested using centrifugation (300 x g, for 5 minutes) and a clarified (2000x g, for 20 min). Supernatant was then aliquoted and stored with light protection at 4º C until further use.

2.6.1.1. Production of rAAV and VLP

Wt (wild type) *Sf9* and *sf-caspase-1* knockout mutant cells were inoculated in biological triplicates at a concentration of 1 x 10^6 cells.mL^{-1} in 250 mL shake flasks (Corning) with a 10 %

working volume. Production of rAAV and VLP was initiated at the target cell concentration of infection (CCI) of 2 x 10^6 cells.mL^{-1}.

For rAAV production, cells were infected using both rBAC-REP/CAP and rBac-CMV/GFP at a multiplicity of infection (MOI) of 0.05 pfu/cell. Production was maintained for 96 hours post infection (hpi) and intracellular rAAVs were sampled at 72 and 96 hpi as established previously [50]. Briefly, cells were harvested by centrifugation (300x g, 4 ºC and 5 min) and lysed using a TNT buffer (20 mM tris hydrochloride (TRIS-HCL) – pH 7.5, 150 mM NaCl, 10 mM MgCl2, and 1 % Triton X-100 (Smith et al., 2009 [51])), supplemented with 0.5 % deoxycholate. After 10 minutes of incubation at room temperature, lysates were clarified by centrifugation (2000x g, 20 min, 4 ºC) and the supernatant stored at -80 ºC until further analysis.

For VLP production, cells were infected at MOI of 1 pfu/cell with rBac-VLP. Production was maintained for 72 hpi and extracellular VLPs were isolated by centrifugation (300g, 5 minutes) and stored at 4 °C until analysis.

Every 24 hours during manufacturing, the number of cells present, and their viability were measured.

2.7. Process Analytics

2.7.1. Cell Concentration and Viability

Viable cell concentration and viability were assessed using a haemocytometer (Brand, Wertheim, Germany) and the trypan blue dye exclusion method.

2.7.2. Baculovirus Titration by MTT Assay

Baculovirus titration was performed by MTT assay for infectious particles, as described elsewhere [53,54]. In short, for each titrated virus three 96-well plates (brand) were seeded with 100 µL of cell suspension (0.5 x 10^6 cells.mL^{-1}). Cells were allowed to settle for 1 hour at 27 °C, to guarantee attachment. In the meantime, serial dilutions of 10-fold dilutions were prepared per each virus replicate, according to the scheme presented in figure x. Sf900II media was used as negative control. Cell culture supernatant of cell plates were removed and 100 of virus dilutions added to each respective well.

Table 2.5 - Example of plate for titration usage, with virus dilution

	1	2	3	4	5	6	7	8	9	10	11	12
A B C D E F G H	Negative Control	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	Negative Control

Cells were then incubated at 27 ºC in a static incubator for 6 days, after which NAD(P)H-dependent cellular oxidoreductase enzymes was revealed by reduction of tetrazolium dye, as was done in method 2.5.3.

2.7.3. Full rAAV Capsid Determination by qPCR

Samples for qPCR were prepared as described elsewhere [50]. In short, intracellular fractions from 72 and 96 hours post infection were diluted and incubated with a final concentration of 60 $U.mL^{-1}$ Benzonase (Merck) for 90 min and afterwards treated with 1 $U.mL^{-1}$ DNAse (Roche) for 30 min at 37 ºC. Enzyme activity was ended by the addition of 6 mg/mL^{-1} Proteinase K with 3 % SDS followed by 30 minutes incubation at 37 ºC and an inactivation step for 20 min at 95 ºC. Samples were diluted 1:50 in 0.1 TE buffer (10 mM TRIS, 1 mM EDTA – pH 8) and stored at -20 ºC until further analysis.

qPCR analysis was performed as established in-house (Virgolini et al., submitted) to estimate the fraction of rAAV capsids which have the gene of interest (*gfp*) inserted into the genome. Full rAAV particles were estimated through assessment of expression of *gfp* (from rAAVs and rBac-CMV/GFP) and compensation of the expression of *gfp* from rBac is done through half of total baculovirus genome copy number, estimated by the quantification of the *ie-1* gene (a ratio of both rBacs of 1:1 was assumed). A master mix containing LightCycler® 480 Probes Master, 0.5 µM (final concentration) forward and reverse primers (IDT) for *gfp* (forw.: 5'-GAACCGCATCGAGCT GAA-3', rev.: 5'-TGCTTGTCGGCCATGAATATAG-3') and *ie-1* gene (forw: 5'-TGCCACACTTGCAGCTC-3', rev.: 5'-ATTTGCATACAACAAGTACACTGC-3'), 0.25 µM (final concentration) PrimeTime® probes (IDT – *gfp:* 5'-TGCTTGTCGGCCATGATATAG-3' / *ie-1:* 5'-ATGTGTGCGCGTTACCACAAATCC-3') for each gene, and PCR grade water was prepared. Copy numbers of the respective genes were obtained by measuring a standard curve of a customized linearized plasmid. The qPCR was performed in a 96-well plate (Roche) in a LightCycler® 480 Instrument II (Roche) according to the PCR conditions specified in Table 2.6.

Table 2.6 - qPCR Program for gene expression quantification

Cycles	Temperature	Time
1	95 ºC	10 min
	95 ºC	10 sec
40	62 ºC	10 sec
	72 ºC	6 sec
1	40 ºC	30 sec

2.7.4. VLP Titer Estimation by Hemagglutination Assay

For estimation of virus-like particle titer, an hemagglutination assay was performed as mentioned elsewhere [55].

In summary, process samples (25 µL) were 2-fold serially diluted in V-bottom 96-well plates (Thermo Scientific) using 1x Dulbecco's phosphate-buffered saline (DPBS) (-/-) solution

(Gibco) and gently mixed 1:1 with 1 % chicken erythrocytes (Lohmann). plates were incubated protected from light for 30 minutes at 4 ºC. HA titers were estimated by evaluating agglutinated wells (homogeneous red suspension) against non-agglutinated wells (clear suspension with single red dot at bottom) and can be estimated by the inverse of the highest dilution of sample that completely inhibited hemagglutination.

3. RESULTS AND DISCUSSION

3.1. CRISPR-Cas9 Implementation

As mentioned previously, CRISPR-Cas9 has been shown to be a highly efficient tool for gene editing, however it has not yet used extensively in Sf9 and High-Five insect cells. At the beginning of this study, only one case reported successful implementation of the system, utilizing a time consuming and tedious plasmid delivery strategy with poor efficiencies, when assessing fragment analysis associated with CRISPR-Cas9 activity [40]. This can be associated with the delivery of the system in a plasmid form, which presents several drawbacks such as low transfection efficiency and low editing capacity (since plasmid still needs to be transcribed and translated). In addition Sf9 cells did not have known promoters for expression of RNA at the time of the study which translated into an inefficient gRNA expression [47]. Thus, our first goal was to further enhance the use of CRISPR-Cas9 in Sf9 insect cells and establish a more efficient and therefore faster CRISPR-Cas9 gene editing pipeline.

To achieve this, the use of a ribonucleoprotein complex (RNP) delivery was evaluated. We decided to use this delivery system since it has been shown to be more specific, reducing time for onset of editing and risk for DNA integration in genome. This was tested by using the already validated gRNA for *fdl,* which is associated to poor glycosylation patterns in insect cells. Two gRNAs were tested, one as reported in literature and one optimized by a commercial algorithm software (see Table 3) [40].

Different transfection methods were tested for the delivery of RNP. Due to its negative charge (similar to DNA) these can be transfected using methods previously developed for DNA. However, since it's a different product being delivered, different reagents and techniques were tested [47]. For insect cells, lipofection using Cellfection™ has been reported in literature to work, and aside from that reagent, TransIT™ and RNAiMAX™ were also tested (all lipofections) [48]. An electroporation procedure was also tested since delivery of RNP using lipofection in a previous work resulted in poor editing efficiency and led to the need for performing 7 rounds of transfection to obtain a population with a high knockout efficiency [56].

3.1.1. Ribonucleoprotein Complexes Formation and Delivery

To evaluate the feasibility of the ribonucleoprotein complex (RNP) delivery for genetic engineering in insect cells, RNP complexes comprising the endonuclease Cas9 and a single guide RNA targeting the previously reported *fdl* gene, were used. 2 different gRNAs were tested, one previously published from Mabashi-Asazuma et al. 2016 [40] (with one more nucleotide) and one in-house design (according to IDT software). Using the three lipofection reagents, TransIT™, Cellfectin™ and RNAiMAX™, and an electroporation protocol, insect cells were transfected in 24-well plates and in duplicates (for the electroporation protocol).

Notably, none of the transfection methods tested for RNP complex delivery show significant toxicity (visual evaluation, no cell debris or floating dead cells) to Sf9 cells, with

electroporation only showing a slightly higher delay in resuming growth, expectably since stress to cells is higher. Interestingly, insect cells unlike many mammalian cell lines exhibit higher resistance to transfection agents, with literature reporting loss of viability in human liver cells up to 70 % using different lipofection reagents [57]. In CHO cells, cell viability loss associated with electroporation varies a lot, since that is dependent on the specific voltage applied and number of cycles used [58] .

To evaluate efficiencies of genome editing of both the respective transfection methods (RNP delivery) and the specificity of the different sgRNAs, a mutation detection assay is necessary. As mentioned in the introduction, after subjected to DNA damage, or in this case nuclease activity, cells go through mainly one of two mechanisms, as shown in figure 3.1. DNA damage repair by NHEJ will create small DNA mutations or deletions in the gRNA target region, creating a heterogeneous cell population in the target DNA locus.

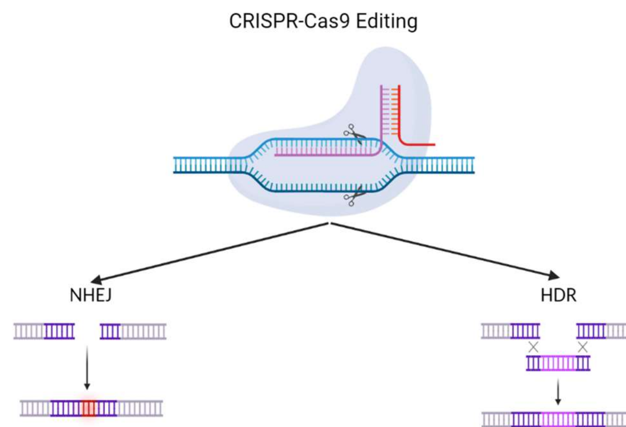


Figure 3.1 - Repair mechanisms to Cas9 editing, non-homologous end joining (NHEJ), and homology directed repair (HDR) (made using BioRender)

The T7 endonuclease assay takes advantage of the capacity of this nuclease to target and cleave non-perfectly matched DNA. After amplification of target gene in the region of interest as shown in figure 3.2, using T7 endonuclease I allows for the identification of defects caused by CRISPR-Cas9 editing. This creates fragments of DNA that represent the gene copies that underwent Cas9 action, and through a simple densitometry analysis it’s possible to determine what fraction of the genomic material of the population was edited. General pipeline for evaluating editing efficiency is presented in figure 3.3.

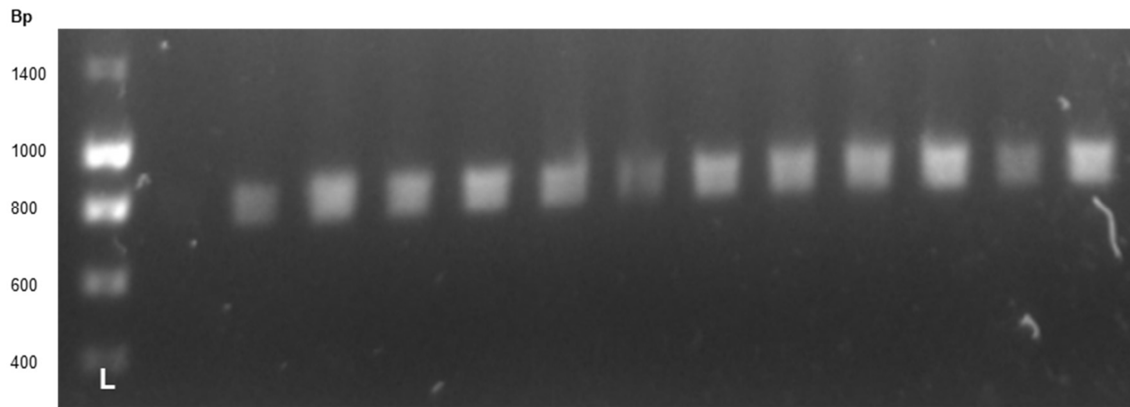


Figure 3.2 - Successful amplification of fdl gene from Sf9, L represents DNA ladder

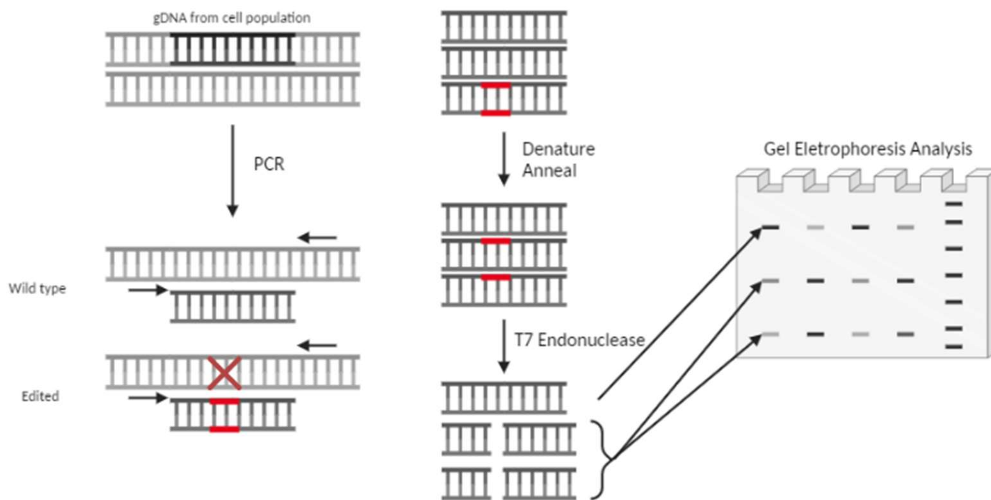


Figure 3.3 – General pipeline towards evaluation of gene editing efficiency (done using Biorender)

Target gene is represented in figure 3.4 with primers, gRNA targets and expected fragments. Evaluation of editing efficiency by fragment analysis of populations transfected with aforementioned RNP complexes resulted in the gel presented in figure 3.5. After densitometry analysis, absolute values for efficiency are presented in table 3.1.

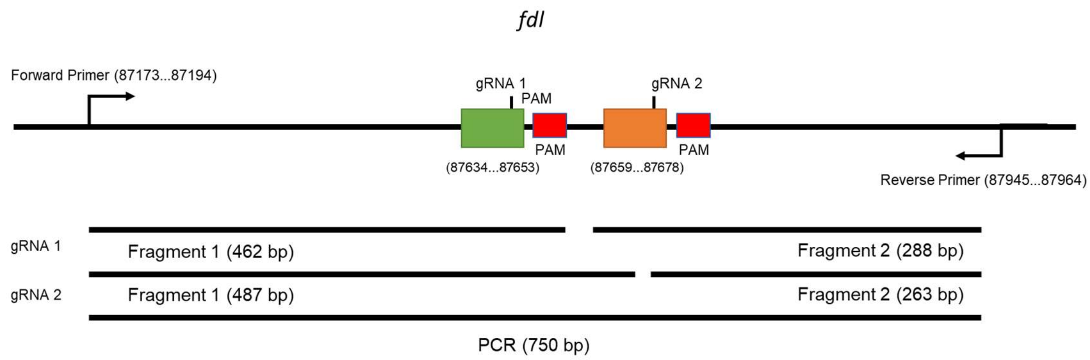


Figure 3.4 – Fdl gene with identification of target for gRNA, primers for amplification and expected fragments from T7 digestion

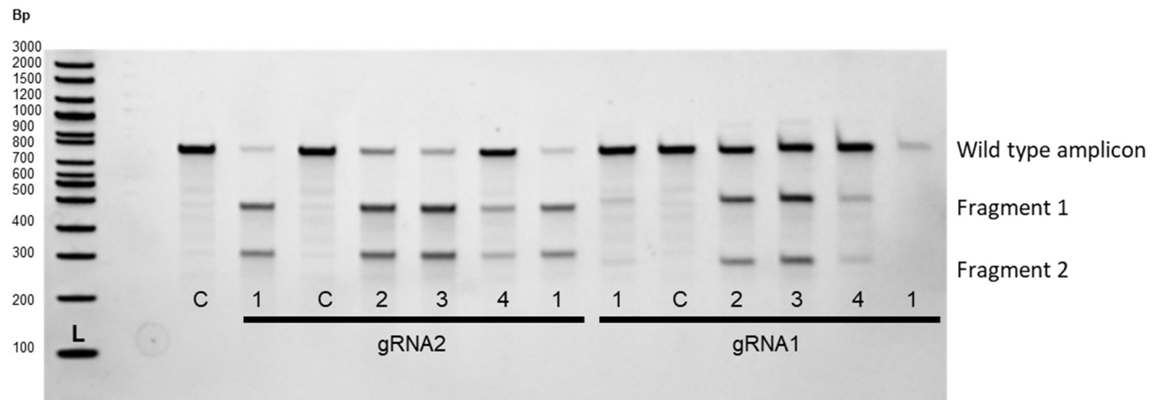


Figure 3.5 - Gel analysis of fragments resulting from T7 digestion; L represents ladder, C represents wild type Sf9; 1-4 are associated with transfection method: 1 – Nucleofector, 2 – Cellfectin™, 3 – RNAiMax™; 4 – TransIT™; gRNA 1 was designed by IDT software; gRNA 2 was adapted from Mabashi-Asazuma et al., 2017 [40]

Through evaluation of the gel in figure 3.5, it is possible to identify what methods present a higher efficiency, due to the fading of the band with the same shift as wild type Sf9 and relative intensity of edited bands. By evaluation of band intensity, it is possible to quantify the editing efficiency of the population, by comparing the wild type Sf9 fragment, the largest fragment, with the edited bands following equation 1.

Equation 1 – $Efficiency\ (\%) = \left(1 - \frac{Wild\ type\ band\ intensity}{Sum\ of\ all\ bands\ intensity\ (wild\ type + fragment\ )}\right) \times 100$

Table 3.1 – Gene editing efficiency for fdl knockout

gRNA1		gRNA2	
Method/Reagent	**Efficiency**	**Method/Reagent**	**Efficiency**
Eletroporation: Nucleofector	0/0 %	Eletroporation: Nucleofector	90/88 %
Lipofection – RNAiMax™	52 %	Lipofection – RNAiMax™	85 %
Lipofection – Cellfectin™	46 %	Lipofection – Cellfectin™	77 %
Lipofection – TransIT™	15 %	Lipofection – TransIT™	26 %

As shown in table 3.1, efficiencies varied a lot depending on the used gRNA and the RNP delivery method employed. gRNA 2 consistently exhibited higher efficiencies (1.5 fold more at least) regardless of the transfection method, showing higher targeting efficiency to the gene. Although electroporation proved to be the most efficient delivery method (90/88 % with gRNA 2), it is harder to upscale and less cost efficient. As a result, and since the efficiencies observed for lipofection were similar than those obtained in electroporation (85 % for RNAiMAX™), this method was chosen for further evaluation.

3.1.2. Establishing Single-cell Clones

To guarantee an homogenous population of knockout cells, single-cell clones were established. To achieve this, single-cell isolation methods are available, such as limited dilution, highlighted in figure 3.6, and single-cell sorting. Limited dilution defines the repeated dilution of a cell pool to obtain single cells inside a specified volume of a microwell. Single-cell sorting uses fluorescence-activated cell sorting (FACS), that automates the introduction of a single cell into each microwell.

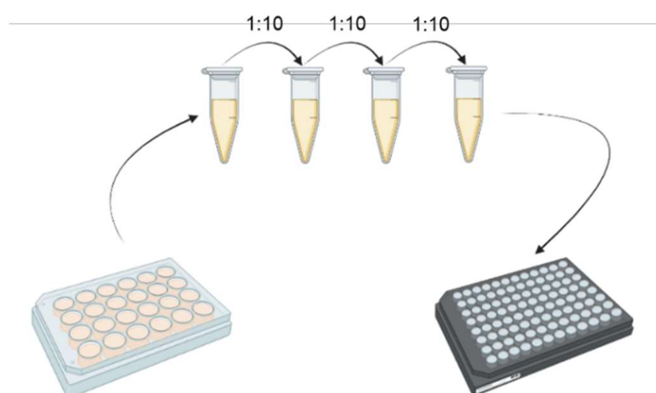


Figure 3.6 - Limited dilution for single cell cloning

Here, the limited dilution approach is used to generate single-cell clones rather than sorting cells, since previous laboratory experience has shown that sorted single cells are more likely to die during the amplification phase. Initially tested conditions for cell amplification were based on previous in-house work [59]. This protocol used a 1:1 ratio between conditioned media (spent cell growth culture media, carrying autocrine cell growth factors and other cell metabolites)

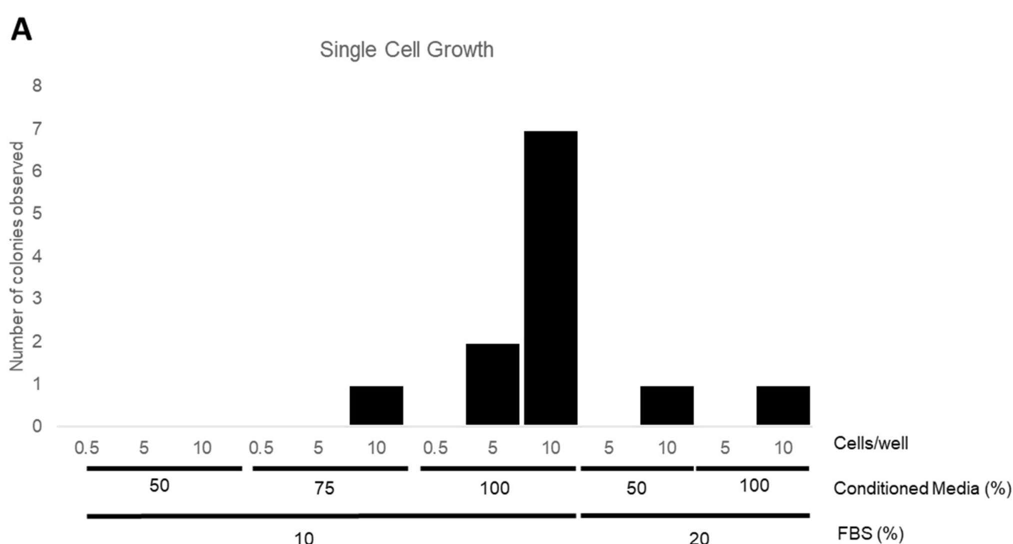
supplemented with 10 % FBS (which is rich in growth factors and both micro- and macronutrients [60]) with fresh growth media. However, this initial condition was unsuccessful to generate single-cell clones. Thus, several conditions were tested to select the optimal growth media to support single cell amplification.

Autocrine growth factors are recognised to be necessary for insect cells to maintain growth, and because they are absent at extremely low seeding quantities, cells don't divide.[61]. To address this issue, several techniques have been described in literature, as shown in Table 3.2.

Table 3.2 - Previously reported methods for generation of monoclonal insect cell lines (adapted from J. Zitzmann et al, 2018) [61]

Method	Source of Growth Factor	Cell Line	Ref.
Limiting Dilution	Conditioned Medium	*Drosophila melanogaster* S2,	62
		Spodoptera frugiperda Sf9	63
	Feeder cells – mitomycin C	*D. melanogaster* S2	64
	Feeder cells – irradiated	*D. melanogaster* S2	65
	Feeder cells – spatially separated	*D. melanogaster* imaginal disk	66
	Feeder cells - untreated, living	*D. melanogaster* S2	67
Soft Agar	Conditioned Medium	*D. melanogaster* S2	68
	Feeder Cells - irradiated	*D. melanogaster* S2	69

Several conditions were assessed for increased yield of growing single-cell clones after limited dilution. Thus, different harvest cell concentrations for conditioned media (2 and 3 × 10^6 cells.mL^{-1}), different FBS supplementations (10% and 20%), different numbers of cells per well (0.5, 5 and 10) as well as different ratios between conditioned and fresh media (1:1, 3:1, 1:0) were tested. The different conditions were used to determine what supplementation would be ideal to obtain the highest number of wells with single-cell colonies. Per condition 30 wells were seeded and grown for 15 days. Actively growing colonies (round shaped aggregates with viable cells) were deemed a positive result. The overall results can be seen in Figure 3.7.



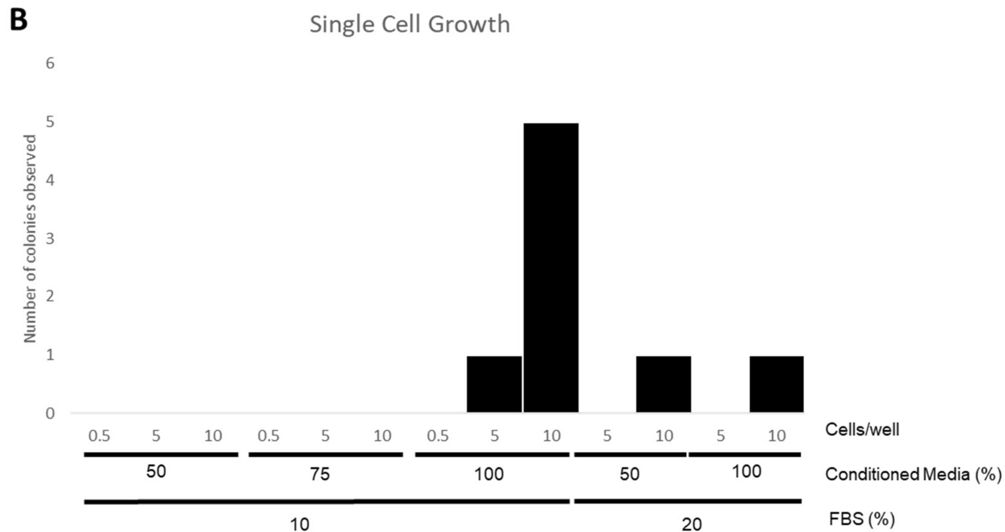


Figure 3.7 - Number of colonies observed per condition (first test); A and B represent media with inoculum at a concentration of 3 and 2 x 10^6 cells.mL^{-1} respectively

After the initial test, no clear difference between harvest cell concentration for spent media was observed. However 100% of conditioned media showed the best results. Regarding FBS supplementation, 20% of FBS didn't seem to allow single-cell growth. Therefore, a second round of testing was done to understand if 10% was indeed the optimal ratio for supplementation or if a reduction would be beneficial for single-cell growth.

As previously mentioned, 20 % FBS was shown to be detrimental for single-cell growth, thus studies on the effects of FBS in the media were conducted at lower concentrations (5% and 0%) using 100 % conditioned media and two different cell concentrations for plating. The conditions that yielded the best results for the first study were used as control for this test (10 % FBS, 100 % conditioned media, 10 cells/well). Results are shown in figure 3.8.

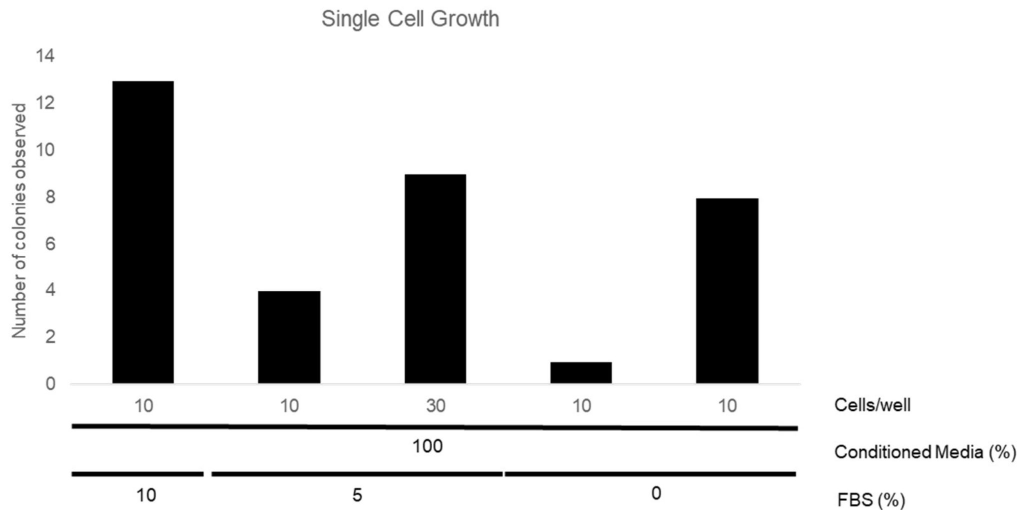


Figure 3.8 - Number of colonies observed per condition (second test); conditioned media harvested at a concentration of 2.2 x 10^6 cells.mL^{-1}

It was concluded that 100% of conditioned media (cell concentration of harvested media between 2 – 3 * 10^6 cells.mL^{-1}), 10 % FBS, and a cell concentration of 10 cells/well (or 100 cells.mL^{-1}) was the best supplementation strategy for single-cell cloning. Since some wells had more than one colony, for future work 5 cells/well will be done simultaneously in different plates to assure single-cell colonies in wells.

3.2. Establishing *Sf-caspase-1* Knockout Clones

Now that the pipeline for gene knockout using CRISPR-Cas9 and the single-cell cloning strategy were established, a literature review was performed to identify engineering targets that might show beneficial effects. *Sf-caspase-1*, previously studied using transient silencing methods such as shRNA, has shown to improve cell stress resistance and prolong survival after baculovirus infection [33–35]. As a result, it was deemed a good knockout target, to evaluate i) the applicability of the established pipeline and ii) if improvements can be observed in the delay of apoptosis (and thus prolonged production times and product titers) compared to gene silencing of the previous studies [33–35].

Sf-caspase-1 represents the insect ortholog of mammalian effector caspase *CASP3* and *CASP7* [70]. Much like other caspases, it is an aspartate-specific cysteine protease that is produced inactivated and becomes autocatalytic after activation, initiating an apoptosis initiation cascade. Interestingly, our unpublished work showed that this gene is overexpressed at later stages of infection, further supporting the gene as a good engineering target.

3.2.1. Knockout Efficiency

The previously established CRISPR-Cas9 pipeline was applied, targeting *Sf-caspase-1* in two consecutive transfections with the objective of increasing efficiency. This was done due to the tetraploid nature of insect cells, where each cell contains 4 copies of the same gene and therefore to increase the likelihood of finding a completely knocked out mutant a higher efficiency was deemed necessary. Target gene is represented in figure 3.9 with primers, gRNA targets and expected fragments. Moreover, initial tests suggested an improved efficiency when both gRNAs were used as shown in figure 3.10.

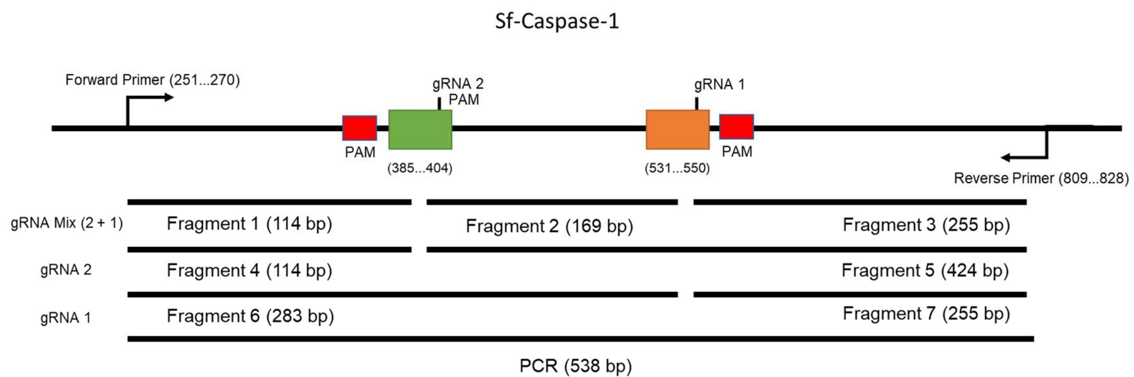


Figure 3.9 - Sf-caspase-1 gene with identification of target for gRNA, primers for amplification and expected fragments from T7 digestion

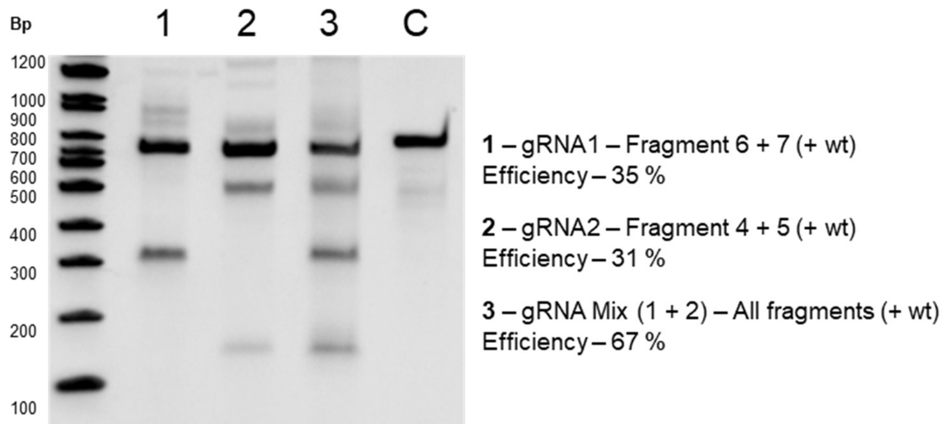


Figure 3.10 - Initial tests for Sf-caspase-1 targeting efficiency of gRNAs, C represents wild type (wt) Sf9

Since more cells were needed to freeze, evaluate, and seed sf caspase-1 edited cells, transfections were carried using a larger volume in 6-well plates (1.5 mL). To assess Cas9 functionality *fdl* gRNA was used as a control. Additionally, to evaluate if the second transfection proved efficient, wild-type *Sf9* was transfected using the same RNP complex solution. Results for PCR amplification shown in figure 3.11 and fragment analysis associated with this experiment are shown in figure 3.12.

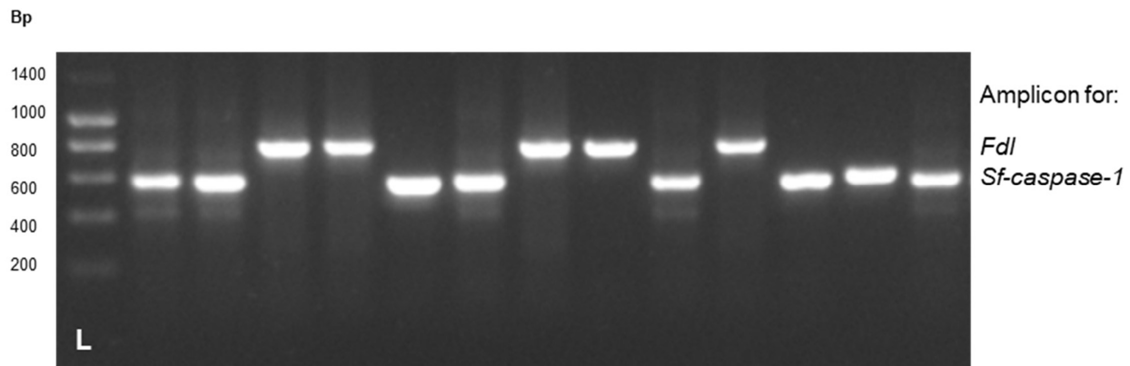


Figure 3.11 - Successful amplification of Sf-caspase-1 gene from Sf9 (and fdl, from control transfections), L represents DNA ladder

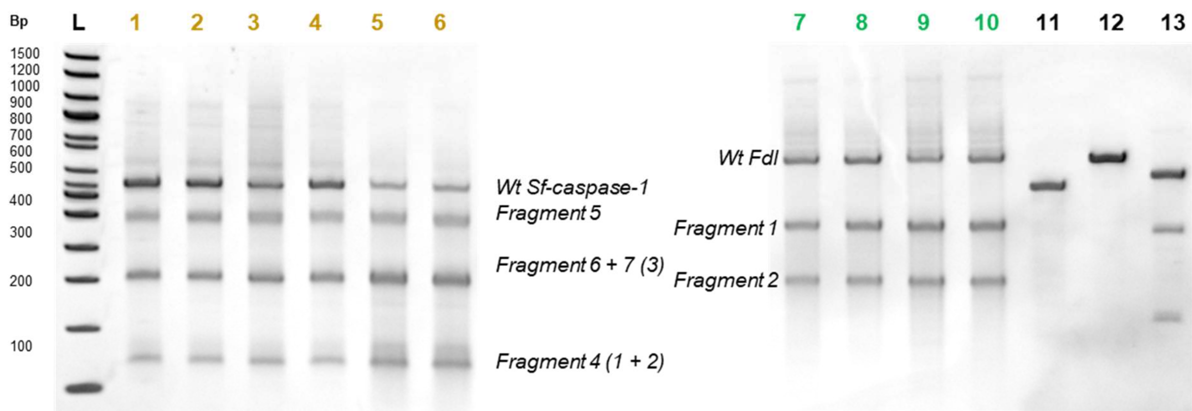


Figure 3.12 - Gel analysis of fragments resulting from T7 digestion; L represents DNA ladder, 11 and 12 represent wild type Sf9 for Sf-caspase-1 and fdl respectively, 13 represents the T7 assay control while the remainder are identified in the table 11, (fragments in brackets represent alleles with both deletions)

Table 3.3 -Gene editing efficiency for Sf-caspase-1 knockout

Sf-caspase-1		***Fdl***	
1 – 1st Transfection	59 %	7 – 1st Transfection	59 %
2 – Control Transfection	61 %	8 – Control Transfection	60 %
3 – 2nd Transfection	77 %	9 – 2nd Transfection	72 %
4 – 1st Transfection (6-well plate)	66 %	10 – 1st Transfection (6-well plate)	65 %
5 – 2nd Transfection (6-well plate)	86 %		
6 – Shake Flask	85 %		

As can be observed in figure 3.10 and 3.12, due to usage of two different gRNA targeting slightly different segments of the gene we have three different fragments at the expected size of around 110 and 420 from gRNA2 and 280/250 from gRNA1 aside from the wild type amplicon for the *Sf-caspase-1* (which has around 540 base pairs). Notably, by comparison of the bands it's possible to see that upscaling transfection gave out similar results to a small scale transfection, which is beneficial for future experiments where a high number of cells can be required.

Despite lower efficiency observed for the *fdl* transfection in comparison to the first testing, these remained consistent throughout *Sf-caspase-1* knockout experiments and a population with a *Sf-caspase-1* knockout efficiency of 86 % was obtained and used for the establishment of single-cell colonies. After 8 passages in a shake flask, knockout efficiency was re-evaluated and kept the same value, indicating no difference in cell growth of edited cells.

3.2.2. Single-cell Cloning by Limiting Dilution

Single-cell clones from transfected cells, limited dilution was applied using the optimized conditions of chapter 3.1.2. Seeding concentration per plate was done at 5 cells/well and 10 cells/well. Selected clones for further study can be seen in figure 3.13 after confirmation of monoclonality (single-cell colony).

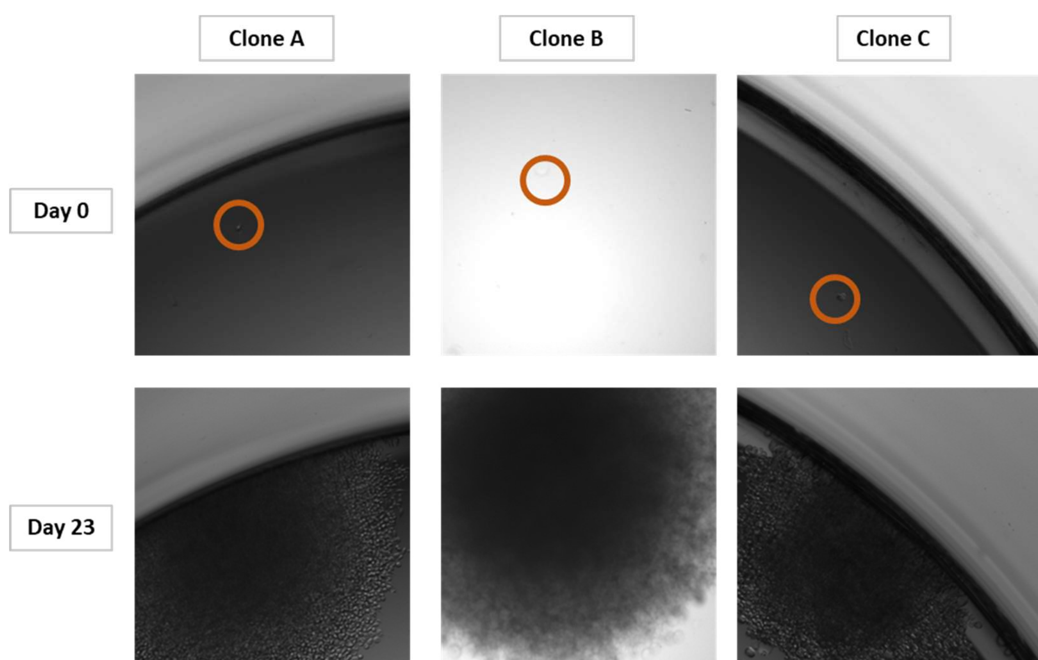


Figure 3.13 – Single-cell growth of selected clones for further study

3.2.2.1. Initial Screening

Initial screening of clones was conducted with 32 clones. As two gRNAs were used deletion of a large fragment is possible, which would translate into a stable knockout, and this would translate into a significant difference in the size of the amplified amplicon (around 170 base pairs less if the fragment between gRNA is deleted) and therefore would be observable in a shift of the revealed band in an agarose gel.

Colony PCR revealed 3 promising clones that show alterations in the *Sf-caspase-1* amplicon size as can be seen in Figure 3.14 (only gels containing clones of interest shown).

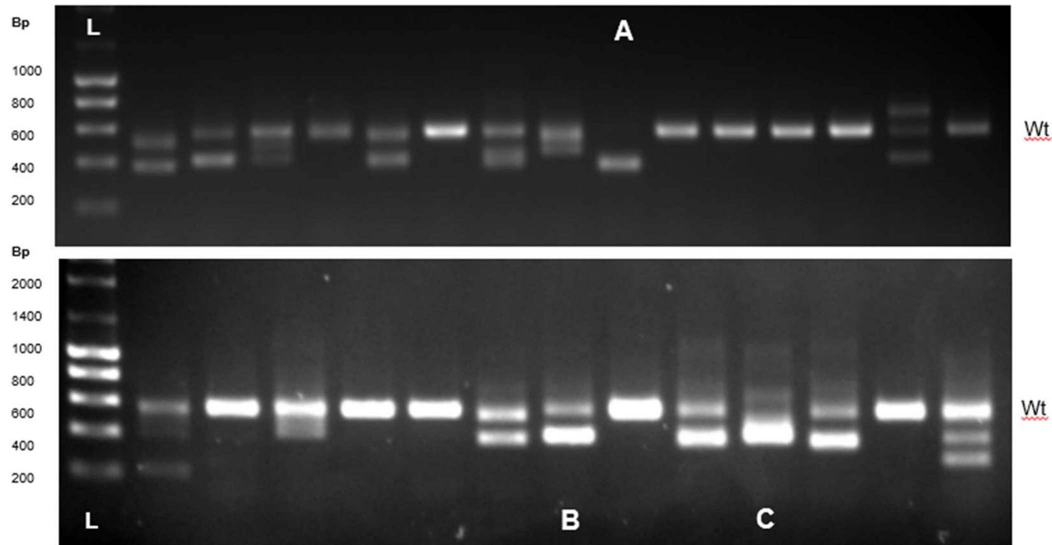


Figure 3.14 - Initial screening of clones derived from single-cell cloning: wt represents wild type Sf9 amplicon for Sf-caspase-1

From potential clones, three were considered for further analysis. Clone C and A presented a complete shift in their band, compared to wild-type *Sf9*. A third clone B was selected since it presents a high intensity of an edited band however still has some of the initial wild-type amplicon. Future characterization will be carried out for clones of interest, namely identification of mutation by sequencing of these amplicons.

3.3. Clone Characterization

3.3.1. Growth Profile

To assess if *Sf-caspase-1* knockout clones show similar growth kinetics compared to wild-type, a cell growth experiment was conducted. Cells were seeded at 1×10^6 cells.mL^{-1} and evaluated every 24 hours for six days, as shown in Figure 3.15.

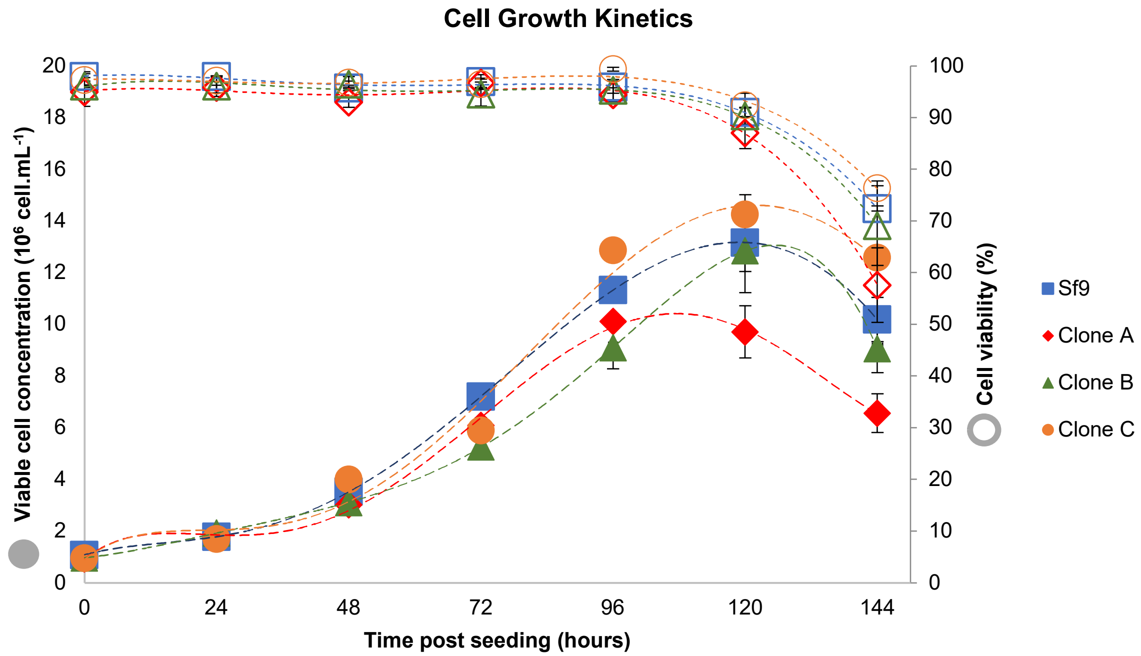


Figure 3.15 - Viable cell concentration and viability profiles for growth assessment of Sf-caspase-1 clones compared to wild-type Sf9 with three biological replicates (n=3, error bars represent standard deviation)

Growth kinetics of clone B and C were comparable to wild-type Sf9, reaching a maximum viable cell concentration of around 13 x 10^6 cell.mL^{-1} and comparable specific growth rates (clone A and C – 0.026 h^{-1} / wt-Sf9 – 0.029 h^{-1}). Clone A showed slightly different growth kinetics with a lower maximum viable cell concentration (10 x 10^6 cell.mL^{-1}) but similar specific growth rate (0.026 h^{-1}). These results suggests that *Sf-caspase-1* mutation does not affect cell growth significantly, corroborating literature data [36]. The difference in maximum viable cell concentration for clone A could arise from a specific unwanted clonal phenotype, regardless of the present mutation. Nevertheless, further evaluation is needed to identify the underlying reasons for the unfavourable phenotype.

3.3.2. Evaluating Stress Response in *Sf-caspase-1* Knockout Clones

A toxicity experiment was carried out to determine if clones had higher resistance to cell stress compared to wild-type Sf9, further establishing the existence of Sf-caspase-1 deletion. Zeocin, an antibiotic that causes double stranded breaks, was used for this in order to activate apoptotic pathways.[71].

Stress response was evaluated with an MTT assay, of zeocin treated cells. After initial assessment, day 4 was deemed the ideal timepoint for assessing toxicity response, achieving the optimal signal for untreated control cells.

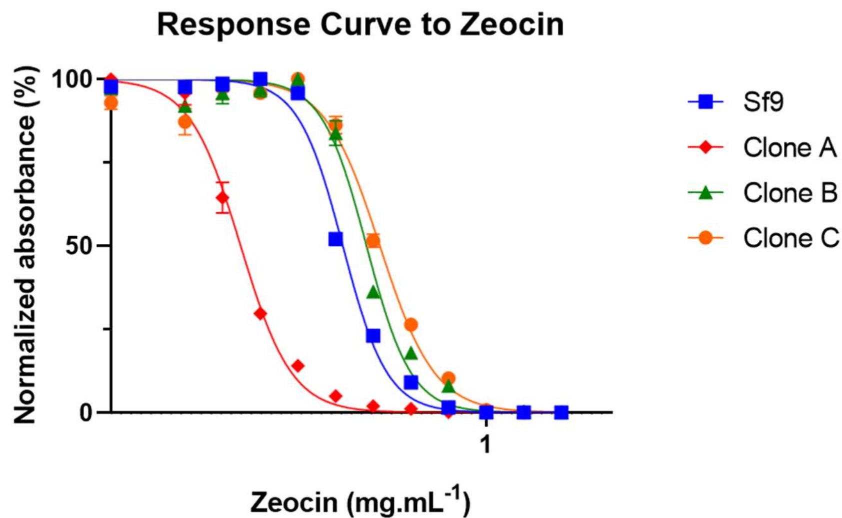


Figure 3.16 - Normalized absorbance of viability assessment after exposure to zeocin, (n = 8, error bars represent standard deviation)

Through this assay, and by observing figure 3.16 it is possible to see that clone A has a mutation which makes it much more sensitive to zeocin (IC50 of 0.01 ± 0.001 mg/mL) when comparing to Sf9 (IC50 of 0.06 ± 0.003 $mg.mL^{-1}$). Regarding clones B and C, these exhibit a higher resistance to apoptosis induced by the zeocin, presenting IC50 values of 0.1 ± 0.006 ($mg.mL^{-1}$) and 0.15 ± 0.01 ($mg.mL^{-1}$).

Despite these values, the assay used isn't ideal since it does not specifically target *Sf-caspase-1* but the entirety of the apoptosis pathway which gets triggered when DNA suffers damage, which can mask the effect this mutation can present in apoptosis resistance, therefore not being directly associated to the knockout of the *Sf-caspase-1* gene. An assay which would be ideal and has been used previously in order to test for the knockout, would be a baculovirus engineered for *p35* deletion, which is a *Sf-caspase-1* inhibitor, as was shown in a study that targeted the same gene for a knockout as this thesis and obtained positive results for a knockout phenotype [36].

In order to further characterize clones, methods for characterizing expression of *Sf-caspase-1* such as RT-qPCR for mRNA quantification and western blot for protein presence identification will be carried out.

3.4. Biopharmaceutical Production

To assess if *Sf-caspase-1* knockout results in beneficial cell phenotype, two model biopharmaceuticals were produced, namely a recombinant AAV2 carrying GFP and virus-like particles (VLP) exhibiting influenza M1 and HA protein. Both have been extensively studied and optimization for infection conditions has already been previously done [50,55].

rAAVs are non-enveloped virus that are being used as gene therapy vectors in the introduction of genetic material in target cells. Despite possessing viral proteins, they lack viral

genes, which makes these non-pathogenic, with low potential for inflammation and a prospective long term gene expression, being therefore safe and efficient therapeutic options [72,73]. Virus-like particles are a new platform for vaccine development. These are highly immunogenic due to the density of antigens displayed in their surface and present several advantages to traditional vaccines, e.g. non-infectious, safe, and having no risk of mutation since they lack genetic material, amongst others [74].

3.4.1. AAV Production

Conditions for AAV production have been previously optimized in the host laboratory, with a CCI of 2 x 10^6 cells.mL^{-1} and a low MOI of 0.05 (per virus) being herein used. Experiment was done in triplicate 250 mL shake flasks.

3.4.1.1. Infection Kinetics

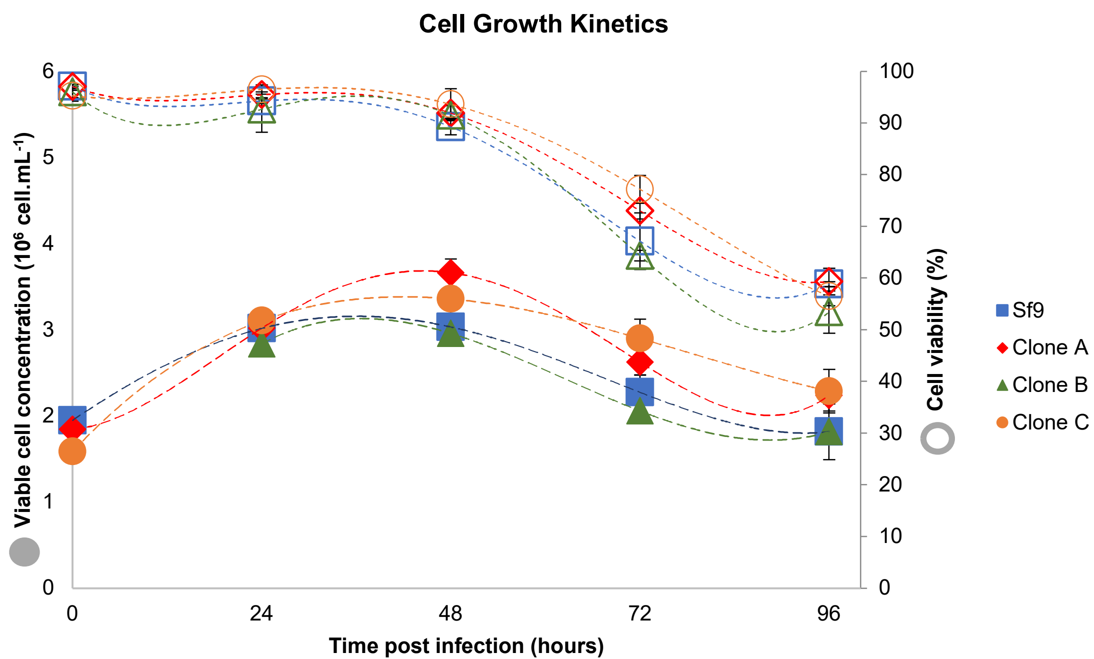


Figure 3.17 - Viable cell concentration and viability kinetics throughout infection for rAAV production for all populations, (n = 3, error bars represent standard deviation)

Figure 3.17 shows the infection kinetics of the AAV production process using a low MOI infection. The results show that potential knockout clones identified previously exhibit equal kinetics to wild type Sf9 during infection with a rBac. Despite increased stress resistance to DNA damage of both clones B and C, which triggers the apoptosis pathway, no significant differences between those clones and wild-type Sf9 were observed, which corroborates results reported previously in literature for the same gene knockout [36].

This contradicts findings from knockdown experiments, which showed an improvement in infection-related viability relative to Sf9 wild type.[33–35]. Theoretically, this can be associated with the mechanism of action of RNAi, where the same vector (shRNA which was used for the study) can be processed by the DICER in different fragments and target different forms of caspases

inside the cells, thereby knocking down not only *Sf-caspase-1* but also different caspases annotated in the genome with some degree of homology. Another suggestion presented by Malmanche et al. 2022 [36] is that this variability could be due to transgene expression, since viability profile during infection is decided not only due to baculovirus infection but also due to the nature of the protein being overexpressed in the vector, since in these studies, products like luciferase and secreted alkaline phosphatase (SEAP) were used, while the knockout study overexpressed β-galactosidase [33,35,36].

3.4.1.2. Intracellular AAV Titers

Due to extraction protocol variability, assays must be repeated to guarantee the robustness of the results obtained and allow for better study of the phenotype observed in each clone.

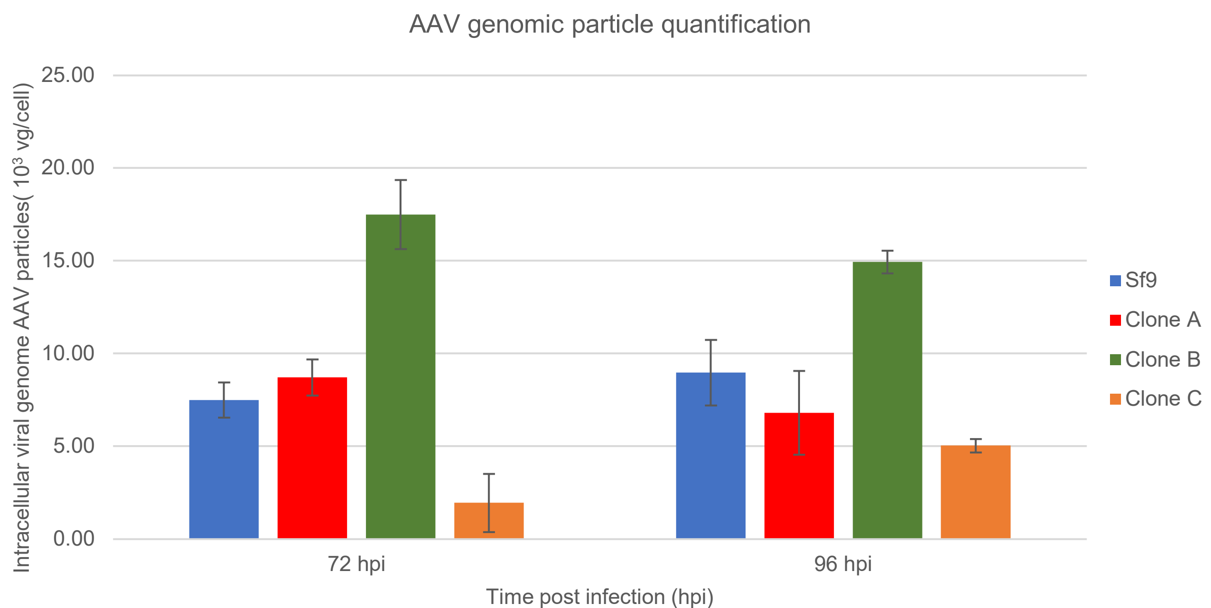


Figure 3.18 - Titer for intracellular genomic particle production throughout infection assessed by qPCR (error bars represent standard deviation)

As can be observed in this figure 3.18, only clone A presents a similar production level when comparing to wild type Sf9, while clone B presents an increase and clone C a decrease. Due to such varying phenotypes, the observed production yields cannot be directly associated with *Sf-caspase-1* knockout. This difference can be associated with the type of mutation present in the gene (which will be studied further by sequencing), the initial wild type population used for knockout of *Sf-caspase-1* and its heterogeneity, and/or off-target effects that aren't yet well understood due to lack of assays. To better understand this difference, additional production runs of rAAVs should be done and titers re-evaluated to observe if phenotype persists.

Overall, the data herein generated suggests that *Sf-caspase-1* knockout has no effect on production processes using IC-BEVS [36].

3.4.2. Influenza HA-VLP Production

To evaluate if the knockout *Sf-caspase-1* effect on productivity was product dependent [36], a recombinant baculovirus carrying HA and M1 genes, necessary for the assembly of influenza virus like particles was used for the production of an alternative product. Experiment was done in triplicate 250 mL shake flasks, with a CCI of 2 x 10^6 cells.mL^{-1}, and a MOI of 1, as these conditions were previously optimized [55].

3.4.2.1. Infection Kinetics

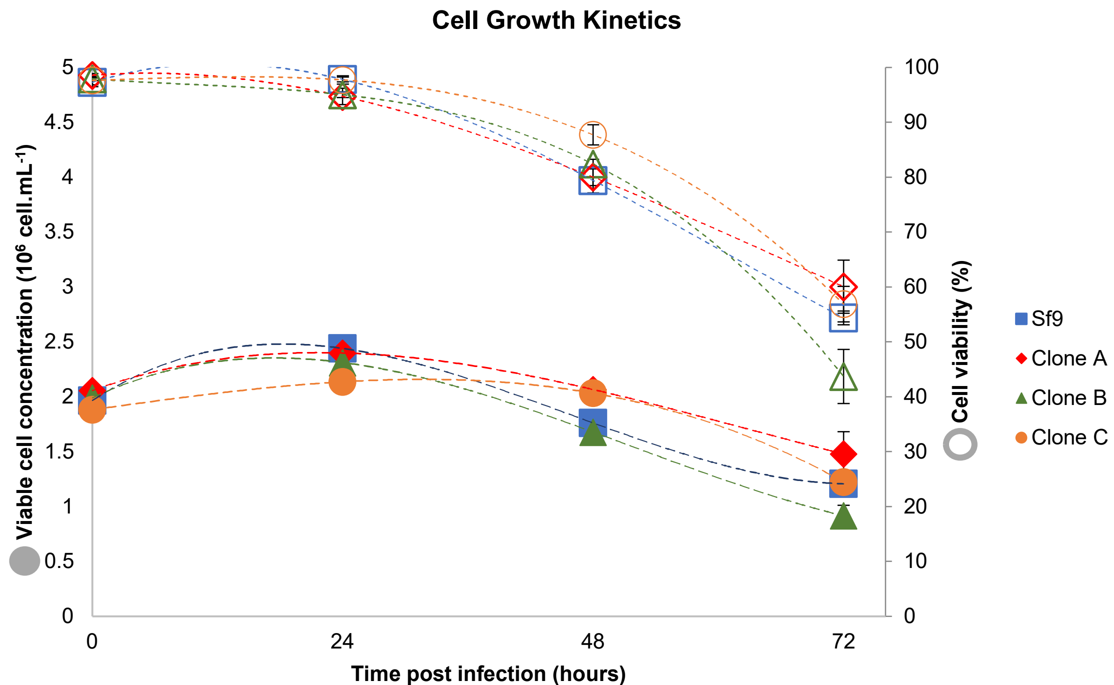


Figure 3.19 - Viable cell concentration and viability kinetics throughout infection for VLP production for all populations, (n = 3, error bars represent standard deviation)

Cell growth and viability profiles of clones and wild type Sf9 are similar, as shown in figure 3.19. Once again these results corroborate literature data and indicates that *Sf-caspase-1* knockout has no effect on delayed apoptosis during baculovirus infection, which can be associated to the fact that most of the effect of this caspase is nullified by the presence of the inhibitor *p35* in the baculovirus [36], and additionally indicates that product type (at least for the two tested in this thesis) wasn't determinant in the delay of apoptosis.

3.4.2.2. Extracellular VLP Titers

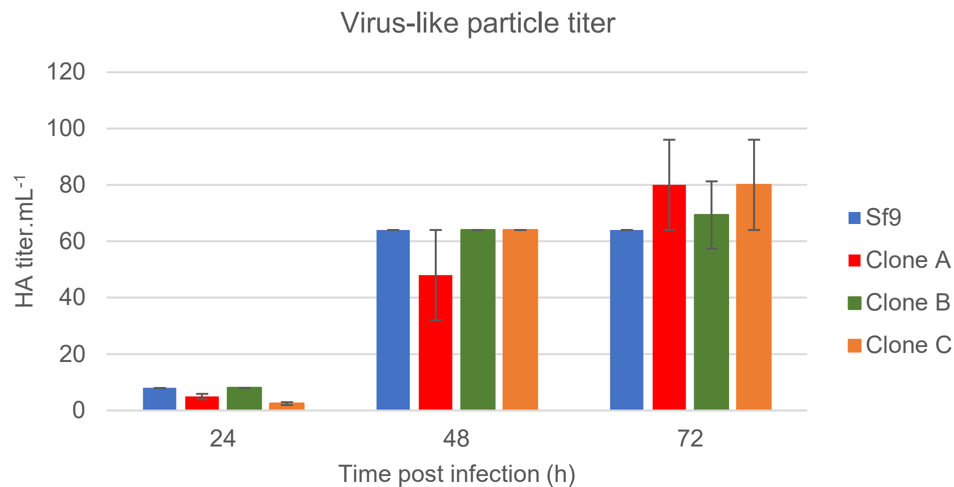


Figure 3.20 - Titer for virus-like particle production throughout infection assessed by hemagglutination assay (error bars represent standard deviation)

As can be seen in figure 3.20, a slight increase in HA VLP production could be observed for clones when compared to wild type *Sf9*. When comparing with previous literature, Sf9 cells are producing below what has been achieved with High Five using the same infection conditions [55,75]. It is important to mention that HA assay presents some drawbacks, since it's not too sensitive to small variations in concentrations of VLP, as shown by Krammer et al., 2010 [75], where despite having differences of >6 fold between cell line produced titers, hemagglutination assays gave out similar results. Due to this, further productions, and titer re-evaluation with other assays such as western blot would be necessary to confirm results.

Unlike suggested in literature, different products gave out the same results for production, which reinforces the idea that knocking out *Sf-caspase-1* in *Sf9* cells is not enough to delay apoptosis during infection, and therefore delay the onset of production [36].

4. Conclusions and Future Perspectives

There is an increasing amount of studies focused on the development of new biopharmaceutical products such as vaccines, recombinant proteins, or even viral vectors for the treatment of genetic disorders. In addition, the rising awareness to threats against human health, pressures the need to develop expression systems capable of producing high titers of high quality products. Insect cells due to the advantages mentioned previously have been used for some veterinary and human products, however, due to limitations in titers and product quality due to post-translational modifications, these aren't capable of producing the high titers necessary in many cases, for example with rAAV, where clinical trials can require up to 10^{14} vg/kg. Therefore, the improvement of the production process and platform are required to achieve market standards for some of the products.

In this thesis, a pipeline for simple and fast knockout of genes in Sf9 cells was established. This was done through the use of a ribonucleoprotein complex comprising the Cas9 protein and the gRNA with homology to target gene, *fdl*, which was previously reported in literature for knockout [40]. Optimization of transfection by testing different delivery systems was done, and the best protocols were chosen for application in the targeting of *Sf-caspase-1*, a gene reported to improve production with the IC-BEVS system by delaying apoptosis. After single-cell cloning optimization and implementation, knockout mutants were identified and tested for production of rAAVs and influenza HA VLPs using IC-BEVS. Results achieved suggest that *Sf-caspase-1* knockout has little to no impact on cell growth and proteins expression kinetics. Nonetheless, the clones established in this thesis still require some characterization to understand what type of mutation is present, and further studies to confirm results shown previously.

By establishing a knockout pipeline and optimizing a single-cell cloning strategy, future studies regarding Sf9 gene editing will be carried out within a reduced timeframe and at a higher cost efficiency, allowing the establishment of mutant cell lines with phenotypes that favour productivity in this cell line, and potentially other insect cell lines, with targets upstream of *Sf-caspase-1* in the apoptosis cascade in insect cells, such as *SfDronc,* already being studied in-house [76].

With the increasing knowledge regarding transcriptomic changes upon infection and genetic traits associated with high productivity in insect cells from in-house data, future studies will tackle different strategies for gene editing in insect cells (aside from knockout) aiming at the improvement of IC-BEVS platform and its full implementation for biopharmaceutical production.

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Table 6.1 - First set of conditions tested for improved colony growth in single-cell cloning

Cell Concentration (Spent Media, cell/mL)	FBS (%)	Conditioned Media (%)	Cell Concentration (Plating, cell/mL)		
$3 * 10^6$	10	50	5	50	100
		75	5	50	100
		100	5	50	100
	20	50	50	100	
		100	50	100	
$2 * 10^6$	10	50	5	50	100
		75	5	50	100
		100	5	50	100
	20	50	50	100	
		100	50	100	

Table 6.2 - Second set of conditions tested for improved colony growth in single-cell cloning

Cell Concentration (Spent Media, cell/mL)	Conditioned Media (%)	FBS (%)	Cell Concentration (Plating, cell/mL)	
$2.2 * 10^6$	100	10	100	
		5	100	300
		0	100	300

Primer

sgRNA1

sgRNA2

Sf-caspase-1

ATGCTGGACGGAAAACAAGACAATGGAAATGTGGATAGTGTTGATATCAAACAAA
GAACCAATGGTGGTGGCGATGAAGGCGACGCTCTGGGCAGTCACAGTTCTTCGC
AACCCAACCGTGTCGCTAGGATGCCAGTTGATAGAAATGCCCCTTATTACAACAT
GAACCATAAACATCGTGGGTTGGCCATTATTTTCAACCACGAGCATTTCGACATTC
ACAGCCTGAAGTCCCGTACTGGCACGAATGTAGACAGCGACAACCTTTCCAAAGT
TCTTAAAAGCTTGGGATTCAAAGTTACGGTGTTCAGTAACTTAAAATCAGAAGAAA
TCAATAAATTTATCCAGCAGACTGCAGAGATGGACCATTCAGACGCTGACTGTTTA
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Fdl

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GCGCGACAGC

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MIGUEL GRAÇA

RATIONAL GENETIC ENGINEERING OF INSECT CELLS TO IMPROVE RAAVS AND INFLUENZA VLPS PRODUCTION YIELD UTILIZING THE BACULOVIRUS EXPRESSION VECTOR SYSTEM

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