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Application of functional genomics in the production of viral biopharmaceuticals: Vaccines and Vectors for gene therapy

Ana Sofia Formas Oliveira

Dissertação

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Master's Thesis in Cell Biology and Biotechnology, Faculdade de Ciências, Universidade de Lisboa, held at the Animal Cell Technology Unit from the Instituto de Biologia Experimental e Tecnológica and Instituto de Tecnologia Química e Biologica, Universidade Nova de Lisboa (IBET/ITQB-UNL) under the supervision of Dr. Ana Sofia Coroadinha.

i. Acknowledgements

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ii. Index

iii. Abbreviations List

bp - base pair

CA - Proteic capsid

CMV - Cytomegalovirus

DMEM - Dulbecco's Modified Eagle's Medium

Env – Coding genes for envelope glycoproteins

FBS - Foetal Bovine Serum

Flp - Flipase

FRT- Flipase recombinase target sites

Gag-pol – Group specific antigen – polymerase: structural and enzymatic viral proteins

GaLV - Gibbon Ape Leukemia Virus

GFP - Green Fluorescent Protein

HIV - Human Immunodeficiency Virus

hPGK - Human phosphoglycerate kinase

hygtk - hygromycin B phosphotransferase/thymidine kinase

IN - integrase

IP - Infectious particles

IRES - Internal Ribosomal Entry Site

kb – kilobase pair

LTR – Long Terminal Repeat

MA - Matrix proteins

MCS - Multiple cloning site

MLV - Murine Leukemia Virus

NC - Nucleocapsid

neo - Neomycin phosphotransferase gene

PEI- Polietilenamine

pol – polymerase

PPT - polypurine tract

PR - proteases

pro – protease gene

PYC2 - Yeast pyruvate carboxylase 2

RCRs - Replication-competent retroviruses

RMCE - Recombinase-mediated cassette exchange

RT - Reverse transcriptase

RV - Retrovirus based vectors

SIN – Self-inactivating vectors

SU - Surface (SU)

SV40 – Simian Vacuolating virus 40

TCA – Tricarboxylic acid cycle

TM - Transmembrane (TM)

TP - Total particles

VLP – Virus like particle

VSV-G - Vesicular stomatitis virus G protein

iv. Preface

This master thesis is within the scope of the project PTDC/EBB-BIO/100491/2008; entitled "Using functional genomics to improve mammalian cells for virus based biopharmaceuticals manufacture" funded by the Portuguese Fundação para a Ciência e Tecnologia (FCT).

The first part of the results described in this thesis contributed for the following publication:

A.F.Rodrigues, A.S.Oliveira, V.S.Bandeira, P.M.Alves, W.S.Hu, A.S.Coroadinha, “Metabolic pathways recruited for the production of a recombinant enveloped virus: transcriptional profiling and experimental validation” (*Submitted to Metabolic Engineering, Elsevier*).

v. Abstract

Retrovirus (RV) based biopharmaceuticals comprise several valuable bioproducts such as gene therapy vectors, recombinant vaccines and replicative-competent particles for oncolytic therapy. However, RV manufacture faces several challenges, particularly the low yields of current producer cell lines and the high content of non-infective particles (an average of 1 in every 100 total viruses produced are infective), contaminating the viral preparations. The identification and manipulation of the metabolic requirements for improved virus production is the scope of this project. In this context, this master thesis proposed to i) validate metabolic pathways recruited under a virus production state – previously identified by transcriptional profiling – by culture medium manipulation and ii) implement a novel method to screen for high-producing clones after gene metabolic engineering.

Medium manipulation enabled increased titers from 1.3 to 2.1 fold upon isolated medium supplementation strategies, reaching a synergistic effect with their combination of 6 fold increase. The improvement in RV production was not associated to increased viral genes expression; it was partially due to higher viral vector preparation quality (enhanced half-life for 2-3 hours and higher infectious particles *per* total particles content) and to an enhanced metabolic status of the producer cell. For the screening method a novel protocol was implemented taking advantage of split proteins technology enabling the isolation of the high-titer clones from the producer population by the assessment of their titers in the first stage of clone isolation - Single Step Cloning-Titration. Validated metabolic pathway will now be object of gene-based metabolic engineering which, resorting to the implemented protocol, can be conducted in a large-scale multi-gene approach, never attempted before.

This work directly contributes for improved cell line development in RV manufacture and encompasses pioneering steps in mammalian cell multi-gene metabolic engineering.

Keywords: Retrovirus based biopharmaceuticals; Packaging cells; Cell culture; Metabolic engineering; Virus titration methods.

vi. Resumo

Os retrovírus (RVs) são vírus com invólucro da família *Retroviridae*. Caracterizam-se por possuírem um genoma de cadeia simples de RNA positivo (7-11 kb), pela capacidade da sua transcrição reversa de RNA para DNA e por possuírem no seu ciclo de replicação uma etapa obrigatória de integração estável do seu genoma num cromossoma do hospedeiro.

No que diz respeito ao seu genoma e composição proteica, os RV possuem várias vantagens que lhes conferem grande sucesso na sua utilização como biofármacos, tais como: (i) genoma bem estudado, passível de ser manipulado por técnicas de biologia molecular, (ii) capacidade de encapsidação de genomas até 9 kb, (iii) elevada eficiência de transdução *in vivo* e *in vitro*, (iv) capacidade de modificar permanentemente o conteúdo genético da célula alvo, possibilitando a expressão a longo prazo do gene terapêutico, (v) a sua baixa imunogenicidade em comparação com outros vectores virais, e (vi) a capacidade para incorporar proteínas de envelope de uma ampla variedade de vírus heterólogos, proporcionando um meio de selecionar as células alvo a serem infectadas ou, para a apresentação de antigénios.

Os vectores derivados de RV têm sido extensivamente utilizados como ferramentas de transferência de genes em terapia génica; partículas sem genoma demonstraram resultados promissores como candidatos a vacinas recombinantes e, mais recentemente, foram desenvolvidas partículas com competência replicativa para serem utilizadas como agentes oncolíticos para a terapia de vários tipos de cancro.

A produção de vectores retrovirais é realizada em células de mamíferos, geralmente com origem de ratinho ou humanas, de forma transiente ou estável. A produção estável para uma produção contínua requer a incorporação sequencial de, pelo menos, três cassetes de expressão independentes (sistema de terceira geração); gag-pol (proteínas virais estruturais e enzimáticas), *env* (glicoproteínas do envelope) e do transgene, que contém o gene de interesse clonado no esqueleto viral. Cada estágio de inserção requer selecção por antibiótico, clonagem e uma análise extensa dos clones para encontrar aqueles com maior expressão e adequada estequiometria dos componentes virais. Assim, o estabelecimento de uma linha celular produtora demora aproximadamente um ano até estar concluído.

Do ponto de vista de produção, os sobrenadantes de vectores retrovirais caracteriza-se por títulos relativamente baixos, cerca de 10^6 partículas infecciosas por ml de meio de cultura. Considerando que a quantidade média necessária para tratar um paciente num ensaio clínico ronda 10^{10} particulas infecciosas, seria previsto cerca de 10 a 100 L de volume de cultura para cada paciente. Adicionalmente, as preparações virais são tipicamente caracterizados por razões baixas de partículas infecciosas/partículas totais

(cerca de 1:100), o que reduz a eficiência terapêutica dos vectores infecciosos. Assim, os sistemas de produção existentes estão longe de satisfazer as necessidades requeridas para a sua ampla utilização terapêutica ou profilática.

A engenharia metabólica surge como uma ferramenta atractiva para melhorar a produtividade celular. Contudo, os incrementos na produtividade em células de mamífero são modestos (cerca de 2 vezes) e, normalmente, baseados em aumentos na produtividade volumétrica em vez de específica (da célula). O metabolismo e as redes metabólicas são redundantes sendo que o mesmo padrão de produção/consumo de um metabolito pode ser originado por diferentes combinações de reacções não conceptualizadas enquanto "via metabólica", no sentido clássico. Assim, manipulações pontuais (um gene) são geralmente absorvidas pela rede e "diluídas" sem efeitos fenotípicos óbvios. Este problema aumenta de dimensão com a complexidade, o elevado grau de regulação e o nível de compartimentação celular das células de mamíferos. Actualmente, realizar engenharia metabólica em células de mamífero é um processo trabalhoso uma vez que envolve a introdução dos genes para as vias alvo e o isolamento e caracterização das centenas de clones isolados.

Em suma, a produção de RV enfrenta dois desafios: aumentar a produtividade celular para dar resposta às quantidades requeridas para uso terapêutico necessitando portanto de estratégias rápidas para a análise da produtividade dos clones obtidos. Para responder a este desafio, esta tese de mestrado teve como objetivos (1) validar resultados de transcriptómica que identificaram vias metabólicas relevantes na produção des retrovírus por manipulações específicas do meio de cultura de células 293 FLEX e (2) desenvolver um método de clonagem e titulação com capacidade de análise *high-throughput*.

As manipulações executadas foram direcionadas para vias metabólicas recrutadas em células produtoras de fácil manipulação, ou seja, vias de consumo de unidades básicas: metabolismo de ácidos nucleicos, metabolismo de aminoácidos, destoxificação de radicais livres e metabolismo das poliaminas. O meio de cultura foi suplementado com uma ou mais combinações dos seguintes suplementos: aminoácidos, nucleósidos, antioxidantes, poliaminas e glutationo reduzido. A produtividade viral aumentou em todas as condições utilizadas. Com suplementos únicos o título viral aumentou 1.3 a 1.9 vezes; com a conjugação de dois suplementos - aminoácidos e nucleósidos, antioxidantes com poliaminas ou glutationo reduzido - aumentou 1.5, 2 e 2.1 vezes. No entanto, o maior efeito a nível de produção foi conseguido pelo *cocktail* de todos os suplementos, atingindo um aumento de 6 vezes. O incremento na produção de RV não foi associado a um aumento da expressão de genes virais; foi parcialmente baseado numa maior qualidade do sobrenadante viral e e num estado metabólico melhorado das células produtoras. Adicionalmente, os suplementos de aminoácidos e *cocktail* aumentaram a estabilidade das partículas virais em respectivamente mais 3 horas e 2 horas.

Estes resultados indicam uma origem metabólica no constrangimento da produtividade viral. Estes resultados demonstraram que a manipulação orquestrada de vias independentes conduz a efeitos sinergísticos de aumento de título evidenciando a necessidade de uma manipulação concertada das vias metabólicas para obter efeitos de maior impacto. Do ponto de vista do bioprocesso, a adição de suplementos no meio de cultura para produção de biofármacos não é interessante uma vez que requere a utilização de sistemas *fed-batch* aumentanto os custos de produção. Assim, é necessário tornar a célula produtora mais robusta e independente de estímulos externos para atingir um estado de elevada produtividade. A engenharia metabólica de múltiplas vias (genes) surge então como uma ferramenta potencial para atingir um estado celular de híper-produtividade. Contudo, não só o estabelecimento de uma linha celular produtora é um processo demorado; as mesmas limitações surgem para populações de células produtoras alvo de engenharia metabólica. Fenótipos híper-produtores são eventos raros, mesmo quando a manipulação se revela benéfica, uma vez que dependem de níveis de expressão altamente regulada dos genes alvo, dependendo do número de cópias inseridas, assim como o seu local de integração cromossómica. O problema cresce exponencialmente para múltiplas manipulações génicas. Para responder a esta necessidade, o segundo objectivo desta tese consistiu no desenvolvimento de um método rápido e eficiente para a clonagem e simultânea titulação de células produtoras, acoplado a um potencial de análise *high-throughput*.

O protocolo implementado usa a recente tecnologia da proteína verde separada (*split* GFP) que consiste na proteína GFP dividida em dois fragmentos (GFP S10 e GFP S11) e é implementado em placas de 96 poços. As células produtoras expressam mCherry e produzem vírus com um transgene que codifica para uma proteína de fusão LacZ-S11. Estas células são plaqueadas a 1 célula por poço (clonagem) e ao sexto dia de cultura, as células alvo, expressando constutivamente a proteína complementar GFP S10, são adicionadas. Durante o restante tempo de cultura é propiciada a infecção das células alvo pela adição de polibereno. Por infecção a proteína GFP S10 da células alvo vai sendo transcomplementada com o fragmento GFP S11 originando sinal de fluorescência directamente proporcional ao número de vírus produzidos. Após 14 dias de cultura as placas de cultura são analisadas num fluorimetro e os poços com maior razão GFP/mCherry são isolados, assegurando assim que os clones seleccionados são os melhores produtores. Os clones podem ser isolados das células alvo por qualquer marca de resistência a antibiótico expressa pelos componentes virais (Zeocina, Blasticidina e Neomicina) e/ou através de puromicina (parte da construção mCherry).

A linha celular alvo foi estabelecida com as células Te671 por infecção com lentivirus que entregaram o gene da *GFP S10*. A linha celular produtora foi criada a partir das células

293 FLEX e 293 CEB 22; a primeira produz vectores retrovirais recombinantes baseados no vírus da leucemia murina (*Murine Leukemia Virus*, MLV), pseudotipados com a proteína do invólucro do vírus da leucemia do macaco gibão (*Gibbon Ape Leukemia Virus*, GaLV) e contendo um transgene codificante para a enzima β-galactosidase (gene *LacZ)*; a segunda é a linha celular percussora das 293 FLEX anterior à inserção da cassete génica para a expressão do invólucro. A tecnologia de troca de cassete mediada pela recombinase (RMCE) permitiu trocar o transgene LacZ-S11 com o anterior até então expresso (LacZ). O clone 32 das células 293 CEB 22 S11 foi utilizado para validar o protocolo após ser submetido a duas etapas de transfecção; a primeira para inserir o marcador celular mCherry e a segunda para introduzir o envelope GaLV. A população obtida foi então sujeita ao protocolo de clonagem e titulação permitindo o isolamento de clones de elevadas produtividades pela quantificação dos seus títulos na primeira etapa de isolamento do clone. Nenhum dos clones 293 FLEX S11 isolados produzia vírus à mesma escala que as células parentais, provavelmente devido a uma baixa eficiência de troca de cassete nestas células; o protocolo estabelecido foi então usado para isolar clones 293 FLEX S11 nesta população, permitindo isolar um clone produtor. Assim, o protocolo de clonagem e titulação desenvolvido proporciona uma ferramenta útil para ultrapassar as limitações impostas pela análise exaustiva de clones por métodos manuais, maximizando a probabilidade de identificar clones com fenótipo híper-produtor.

As vias metabólicas identificadas por dados de transcriptómica e validadas neste trabalho serão alvo de engenharia metabólica por manipulação combinada de múltiplos genes, apenas possível devido à implementação do protocolo de clonagem e titulação durante a presente tese.

Palavras-chave: Biofármacos derivados de retrovírus, células produtoras de retrovirus; cultura celular, engenharia metabólica, métodos de titulação de vírus.

1. Introduction

1.1 Retrovirus based biopharmaceuticals

Retroviruses (RVs) are animal enveloped viruses of the *Retroviridae* family, harboring two copies of a positive-stranded RNA genome with the size of 7–11 kb; they are mainly characterized by the ability of reverse transcribing it from RNA to DNA, followed by stable integration into the host chromosomal genome [1,2]. Virions measure 100-120 nm in diameter and the genome is complexed with the nucleocapsid (NC) proteins, enclosed in a proteic capsid (CA) containing the enzymatic proteins, namely the reverse transcriptase (RT), the integrase (IN) and proteases (PR), required for viral infection. The matrix proteins (MA) form a layer outside the capsid core that interacts with the envelope, a lipid bilayer derived from the host cellular membrane, which surrounds the viral core particle. Anchored on this bilayer, there are the viral envelope glycoproteins (Env) responsible for recognizing specific receptors on the host cell and initiating the infection process. Envelope proteins are formed by two subunits, the transmembrane (TM) that anchors the protein into the lipid membrane and the surface (SU) which binds to the cellular receptors. (Fig. 1 A) [3,4].

Based on the genome structure, retroviruses are classified into simple (e.g. MLV, Murine Leukemia Virus) or complex retroviruses (e.g. HIV, Human Immunodeficiency Virus). Both encode four genes: *gag* (group specific antigen), *pro* (protease), *pol* (polymerase) and *env* (envelope). The *gag* sequence encodes the three main structural proteins: MA, CA, NC. The *pro* sequence, encodes a protease (PR) responsible for cleaving Gag and Gag-Pol during particles assembly, budding and maturation. The *pol* sequence encodes the enzymes RT and IN, the former catalyzing the reverse transcription of the viral genome from RNA to DNA during the infection process and the latter responsible for integrating the proviral DNA into the host cell genome. The *env* sequence encodes for both SU and TM subunits of the envelope glycoprotein. Additionally, retroviral genome presents non-coding *cis*-acting sequences such as: two LTRs (long terminal repeats), which contain elements required to drive gene expression, reverse transcription and integration into the host cell chromosome; a sequence named packaging signal (ψ) required for specific packaging of the viral RNA into newly forming virions, and a polypurine tract (PPT) that functions as the site for initiating the positive strand DNA synthesis during reverse transcription. Additionally to *gag*, *pro*, *pol* and *env*, complex retroviruses, such as lentiviruses, have accessory genes including *vif*, *vpr*, *vpu*, *nef*, *tat* and *rev* that regulate viral gene expression, assembly of infectious particles and modulate viral replication in infected cells (Fig. 1 B) [3]. The main difference between the simple and complex retrovirus is the ability of the latter to transduce non-dividing cells [4].

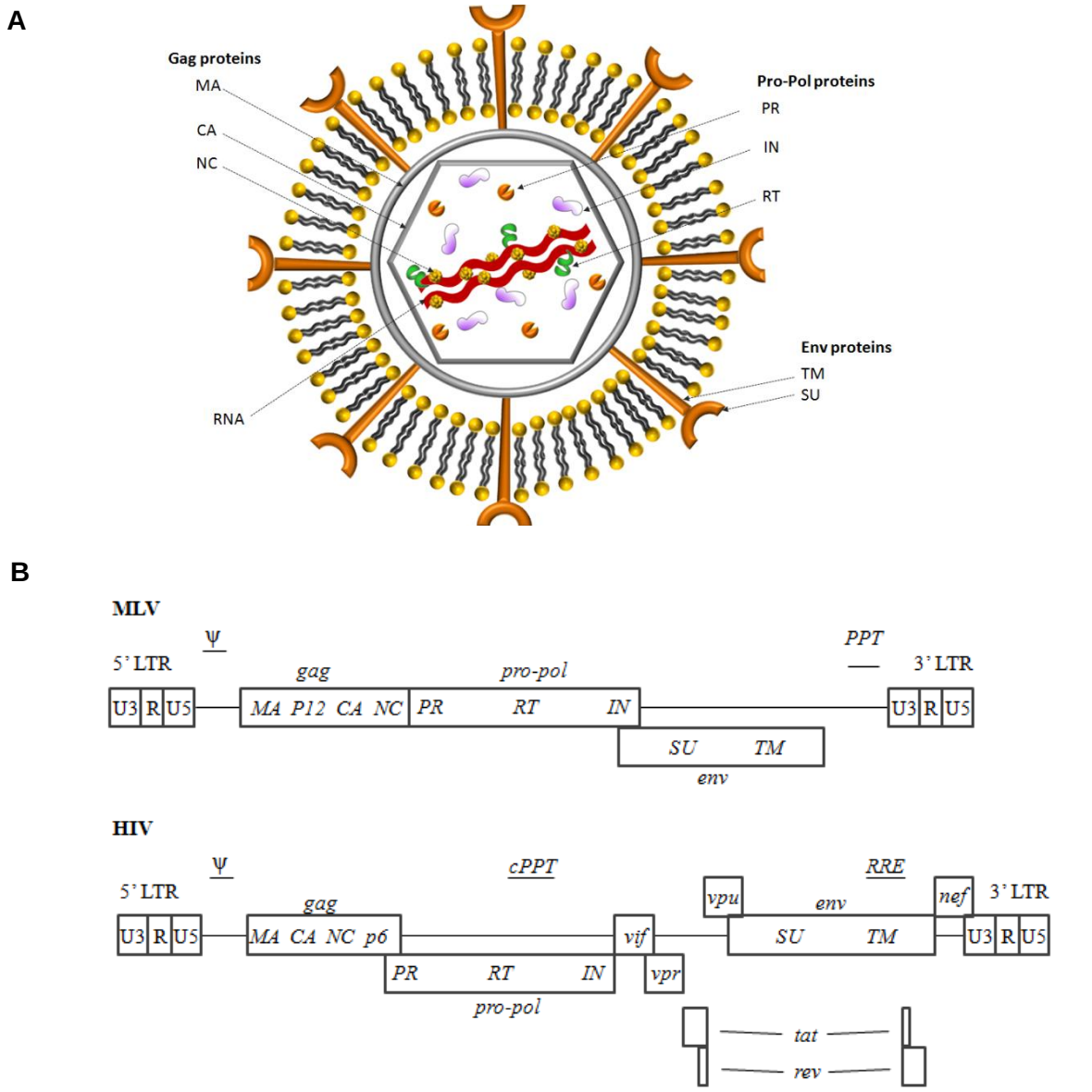

Figure 1 – A) Schematic representation of a retrovirus; B) genome organization of MLV and HIV [4]. Murine Leukemia Virus (MLV); Human Immunodeficiency Virus (HIV); nucleocapsid (NC); proteic capsid (CA); reverse transcriptase (RT); integrase (IN); proteases (PR); matrix proteins (MA); envelope glycoproteins (Env); transmembrane (TM); surface (SU); group specific antigen (*gag*); protease (*pro*); *pol* (polymerase); envelope (*env*); protease (PR); long terminal repeats (LTRs); packaging signal (ψ); polypurine tract (PPT).

Several aspects of retrovirus biology make them exceptionally apt as vectors for different biotechnological applications. Replication-incompetent vectors, have been extensively used as gene transfer tools in gene therapy protocols [5]; genome free particles have already demonstrated promising results as immunogen display platforms in the vaccinology field and more recently, replication-competent particles (RCP) were developed and efficiently used as oncolytic agents for cancer therapy [6].

Approved gene therapy clinical protocols using replicative deficient retroviral vectors for gene delivery include a wide range of diseases, e.g. haemophilia, Human Severe Combined Immunodeficiency (SCID), HIV infection, Gaucher's disease, β-thalassaemia, muscular dystrophy and Parkinson's disease. MLV were the first viral vectors used in gene therapy clinical trials and account for the second highest number of successful protocols [5]. Regarding oncolytic therapy, replicative competent RV vectors are suited for this proposes since can only integrate and replicate in dividing cells, providing an inherent means for targeting malignant cells that divide actively, while surrounding normal cells that are generally quiescent. Furthermore, after transduction, each tumor cell acquires the capability to produce more vectors that will improve efficient transduction of the surrounding malignant cells [6]. Hence, these vectors have been investigated for the treatment of a wide range of cancers including ovarian cancer, breast cancer, brain tumors and lung cancer [7]. Virus-like particles (VLPs) are genome-free particles with the ability to mimic a virus structure and display specific envelope proteins or an immunogen of interest. Thus, they are safer alternative to classical attenuated vaccines with minimum concerns of virus integration/replication. Retro VLPs can be pseudotyped with glycoproteins of several virus families, such as lymphocytic choriomeningitis virus, spleen necrosis virus, vesicular stomatitis virus, hepatitis C virus, yellow fever virus, West Nile virus and influenza virus. Considerable efforts have been gathered to develop a VLP vaccine based in simple RV, such as MLV, to target HIV and preliminary animal studies have already shown its potential [6].

1.2 Manufacture platforms

RV based vectors are essentially produced in mammalian cells, typically murine or human derived cell lines. This can be based on a short-term transfer of the viral constructs, known as transient production, into exponentially growing cells followed by 24-72 hours vector production and harvesting, or by their stable integration and constitutive expression into the host cell genome, named packaging cell lines, for continuous production [4].

The establishment of retroviral packaging cell lines has been based on the physical separation of the viral genome into different transcriptional units, reducing the risk of generating replicative competent retrovirus (RCRs) [2,4]. For both retroviral and lentiviral vector production, different packaging systems, named generations, have been developed. Each new generation aimed at minimizing and reducing the risk of RCRs formation face to the previous one. Currently, third generation is applied for the production of RV derived vectors, requiring the transfection of three independent expression cassettes encoding *gag-pol*, *env* and transgene functions (Fig. 2 A) [4].

Since the third generation of MLV vectors requires the expression of three genetic cassette it is necessary to perform three transfections and clonal selection steps. This process for establishing a producer cell line takes an average time-frame of around one year. To face this challenge, a new generation of retrovirus packaging cells (modular cell lines) based on cassette exchange systems that allow for flexible switch of the transgene and/or envelope, as well as selectable marker(s) excision, was developed (Fig. 2 B and C) [8,9].

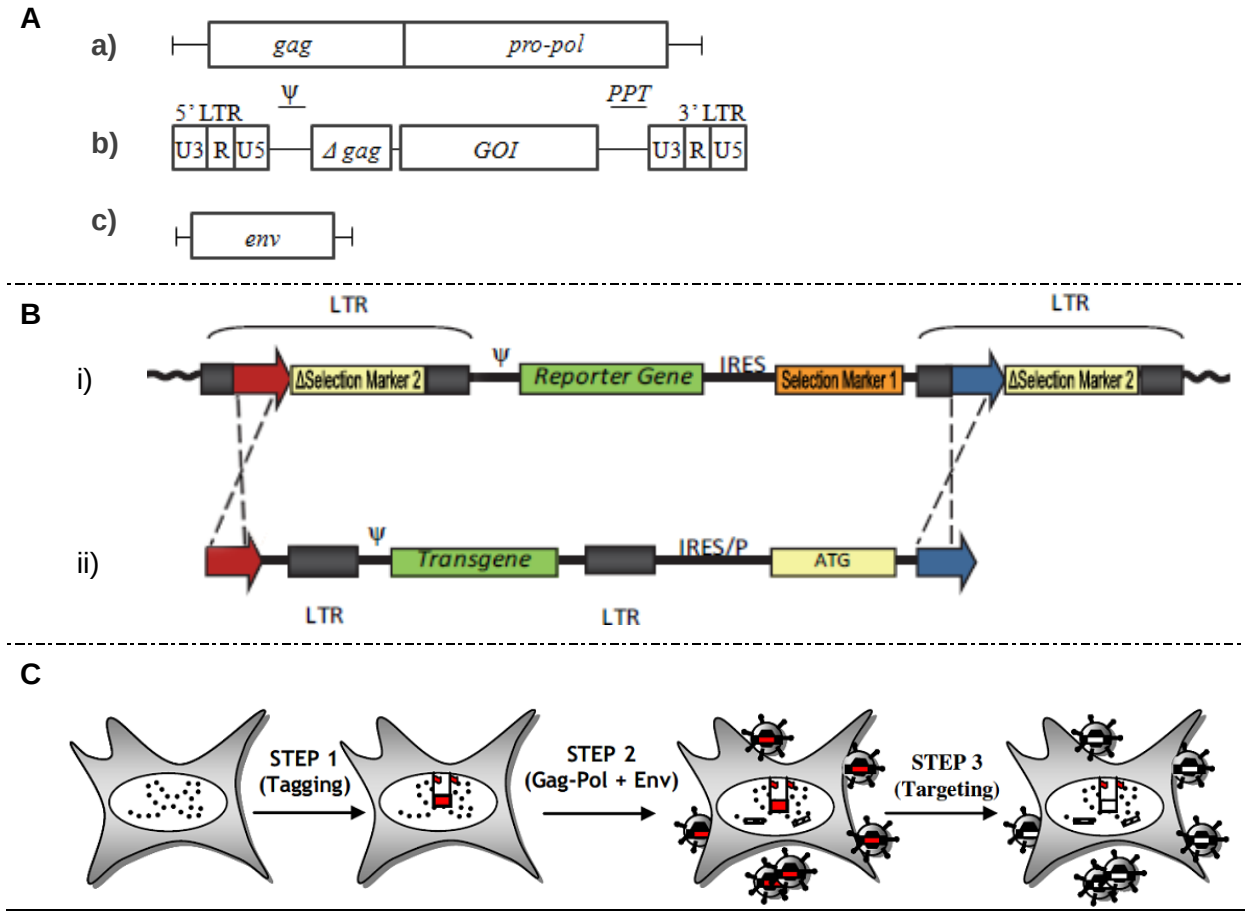

Figure 2 - Representation of RV based vector development: A) third *generation* split genome for replicative deficient RV production (a) packaging functions, (b) transgene and (c) envelope [4]; B) Schematic representation of the modular cell lines transgene based on the RMCE technology (i) Integrated retroviral transgene cassette harboring a marker gene and (ii) targeting therapeutic transgene plasmid allowing a fast exchange and establishment of a new retroviral producer cell [4]; C) Strategy for the generation of the retroviral vector producer cell line with exchangeable transgene [9].

A favorable chromosomal site for stable and high retroviral vector production is first identified and tagged. Due to the presence of two heterologous non-compatible flipase recombinase target (FRT) sites flanking the tagged retroviral genome, the subsequent re-use of this defined chromosomal site by means of recombinase-mediated cassette exchange (RMCE) is then performed to express a therapeutic gene (Fig. 2 B and C). In order to select cell clones that underwent correct targeted integration reaction, the targeting viral vector

contains a start codon that complements a transcriptionally inactive ATG-deficient selection marker 2 after recombination. An example of a modular producing cell line is 293 FLEX, developed by Coroadinha *et al* (2006) [9], and currently used in our group. This cell line contains a LacZ transgene tagging the chromosomal locus of high expression that can be exchanged through RMCE technology, MLV gag-pol packaging functions and Gibbon Ape Leukemia Virus (GaLV) ecotropic envelope. The modular producer cell lines present several advantages: they are safer since integration of the vector within the packaging cell line was identified, the duration of the entire development process is reduced as there is no need for screening and, in addition, production conditions are favorable due to the possibility of pre-adaptation of the master cell line to culture conditions and media. Thus, therapeutic virus production from bench to bedside becomes safer, faster, and cheaper [10].

The development o stable retroviral vector producer cell lines is a tedious and time consuming process. However, it is compensated by obtaining continuously producing and highly consistent cell systems, prone to single-effort bioprocess and product characterization, a critical consideration for market approval. Retroviral vector manufacture, including those used in clinical trials, has been making use of stable and continuous cell lines for more than 10 years [4].

From the manufacture point of view, retroviral vector production is characterized by relatively low titers, around 10^6 infectious particles per mL of culture medium [7,11]. Considering the average amount needed to treat a patient in a clinical trial, in the order of 10^{10} infectious vectors, around 10-100 L of culture volume can be predicted for each patient. Also, viral preparations are typically characterized by low ratios of infectious particles to total particles (around 1:100) which further reduces the therapeutic efficiency of the infectious ones [4]. Several strategies have been attempted to circumvent these limitations. Understanding the metabolic features that influence titer performances has been one important work field [4,10].

Serum supplementation is a major concern in RVs production; in one hand its use difficults product approval by the regulatory agencies quality requirements and increases product manufacturing and purification costs, on the other hand serum removal results in low titers and reduced vector stability. Overall, the major metabolic hinge between serum and high titers has been demonstrated to be the lipids and cellular lipid metabolism [12,13]. Glucose, the traditional sugar source used in animal cell cultures, is present in most of the commercially supplied media. Glucose, along with glutamine, represents the major energy and carbon sources for cells in culture. However, it is rapidly consumed and inefficiently metabolized, being the majority converted to lactate which *per se* inhibits the cell growth. The use of alternative sugar sources, such as fructose (alone or in combination with glucose), has a beneficial effect on vector production, that was correlated with an increase in the

intrinsic MLV retroviral vector stability which was associated with reduced sugar oxidative metabolism and increased lipid synthesis [14,15]. This observation was also correlated with an increase in the medium osmolarity, in which the addition of osmolytes or high sugar concentrations, was found to be a good approach to improve viral production [16].

In fact, considering that mammalian cells are severely affected by nutrient and by-products environment [10], medium manipulation and further metabolic engineering is a promising approach to achieve superior titers.

1.3 Metabolic engineering as a tool for cell line improvement

Since middle 80's, biotechnologists realized it was possible to change cellular phenotype towards improved yields of particular bioproducts targeting specific metabolic genes, by over-expression and/or down-regulation [17,18]. Since then, bacteria cell factories have seen their specific productivities increase by several orders of magnitude. Metabolic engineering in these simple organisms has become relatively straight forward and, with the appearance of genome-scale metabolic models in the end of the 90's, achieved high levels of predictability [19,20]. Contrarily to prokaryotic's, eukaryotic cells, particularly mammalian's, were found to be difficult to manipulate by gene metabolic engineering with similar levels of predictability, resulting in modest improvements in cellular phenotype and product yields.

Table 1 summarizes the main achievements in mammalian metabolic engineering. Targeting apoptosis and by-product formation have been the blockbusters of mammalian metabolism manipulation.

Table 1 – Examples of metabolic engineering targets in mammalian cells.

Metabolic Pathway	Genes	Cell Line / Product	Output	Ref.
Apoptosis	*E1B-19, Aven*	CHO / mAb	1.5 fold titer increase in bioreactors	21
	bcl 2	NS0 / mAb	1.4 increase in final antibody titer	22
	siRNA: Bax, Bak	CHO / IFN-γ	1.4 fold improvement in productivity	23
By-product Formation	*Ldh*	CHO / mAb	3 fold higher production (30% improvement in cell density and cell viability)	24
	Glut-5	CHO / mAb	Only clones with low expression of GLUT5 transporters exhibited reduced lactate production	25
	Pyc 2	HEK-293 / interferon-α2b	1.3 fold increase in product yield	26
Protein Processing and Secretion	*Pace*	CHO / Coagulation factor IX	3 fold improvement in the product specific activity	27
	Hsp70	NS0 / mAb	Improved stability of secreted product in culture supernatants	28
	PDI	CHO / IgG	1.3 fold increase in cell specific productivity	29
	CHOP	CHO / hTRA-8	Combined with UPR genes or chaperones enhanced antibody production in 3 fold	30
Redox Balance	*VKOR*	BHK / Coagulation factor IX	14-fold increase in carboxylation rate	31
	PDI, ERO1L	CHO / IgG	1.3 (ERO1L) and 1.5 (ERO1L and PDI) fold increase in specific productivity	32
Cell Cycle Control	*p21, p27, or p53175P*	CHO / SEAP	10-15-fold increase in specific production	33
	$p21^{CIP1}$	NS0 / IgG 4	4 fold higher production in induced cells	34
	mTOR	CHO / rituximab IgG	4 fold increase in antibody titers	35
Lipid Metabolism	*Hsd17b7*	NS0 / -	Cholesterol autotrophy	36
	CERT	CHO / HSA, IgG	1.5 fold increase in specific productivity	37
	XBP1(S)	CHO / SEAP, SAMY	SEAP - 6 fold; SAMY - 4fold production increases occurred at the post-translational level	38
Glycosylation Machinery	Sialidase anti sense RNA	CHO / DNase	1.3 fold increase in sialic acid content	39
	GnTIII	CHO / chimeric anti-CD20 IgG1 antibody	The killing efficiency of CD20-positive target cells was improved in 10-20 fold	40
	Bombyx mori 30Kc19	CHO / EPO	2.5-fold increase in productivity	41

mAb - monoclonal antibodies; IgG - human imunoglobulin G; hTRA-8 -humanized death receptor 5 antibody; UPR - unfolded protein response; SEAP - human model glycoprotein (human placental secreted alkaline phosphatase); SAMY - synthetic reporter protein (*Bacillus stearothermophilus*-derived α-amylase); ADCC - antibody dependent cellular cytotoxicity; EPO – Erythropoietin; *E1B-19K* - antiapoptotic adenoviral gene; *Aven*/ *bcl2* - anti-apoptosis genes; *Bax/ Bak* – pro-apoptotic genes; *Ldh* – lactate dehydrogenase; *Glut-5* - fructose transporter; *Slc2a5 gene*; *Pyc2* - yeast pyruvate carboxylase 2; *Pace* - paired basic amino acid cleaving enzyme; *Hsp70* - heat shock protein 70; *PDI* - protein disulfide isomerase; *CHOP* - transcription factor C/EBP homologous protein; *VKOR* - vitamin K oxidoreductase; *ERO1L* - essential oxidoreductase; *p21, p27, or p53175P* - tumor suppressor genes; *mTOR* - mammalian target of rapamycin; *Hsd17b7* - hydroxysteroid (17-beta) dehydrogenase 7; *CERT* - ceramide transfer protein ; *XBP1* - X-box-binding-protein 1; *GnTIII* - rat glycosylation enzyme β1–4-N-acetylglucosaminyltransferase III; *30Kc19* - 30Kc19 protein.

Lactate accumulation is a major concern in mammalian cell culture, since it adversely affects cell metabolism, growth and final product quality. This phenotype is connected to the Warburg effect-like scenario occurring in cultured mammalian cell lines and is associated with a high glucose consumption and accumulation of significant amounts of lactate leading to culture medium acidification [42]. The main cause of the lactogenic phenotype has been associated to the low activity of pyruvate dehydrogenase (PDH) leading to reduced channeling of pyruvate into the citric acid cycle (TCA) and consequently impairing energy metabolism. In 1999, a recombinant yeast pyruvate carboxylase 2 (PYC2) expressed in the cytoplasm of BHK-21 cells was shown to reconstitute the missing link between glycolysis and TCA [43]. Later, 2002, these metabolically engineered cells have been additionally transfected with a plasmid bearing the gene for human erythropoietin. PYC2-expressing clones showed a 2-fold higher product concentration in a continuously perfused bioreactor. These cells became more resistant to low glucose concentrations in the culture medium, which enabled a more prolonged production phase in bioreactors (80% cell viability, 2 days (30%) longer production time) [44]. CHO cells producing recombinant human granulocyte macrophage colony stimulating factor were as well target with PYC2. The PYC2-expressing cell clones showed a decreased cell growth, lower maximum cell concentration, only 65% lactate accumulation and the product yield was 3 fold higher when compared to the control [45].Wlaschin and Hu (2007) [25] evaluated the efficiency of the fructose-specific transporter (GLUT5)-transfected CHO cells for utilizing fructose as an alternative to glucose and its effectiveness on reducing lactate production. GLUT5-overexpressing clones could utilize fructose as a carbon source and sugar consumption was reduced even when cells were cultured in moderate concentrations of fructose. Only clones with low expression of GLUT5 transporters exhibited reduced lactate production and it was not correlated with production levels. Last year, the expression of Lactate dehydrogenase A (LDH-A) and PDH Kinase 1, 2, and 3 was down-regulated simultaneously using a short interference RNA reducing lactate level and increasing specific productivity and volumetric antibody production by approximately 90%, 75% and 68%, respectively [46]. In this approach, pyruvate conversion into lactate by LDH-A was suppressed and pyruvate dehydrogenase activity, which is inhibited when phosphorylated by PDH kinases, was restored.

Controlling cell death and extend cell life enables extending the production. In general, cell cultures are often terminated because of apoptotic cell death that may be caused by one of several factors including nutrient depletion, metabolic by-product accumulation, excessive shear forces or hypoxia [47]. Engineering apoptosis network by changing the expression of its regulators has shown great impact on culture viability and productivity. Genetic strategies involve the over-expression of anti-apoptotic genes or down-regulation of pro-apoptotic genes. The manipulation of the expression of the corresponding

proteins inhibits the release of pro-apoptotic molecules from the mitochondria and may prolong the viability of the cell [48,49]. *Bcl-2* over-expression constitutes the most common manipulation reported in the literature. The mechanism of action of Bcl-2 protein is broad. For instance, it can heterodimerize with *Bax* (death inducer), inactivating it. It also prevents the release of cytochrome c from the mitochondria, an important step in the apoptotic cascade which leads to the activation of the caspases (proteases which are largely responsible for the destruction of the cell) through the cytochrome c/ Apaf-1/ caspase 9 apoptosome or apoptosome independently [47]. *Bcl-2* was shown to prevent apoptosis in a number of commercially relevant cell lines, including CHO [31], NS0 [22], hybridoma [52,53], and BHK [51,54,55] , although, it failed to increase cell specific productivity [56]. Indeed, in the majority of apoptosis controlling strategies, specific cell productivity does not increase; instead, lifespan does, consequently enhancing production time and volumetric productivity [47].

Table 1 illustrates a striking problem in mammalian cell metabolic engineering: the productivity improvements are modest (in the order of 2-fold) and, most of the times, are based on increases in volumetric rather than specific (cell) productivity. Metabolism and metabolic networks are redundant [57] and the same metabolite production/consumption pattern can be generated by different combinations of reactions not conceptualized has a "pathway", in the classical sense. The implications for this are substantial as point manipulations are often absorbed by the network and "diluted" without obvious phenotype effects. The problem gains enormous relevance with the complexity, tight regulatory control and high level of cellular compartmentalization of mammalian cells. For instance, targeting the Warburg effect induced lactogenic phenotype spans across cytosol and mitochondria, involves several glycolytic and TCA enzymes and a transcription factor (HIF1) [58]. Manipulating mammalian cell metabolism towards a hyper producing phenotype may reveal to be a daunting challenge.

The datasets available so far suggest that hyperproductivity is conferred by a collection of many positive characteristics encompassing changes of many genes with a wide range of functions, rather than a transformation caused by a set of "master controllers" (Fig. 3) [59]. Although the pyramid represented in figure 3 is a general/theoretical concept, multi-gene engineering technology, enabling over-expression and/or silencing of combinatorial target genes has already proven its potential in the pursuit of hyperproductivity.

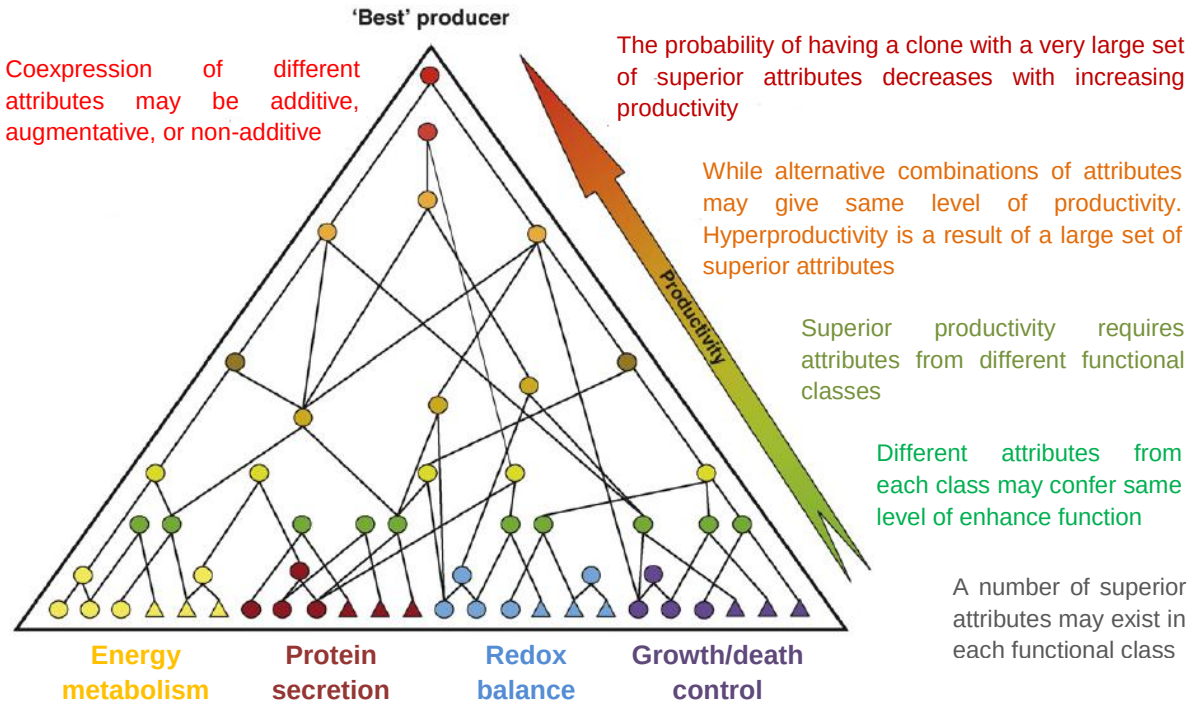

Figure 3 – Multiple routes to hyperproductivity. Hyperproductivity requires the accumulation of multiple superior characteristics. While the productivity is low, acquiring some elements from one or more favorable functional classes (only four potential classes are shown) will enhance the productivity. Thus, many different routes can give the same level of productivity. To achieve a higher productivity level a large number of attributes, arising from multiple favorable pathways may be needed. Hyperproducers are rare, as they must acquire many attributes. In addition, there are possibly multiple routes that lead to the top [59].

The most outstanding achievement was accomplished by Fussenegger *et al* [60], a productivity increase of 30-fold in CHO cells producing with a secreted alkaline phosphatase. This was achieved by co-expressing Bcl-xL survival gene with a cell cycle regulator gene, cyclin-dependent kinase inhibitor (p27), which enabled CHO cell-cycle arrest predominantly in G1 phase. Such cessation of proliferation at high cell density was shown to be extremely important to allow an extended period of high production.

Current strategies for metabolic engineering manipulation involve numerous, labor-intensive steps, from the introduction of the target genes to the isolation and characterization of candidate clones. For a single gene, the process takes several months and requires screening of several hundreds of clones [59]. To change the paradigm of mammalian cell metabolic engineering from single to multiple gene manipulation it is crucial to accelerate clone screening time.

1.4 Titration methods for viral particles quantification

Viral titration – quantification of total and/or infectious particles – is crucial in virus based biopharmaceuticals production as the balance between the therapeutic and adverse effects of using viral particles lays on precise measurements of particles (total and infectious) content. Additionally it is an essential step of packaging cell line development to assess the titer and the transduction efficiency of the generated vector preparation allowing the selection of the best producing clones. Two types of titration methods are considered: functional and non-functional [61,62].

The non-functional titration assays rely on the quantification of constitutive viral proteins (such as the HIV-1 capsid protein p24 by immunological titration or by determining reverse transcriptase activity) or on the assessment of viral genome (RNA or DNA) in the cell culture supernatant [63]. In general, non-functional assays do not reflect infectious viral particles content produced by the cells due to the presence of defective interfering particles, transduction inhibitors, and contaminant genomic DNA from vector production in the preparation. Additionally, genome quantification is also not the most accurate method for viral quantification, when viral production involves a transient transfection, supernatant may be contaminated with plasmid DNA over estimating the titers [64].

Functional titration methods involve the transduction of a target cell and further quantification of vector reporter gene expression or its integration in transduced cells [63,64]. Reporter genes can be a selectable marker, fluorescent proteins or enzymes and titer is assessed by resistant colonies after selection, flow cytometry or fluorometer assays and enzymatic activity quantification, respectively.

The establishment of a producer cell line is a time consuming process not only due to the time spent in the insertion of viral expression cassettes, allowing the cells to produce the viral particles, selection of the producer cell population, but also, and mainly, for the time spent in screening the high titer producer clones [61]. The same limitations arise for a metabolically engineered population. Hyper-producing phenotypes are rare events, even when the manipulation reveals to be beneficial, as they rely on tight expression levels of the targeted genes, dependent on the number of inserted copies as well as their chromosomal integration site. The problem grows exponentially for multiple gene manipulation. In order to reduce the time needed for this process it is necessary to develop fast, and with high throughput capacity, titration methods.

Implementing screening schemes with high throughput potential to accurately assess growth and productivity at earlier stages, ideally during cloning procedure, would certainly accelerate the process.

In 2002 Green and Rasko developed a “Rapid Screening for High-Titer Retroviral Packaging Cell Lines Using an *In Situ* Fluorescence Assay” [61] involving the detection of high-titer producer clones containing fluorescent reporter genes (eGFP, eCFP and eYFP). The method consisted in removing the clone supernatant at early cloning stage (96 well plate), and transfer it to infect target cells also in 96 well plate. Upon transduction, target cells express the fluorescent protein that was detected in multichannel fluorescent reader. The correlation of cell fluorescence between the fluorescent plate reader assay and flow cytometric assessment was high (r^2 = 0.96). Simultaneous cell density analysis using alamarBlue fluorescence was proportional to cell number per well (r^2 = 1.0) [61]. The greatest contribute of this method was the ability to select rare high producing clones (up to 7 fold) by adding a screening step at cloning stage, which allowed reducing considerably the time spent in clone analysis.

2. Aim and strategy

The aim of this master thesis project was to target metabolic pathways of retrovirus producing cells to improve product yields. These pathways were chosen based on previous performed transcriptional profiling analysis which identified relevant networks recruited when establishing a retrovirus producer cell line (Fig. 4). In this work we aimed at validating transcriptional profiling results and implementing a novel titration method allowing for a fast and high-throughput screening of combinatorial gene metabolic engineering embracing the genes highlighted as potential targeted candidates.

This work is, then, divided in two parts. In the first part the metabolic pathways identified by transcriptional profiling were validated by media manipulation. Viral productivity response was quantified in culture medium containing one or a combination of supplements.

In the second part a single step cloning-titration method was implemented for high throughput screening of high-producing clones (Fig. 5). This method takes advantage of a transcomplementation assay based on the split-Green Fluorescent Protein (split-GFP). A packaging cell population expressing a transgene encoding for the GFP S11 fragment is co-cultured with a target cell line harboring the GFP 1- 10 fragment. The viral vectors produced by the clone containing the GFP S11 fragment infect the target cells present in the well reconstituting a fluorescent GFP protein. Only the clones yielding highest GFP signal will be isolated for posterior studies. To give high throughput potential, the method was established using 96 well plates analyzable by a fluorometer plate reader (Fig. 5).

After method implementation and validation it will be used to isolate high-producing clone from metabolically engineered cells with combinatorial target genes (a preliminary study was already conducted for this thesis).

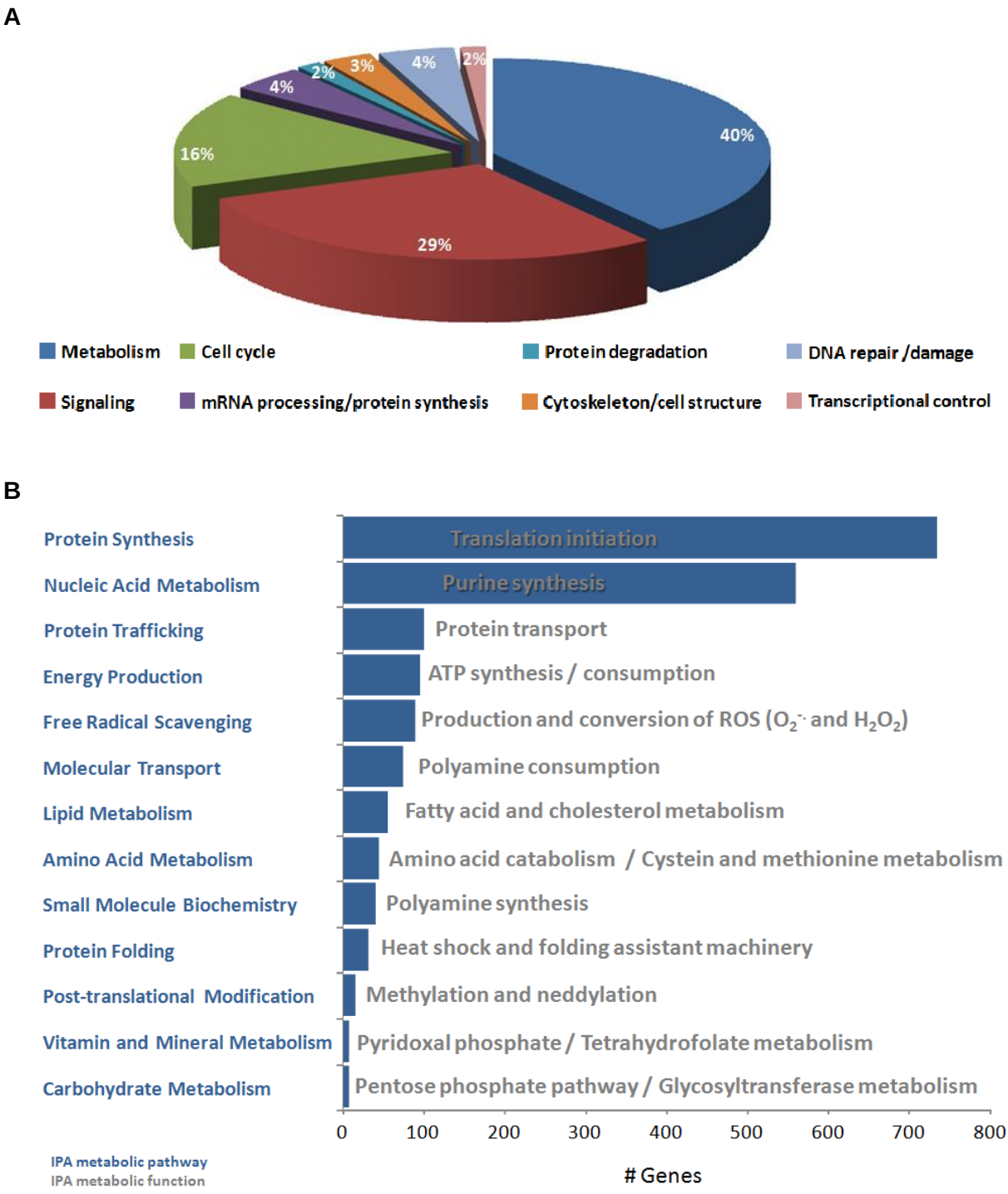

Figure 4 – Transcriptional profiling data of a RV producer cell line compared to non producer. A) Pathway category distribution; B) Function and number of genes per metabolic pathway. From [65].

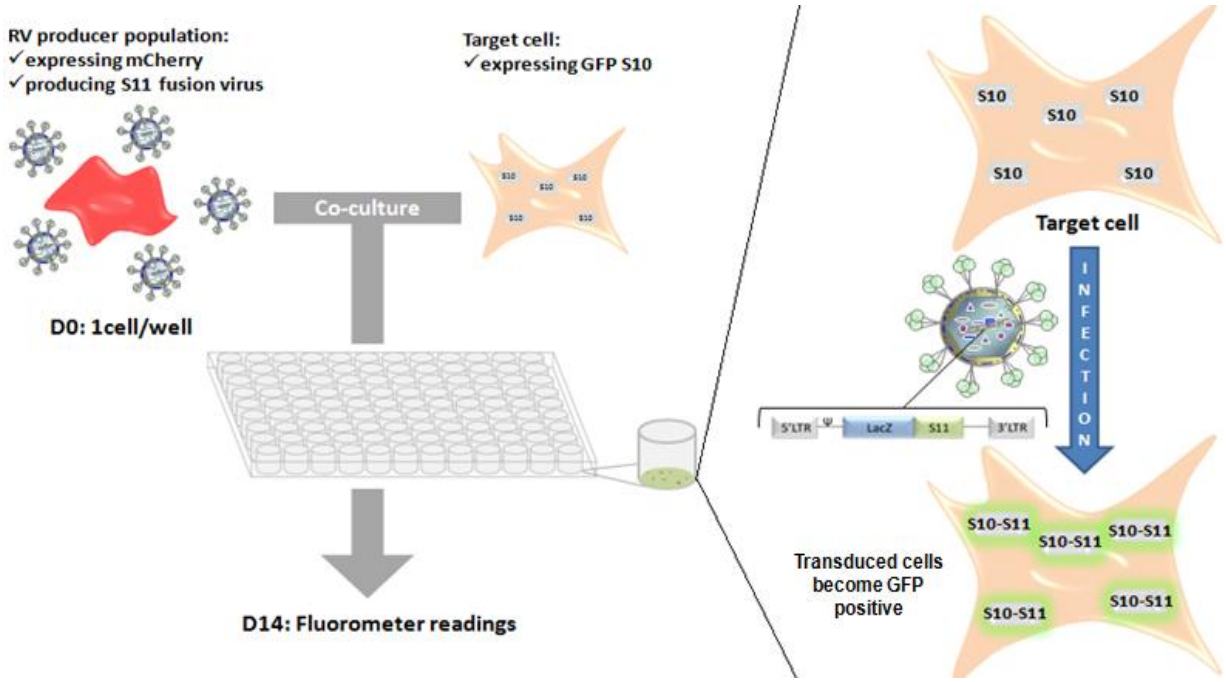

Figure 5 – Schematic representation of single step cloning-titration protocol. Clones from metabolically engineered cell population producing virus harboring GFP S11 transgene are co-cultured with GFP S10 transcomplementing cells. During the expansion period (14 days) target (transcomplementing) cells are infected with the produced viruses yielding (reconstituting) GFP signal. High producing clones originate the highest GFP signal; mCherry signal (from producer clone) allow for cell growth normalization providing an estimate of specific productivity.

3. Materials and methods

3.1. Plasmids

For all the vectors constructed in this work the cloning sites, primers and templates are listed in Table S1. A schematic representation can be found in Fig. S1. Underlined plasmids indicate vectors constructed in this work.

pCMV mGFP S10 and pCMV mGFP S11 contain a mammalian codon-optimized version of the GFP under the control of a CMV promotor, split in *GFP S10* coding for the first 214 amino-acids, and *GFP S11* for the last 15, respectively. These plasmids were acquired trough SandiaBiotech (Albuquerque, NM, U.S.A) and were used for several constructions further explained below.

pIRESGALEO is a murine stem cell virus (MSCV) based retroviral vector where *LacZ* is under the control of a LTR promoter and the fusion protein gene *hygromycin B phosphotransferase/thymidine kinase* (*hygtk*) is under the control of the encephalomyocaerditis virus-internal ribosome entry site (EMCV-IRES). This vector contains two FRT sites in the U3 of 3'LTR, a wild type FRT site (*wt*) and a spacer mutant FRT site followed by an ATG defective neomycin phosphotransferase gene (*neo*) [66].

pEmMFG is a recombinase mediated cassette exchange targeting vector containing a FRT *wt*, an MLV based retroviral vector MFG-LTR that drives the *eGFP* gene followed by an EMCV-IRES element next to an ATG and F5 FRT site. ATG sequence and the F5 mutant FRT site complementing the *neo* gene in pIRESGALEO after targeting and subsequent cassette exchange [9].

pCMV mGFP LacZS11 codes for a fusion protein LacZ-S11 under the control of a CMV promoter and was obtained by cloning *LacZ* gene (from pIRESGALEO) into the multiple cloning site (MCS) of pCMV mGFP S11. *LacZ* was amplified by PCR and introduced into the MCS at AgeI site.

pTAR LacS11 is a RMCE targeting vector derived from the pEmMFG backbone in which *eGFP* was replaced by *LacZ-S11* obtained from pCMV mGFP LacZS11. Both retroviral backbone and *LacZ-S11* were amplified by PCR, using primers with NotI recognition site.

pRRLsin LacZS11 and *pRRLsin S10* were derived from pRRLsin eGFP backbone, kindly provided by Didier Trono from the Swiss Federal Institute of Technology (EPFL) through Addgene plasmid repository (Cambridge, MA, USA). This backbone is a third generation lentiviral transgene vector, driving the expression of *eGFP* from the hPGK promotor (human phosphoglycerate kinase) as described in Dull *et al* (1998) [67]. For pRRLsin LacZS11 and pRRLsin S10, *eGFP* was replaced by *LacZ-S11* or *GFP S10* obtained from pCMV mGFP LacZS11 or pCMV mGFP S10, respectively, isolated by PCR with primers carrying the NheI restriction site. pRRLSin backbone was amplified in two fragments by PCR, with primers carrying NheI and PciI restriction sites. The cloning step involved three fragments: two fragments corresponding to the vector (PcII cloning site) and the insert (NheI cloning site).

*Lentiviral vectors for metabolic engineering***,** the lentiviral backbone was isolated from pRRLsin LacZS11, removing *LacZ* by restriction with AgeI. Each metabolic gene (isocitrate dehydrogenase 1 and 2, glutathione synthase and inhibitor of hypoxia inducible factor 1) was amplified by PCR from the original plasmids obtained from DNASU Plasmid Repository (Biodesign Institute, Arizona State University) and cloned into AgeI site. Mock vector was obtained by religating lentiviral backbone after *LacZ* removal.

pPuro mCherry was derived from pSelect Puro (Invivogen, San Diego, CA, U.S.A.) that contains a multiple cloning site (MCS) downstream of the EF1 promoter and a puromycin selectable marker. The construction contains a *mCherry* gene, amplified by PCR from pRSET B (described in Shaner *et al* (2004) [68]) cloned into the MCS with NheI.

pSVFLPe contains FLP recombinase gene under a SV40 promoter (a kind gift of Dr F. Stewart).

pCeB is a vector containing MLV gag-pol and a blasticidin resistance gene (*bsr*) both driven by the MLV 5'LTR as described in Cosset *et al* (1995) [69].

pGaLV expresses the GaLV envelope protein and a zeocin (*Zeo*) resistance marker under the control of a CMV and kindly provided by Dr. Otto Merten (Genethon, Department of Bioprocess Development, France) .

pMD$_2$G expresses the envelope G glycoprotein of the vesicular stomatitis vírus (VSV G) under the control of CMV promotor (kindly provided by D. Trono through Addgene).

pMDLg/pRRE is 3rd generation LV packaging plasmid codifing for gag - virion main structural proteins - pol, responsible for the retrovirus-specific enzymes; and RRE, a binding site for the Rev protein which facilitates export of the RNA from the nucleus (kindly provided by D. Trono through Addgene).

pRSV-REV is a 3rd generation LV packaging plasmid containing the second and third exons of HIV-1 rev under the transcriptional control of RSV U3 promoter (kindly provided by D. Trono through Addgene).

3.2 Cell Lines and culture media

HEK 293 is a Human Embryonic Kidney derived cell line (ATCC CRL-1573) and was used to produce conditioned medium for limiting dilution of all HEK 293 derived cell lines.

Te671 is a Human rhabdomyosarcoma derived cell line (ATCC CCL-136) used to develop S10 Target cell line, and as target cells to titrate the infectious RV particles. These cells were additionally used to produce conditioned medium for limiting dilution of all Te671 derived cell lines.

HEK 293T is a HEK 293 derived cell line expressing large T antigen from SV40 (simian vacuolating virus 40) used to produce LV vectors in a transient manner and to test the newly generated plasmids functionality.

293 FLEX cell line is a HEK 293 derived cell line producing MLV based recombinant retroviral vectors expressing GaLV ecotropic envelope and harboring a LacZ reporter gene [9].

293 CEB 22 is a HEK 293 derived cell line, the precursor cell line of 293 FLEX prior to envelope insertion as described in Coroadinha *et al* (2006) [9]. These cells produce envelope-free MLV-derived virions harboring a *LacZ* transgene. 293 CEB 22 and 293 FLEX were used to establish retroviral vector producer cells producing retroviral vectors harboring a *LacZ-S11* transgene by RMCE.

293 FLEX GFP cell line is derived from 293 FLEX 8 as described in [9] and was used for the production of GFP expressing RV.

DK cell line (E1-transcomplementing dog kidney cells), provided by Dr. Eric Kremer (Institut Génétique Moléculaire Montpellier, France), were used for fluorometer tests involving mCherry signal.

All cells were maintained in Dulbecco's modified Eagle's medium, DMEM, (Gibco, Paisley, UK) with 25 mM of glucose, 4 mM of glutamine, supplemented with 10% (v/v) Foetal Bovine Serum (FBS) (Gibco). In the case of fluorometer assays, DMEM without phenol red was used. For supplemented medium studies, Advanced DMEM with 25 mM of glucose supplemented with 4 mM of glutamine and 10% (v/v) Foetal Bovine Serum (FBS) (Gibco)

was used. All cells were cultured under adherent conditions (T-flask) (Starstedt, Nümbrecht-Rommelsdorf, Germany) at 37 ◦C in a humidified atmosphere containing 7.5% CO_2.

3.3 Bacterial strains

Escherichia coli (*E. coli*) Library Efficiency® DH5α™ and One Shot® Stbl3™ (Invitrogen, Carlsbad, CA, U.S.A) competent cells were used for the production of the DNA plasmids. Transformation procedures were carried under manufacturer instructions.

The liquid bacterial cultures were performed with *Terrific Broth* media (TB) (Fast-Media® TB from Invivogen) supplemented with the appropriated antibiotic (Ampicilin, Kanamycin or Puromycin). The media was prepared using ultrapure water (Millipore, Billerica, MA, U.S.A.), according to the manufacturer's instructions.

3.4 Cloning procedures

All PCR reactions were performed with proof-reading *Phusion* High-Fidelity DNA Polymerase (Finnzymes OY, Espoo, Finland), using the following conditions: initial denaturation step (30 seconds at 98ºC), followed by 30 cycles of denaturation (10 seconds at 98ºC), annealing (30 seconds at temperature of melting (Tm)) and elongation (72ºC – Time according fragment lenght) and lastly by a final elongation step (6 minutes at 72ºC). The oligonucleotides used for PCR were custom-made by Sigma Aldrich (St.Louis, MO, U.S.A). Restriction reactions were incubated 2-4 hours (h) at 37°C. The enzymes in Table S1 (New England Biolabs, Ipswich, MA, U.S.A) were used with the appropriate buffers and supplements according to the manufacturer instructions. All the generated fragments were isolated by agarose gels (Lonza, Basel, Switzerland) 0.7% (w/v) and subsequently purified with illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). Ligation reactions were performed using T4 DNA ligase (New England Biolabs) and incubated overnight following manufacturer's instructions.

3.5 Plasmid purification and quality control

Plasmid purification was performed in different scales. Small-scale purification (yields around 20 µg of DNA) was performed with QIAprep® Miniprep (Qiagen, Hilden, Germany). A larger scale purification (yields around 500 µg of DNA) was performed using Genopure Plasmid Maxi Kit (Roche, Basel, Switzerland) following manufacturer's instructions. Working DNA banks for each plasmid were generated and stored at -20 ºC.

The DNA concentration was determined in a spectophotometer (*Nanodrop 2000C Spectophotometer,* Thermo Scientific, USA). Plasmid purity was determined by the Abs_{260nm}/Abs_{280nm} ratio and plasmid integrity (with and without enzymatic restriction)

assessed in 0.7% (w/v) agarose gels (Lonza, Basel, Switzerland) prepared in TE buffer. All plasmids were sequenced using Macrogen Europe services (Amsterdam, The Netherland).

All generated plasmids were tested for their functionality transfecting HEK 293 T cells (described in section 3.8).

3.6 Determination of cell concentration and viability

Cell concentration and viability were determined by the trypan blue exclusion method using a 0.1% (v/v) solution prepared in Phosphate Buffer Saline (PBS, Gibco) and counting cells in a Fuchs-Rosenthal hemacytometer (Brand, Wertheim, Germany). Each counting was performed at least twice.

3.7 Cloning by limiting dilution

For clone isolation, serial dilutions were preformed to achieve 0.5 cells per well in 96 well plates. Limiting dilution medium was composed by 50% conditioned (parental cells supernatant) and 50% fresh medium, 20% FBS and half concentration of antibiotic (specific for each cell). At day 14, wells with single colonies were trypsinized and amplified for cell banking and posterior analyzis.

3.8 Cell transfection

For transfection procedures cells were seeded in 6-well plates (Nunc, Rocherster, NY, U.S.A) at $5x10^4$ cell/cm^2. 24 hours later transfection was carried using polyethylenimine (PEI, Linear 25 kDa from Polysciences, Eppelheim, Germany) at 1:3 (m/m) ratio of DNA:PEI) [8] or by calcium phosphate precipitation method. 5 µg of DNA per $1x10^6$ cell were used. PEI transfection solution was prepared in fresh serum-free-media

The calcium phosphate precipitation method (CAPHOS SIGMA - Calcium Phosphate Transfection Kit, Sigma) was executed according to manufacturer instruction and was only used for RMCE.

3.9 Viral vectors production and titration

3.9.1 Production

For lentiviral transient production a third generation of lentiviral system was used [67]. HEK 293T cells were seeded at $8x10^4$ cell/cm^2. PEI transfection was carried out 24 hours later as described above with a mixture of: pREV and pMDLG RRE (providing the packaging functions), pMD_2G (for the envelope) and pRRLsin derived vectors (section 3.1) providing the transfer vector (transgene). The DNA ratio used was 1:4:3.6:10 [67]. Except for transfer vectors, all plasmids were kindly provided by D. Trono through Addgene (Cambridge, MA,

U.S.A). After 24 hours, the medium was replaced with 2/3 of the original volume to concentrate lentiviral particles stock. The medium containing the viral vectors was collected after an additional 24 hours production period.

RV particles harboring a *LacZ-S11* reporter gene were obtained by transient production following an identical procedure described above using pCEB to provide the packaging functions, pMD_2g for the envelope and pTAR LacS11 for the transfer vector (transgene). The DNA ratio used was 2:1:3.

Retroviral vectors with GFP transgene were obtained by stable production using 293 FLEX GFP cells.

All viral vectors supernatant were filtered through 0.45 µm cellulose acetate filter for clarification, aliquoted and stored at -80ºC.

3.9.1.1 Retroviral vector production in supplemented culture medium

For retroviral vector production in supplemented medium a 48+24 hours production protocol, with medium exchange after the first 48 hours, was used (Fig. 6). 293 FLEX cells were inoculated at $8x10^4$ cells/cm^2 in media containing one or a combination of the following supplements: i) a amino acid solution (Sigma); ii) a nucleoside supplement (Millipore, Billerica, MA, U.S.A); iii) an anti-oxidant supplement (Sigma); iv) a polyamine supplement (Sigma); v) reduced glutathione (Sigma). A non-supplemented control was also prepared. A detailed schematic representation of this experimental design, final concentrations and combinations of supplements used can be found in Fig. 6 and supplementary material (Table S2). Three biological replicates were used (corresponding to three independent freezing batches), each of them prepared in four technical replicates. At 30 hours post-seeding, total cellular RNA was extracted from the first technical replicate. At 48 hours, culture supernatant of the second technical replicate was harvested, filtered through 0.45 µm for clarification, aliquoted and stored at -80ºC until analysis. Cell concentration and viability was determined by the trypan blue exclusion method; for the third and fourth technical replicate, medium was exchanged. After an additional 24 hour period of production, the same procedure described above was applied to the third technical replicate. The fourth technical replicate was used to assess retroviral vector decay kinetics.

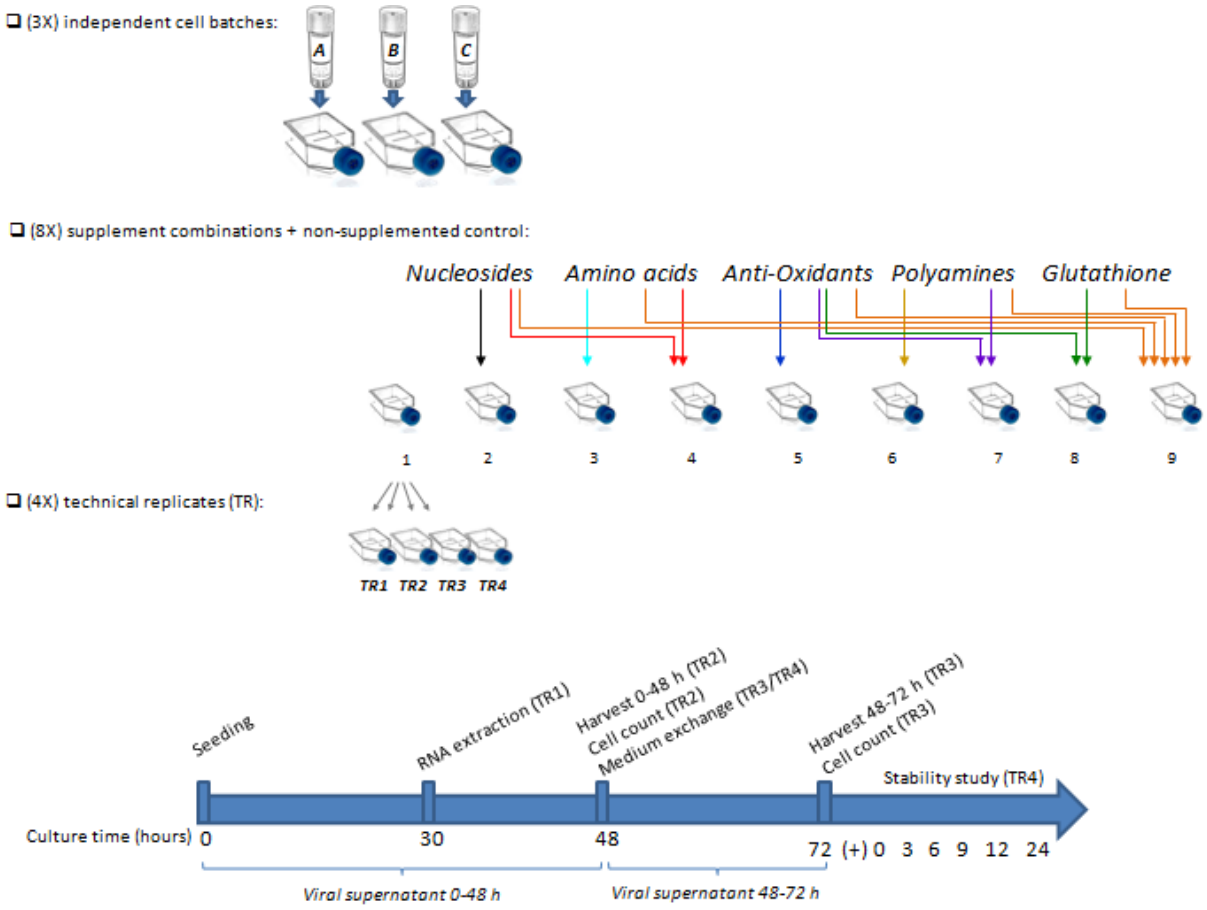

Figure 6 – Experimental design of retroviral vector production in supplemented medium.

3.9.2 Titration of infectious particles

For LacZ containing virus, Te671 cells were seeded at $5x10^4$ cells/cm^2 in 96 well plates and incubated overnight. After 24 hours, the supernatant was removed and cells were infected with 50 µl of viral suspension at several dilutions performed in DMEM 10% (v/v) FBS containing 8 µg/ml of polybrene. After 4 hours incubation at 37 °C to promote virus absorption, 150 µl of fresh medium were added to each well. Two days after infection, cells were fixed. For that purpose, the culture supernatant was removed and cells were washed with PBS and incubated with formaldehyde at 0.3% (v/v) and glutaraldehyde at 1.35% (v/v) in PBS for 5 min. After a washing step with PBS, staining was carried out using a solution of 0.2 mg/ml x-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Stratagene, La Jolla, USA), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)6$, and 1 mM $MgCl_2$ in PBS. The viral titer was determined by counting the stained blue cells using a phase contrast inverted microscope and multiplying by the dilution factor and volume of supernatant.

For GFP transgene vectors titration, Te 671 cells were seeded at $5x10^4$ cells/cm^2 in 24 well plates one day before infection. Cell concentration was determined at the time of infection. Transduction was performed in duplicates by removing the cell supernatant and infecting with 0.3 mL of viral suspension using several dilutions in fresh DMEM with 10% (v/v) FBS and 8 µg/ml of polyberen. Cells were incubated at 37ºC overnight after which 1 ml of fresh supplemented DMEM was added. Two days after infection, cells were harvested and analyzed for GFP fluorescence by Flow Cytometry (CyFlow-space, Partec GmbH).

Titration of virus containing split GFP reporter proteins was carried out has described above but with double infection steps and lower cell inoculums. The cells were seeded in 24 well plates at the density of $2x10^4$ cell/cm^2. The day after, first transduction was performed with 500µl of LV supernatant carrying the *LacZ-S11* transgene (saturating multiplicity of infection (MOI)). After 4 hours, viral adsorption time, supernatant was replaced by 1 ml of fresh medium. Second transduction was done 24 hours after with 300µl of serially diluted viruses of viral supernatant carrying the transcomplementing transgene, *GFP S10*. At this time point, cell concentration was determined and 48 hours after cells were analyzed for GFP transcomplementation signal. This double infection protocol was only used while the Target (GFP S10 expressing) cell line was not established. Upon the establish of Target cell line, viral particles harboring *LacZ-S11* transgene were titrated using Te 671 S10 cells in a single infection protocol.

The GFP titer was determined taking into account the percentage of GFP positive cells, the cell concentration determined at infection time and the dilution factor. Infections that rendered 2-20% of infected cells were considered for titer calculations. Viral titers obtained by flow cytometry method, defined as Infectious particles per mililiter (IP/mL), were calculated using the following formula:

$$\mathrm{IP/ml} = \frac{\%\text{GFP positive cells x number of transduced cells x dilution factor}}{100 \text{ x transduced volume (ml)}}$$

3.9.3 Titration of genome containing particles

Genome containing particles were titrated using a protocol implemented in our laboratory and described in Carmo *et al* (2004) [70] with the following modifications: the primers used for cDNA synthesis were LacZ reverse and GFP reverse, in Table S3.

The primers used for PCR were LacZ (forward and reverse), given in Table S3.

3.9.4 Retroviral vector decay kinetics

To evaluate vector decay kinetic, the 24 hours viral vector productions from supplemented cultures was used. Culture supernatant was harvested and filtered through a 0.45 µm filter. Part of this supernatant was 1:10 diluted in Advanced DMEM with 10% (v/v) FBS (neutral medium) and the remaining was not diluted. The viral suspensions were then incubated at 37 °C; samples were taken at 0, 3, 6, 9, 12, and 24 hours and stored at −80 °C for viral titer determination. Decay rate constants and half-lives were calculated according to a first order exponential decay model [71].

3.10 Cell line development

3.10.1 Target cell line

The Target cell line was developed by stable expression of GFP S10 gene into Te671 using LV infection. Since the transgene of LV S10 does not contain a selectable marker, a MOI of 30 – to assure synchronous infection and multiple copies per cell – was used. Cells were cloned by limiting dilution and clones were analyzed for GFP transcomplementation after infection with retrovirus and lentivirus harboring LacZS11 transgene.

3.10.2 Cassette exchange

For site-specific recombinase mediated cassette exchange tagged 293 FLEX and 293 CEB 22 cell lines were seeded at $5x10^4$ cells/cm^2 in 6 well plates and co-transfected in the next day using calcium phosphate precipitation method with 4 µg of targeting plasmid, pTAR LacS11, and 12 µg of pSVFlpe recombinase expressing plasmid. 48 hours post-transfection, Neomycin selection was started with G418 (1000 µg/ml; Invivogen). Medium was regularly exchange during 21 days after which limiting dilution method was applied, in the presence of ganciclovir (5 µg/ml; Invivogen), to eliminate non-exchanged clones. The clones were amplified and analysed for cassete exchange and number of pTAR LacS11 copies integrated.

To evaluate cassette exchange efficiency and number of targeting copies integrated into the cell genome, genomic DNA was isolated using the "QIAamp® DNA Mini and Blood Mini Kit" (Qiagen, Valencia, CA, USA) according to manufacturer's instructions.

PCR with specific primers for *LacZ* and N*eo* gene (Table S3) was performed and PCR amplification products were separated in agarose gel. Depending on the PCR product size it was possible to confirm if cassette exchange was successful (Fig. S2 and Table S4).

To estimate the number of copies per cell, genomic DNA was quantified by qRT-PCR using primers for LacZ gene (Table S3) on a thermocycler "LightCycler ® 480 Real-Time

PCR System" (Roche Applied Science) using "LightCycler® 480 SYBR Green I Master" (Roche Applied Science) PCR kit. The number of copies per cell was quantified relatively to a single copy control (293 FLEX) after normalization to a control gene (RPL22) using $2^{-\Delta\Delta Ct}$ method [72,73]. Single copy was considered for values below 1.4 [73].

3.10.3 mCherry and GaLV insertion

293 CEB 22 S11 clone 32 was chosen to insert mCherry by PEI transfection (described in section 3.8). After 15 days of Puromycin selection (Invivogen), cells were sorted (MoFlo High-Speed Cell Sorter, Dako Cytomation, Glostrup, Denmark) to isolate a population of cells within a narrow range of mCherry intensity of expression (clones in the same range were additionally isolated). The sorted mCherry population was transfected (PEI) with phGaLV. After 15 days of Zeocin selection (Invivogen), this GaLV population was used for protocol validation.

3.11 RNA extraction and gene expression quantification by Real-Time PCR

To analyze viral components expression in supplemented medium studies total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer instructions.

For real time PCR, RPL-22 was chosen as a control gene. Forward (F) and reverse (R) primer sequences for control and virus component genes are given in Table S3. The reverse transcription of total RNA was performed according to Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science, Mannheim, Germany) protocol for cDNA synthesis using 2 µg of total RNA and oligo dT primer for total mRNA reverse transcription. The reverse transcribed (RT) product was aliquoted and stored at -20ºC until further processing. SYBR Green I dye chemistry was used to detect the PCR products using LightCycler® 480 SYBR Green I Master (Roche Applied Science) according to the manufacturer instructions using LightCycler® 480 Real Time PCR System (Roche Applied Science). cDNA samples were run along with a no RT-reaction control and a no cDNA template sample. Three independent biological replicates were analyzed, each of them run in three (technical) replicates.

3.12 Single-step cloning-titration method implementation and validation

3.12.1 Plate manufacture and culture conditions

In order to establish the best culture conditions for the co-culture, different black-wallet 96 well plates with clear bottom from three manufactures were tested (Corning, New

York, NY, USA/ Nunc/ Perkin Elmer, Waltham, MA, USA). In this assays, DMEM without phenol red was used.

3.12.2 Fluorescence signal detection and calibration curves

For fluorescence signal detection and calibration curves Te671 cells were infected with serial dilutions of retrovirus codifying for GFP. Cells were seeded at 5×10^4 cells/cm^2 in the three 96 well plates with clear bottom mentioned above and incubated overnight. After 24 hours, the supernatant was removed and cells were infected overnight with 100 µl of viral suspension at multiplicity of infection (MOI) ranging from 5 to 0. 48 hours post-infection medium was replaced by 50 µl of PBS 5% (v/v) FBS to avoid fluorescence signal interference. Fluorescence reading was performed using plate fluorometer reader FLx800 (Biotek, Vermont, USA) using the 485/20 emission filter and 528/20 absortion filters (Biotek).

For red fluorescence signal detection and calibration curves DK cells were infected with serial dilutions of mCherry CAV (canine adenoviral vectors), kindly provided by Paulo Fernandes (ACT Unit IBET/ITQB, Oeiras, Portugal). The signal was detected 24 hours post-infection using the 560/20 emission filter and 645/40 absortion filter (Biotek), readings were performed as described above.

3.12.3 Method validation

For the method validation 293 CEB 22 S11 Cherry sorted population with GaLV was submitted to a limiting dilution (Corning plate) and 6 days after Target cell line was added at 500 cell/well. Until the last day of the cloning protocol (day 14) three infection rounds were performed by the addition of polyberen (final concentration of 10 µg/ml) proceeded by 4 hours of agitation, with 48 hours interval in between.

At day 14, GFP and mCherry signal was quantified using a fluorometer reader as previously described (section 3.12.2). Wells with the highest GFP/mCherry ratio were trypsinized and clones were amplified and isolated from Target cell line by Puromycin selection.

4. Results

Part I – Experimental validation of transcriptional profiling by culture medium manipulation

In the first part of this work, metabolic pathways recruited for virus production previously identified by microarray analysis [65] – comparing producer cell lines with their corresponding parental cells – were evaluated as targets to improve vector production. From the highlighted pathways (Fig. 4), were selected those that could be manipulated by medium supplementation strategies with minimum off-target or growth inhibition effects. Within this aim, the pathways from Fig. 4 B can be divided in three categories: easy to manipulate, difficult and intermediate/mixed.

Easy: primary building blocks consumption pathways. These pathways are relatively easy to target by culture medium manipulation, simply by the addition of the consumed molecules. They include *Nucleic Acid Metabolism*, *Amino Acid Metabolism, Free Radical Scavenging, Polyamine Metabolism, Vitamin and Mineral Metabolism and Lipid Metabolism*. These categories were chosen for culture medium manipulation, containing one or a combination of the following supplements: amino acid solution, nucleoside supplement, anti-oxidant supplement, polyamine supplement and reduced glutathione.

Difficult: pathways of assembly and modification of intracellularly synthesized molecules. These pathways are very difficult to target by culture medium supplementation as they rely on intracellularly synthesized molecules, many non-transportable through the cell membrane, and specific enzyme modification machinery. These included *Protein Trafficking, Protein Folding and Post-translational Modification*. These pathways can be targeted by the addition of chemical inhibitors and activators. However, this typically results in a myriad of off-target effects, very difficult to control and often associated to cell growth inhibition.

Intermediate/mixed: pathways that share “easy” and “difficult” features. They include: *Pentose Phosphate Pathway* since it proceeds through phosphorylated intermediates – cell membrane impermeable and thus, not susceptible of addition to the culture medium – *Protein Synthesis,* and *Energy Production*. They can, however, be indirectly targeted by medium supplementation through their precedent “building blocks consumption pathways” although the interpretation of the results is not straightforward. Thus, amino acid supplementation also targets *Energy Production,* as these are major substrates to feed TCA cycle reactions, and *Protein Synthesis* as increased protein synthesis necessarily requires amino acid consumption. Reduced glutathione supplementation targets *Pentose Phosphate Pathway*, since during its oxidation $NADP^+$ is produced, pushing G6PD (glucose-6-

phosphate dehydrogenase) activity from glucose 6-phosphate to 6-phospho-glucono-δ-lactone. This is the first and rate-limiting step of *Pentose Phosphate Pathway* and G6PD activity is known to be controlled by the NADP+/NADPH ratio [74].

To evaluate the effects on viral production in response to culture medium manipulation, infectious particles, total particles, vector infectivity kinetics and viral component gene expression were assessed (Fig. 7 and Fig. 8). Infectious vector production was improved 1.3 to 2.1 fold upon all medium supplementation strategies (Fig. 7 A), reaching a synergistic effect with their combination of 6 fold increase. Vector preparation quality, infectious particles per total particles content, was also improved with no significant changes in vector components expression (Fig. 7 A and B respectively).

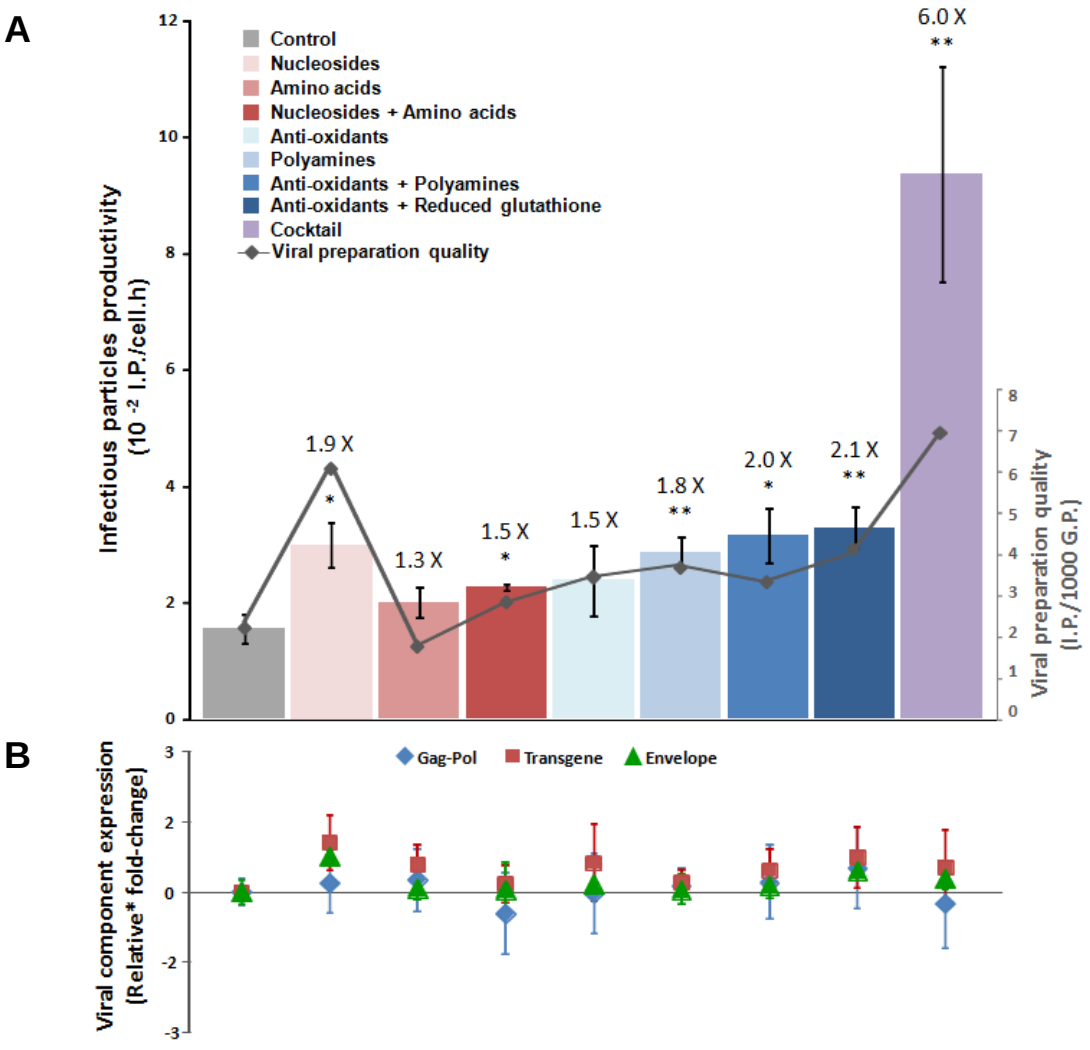

Figure 7 – Effect of culture medium supplementation in viral production of 293 FLEX. A) Viral particles specific productivity. Values are shown as fold change relatively to the control (non supplemented medium). Error bars represent standard deviation of 3 biological replicates, * p<0,05 and ** p<0,005 given by a non-paired two-tailed t-test. The grey line (right y-axes) specifies the amount of infectious particles per 1000 total particles. B) Quantification of viral gene expression, Gag-pol, transgene and envelope, by qRT-PCR; horizontal axe shares the order of conditions represented in A).

Retroviral infectivity is rapidly lost at culture temperature (37ºC), thus, increases in vector stability have been found to be one of the main reasons leading to improve infectious titers [75,76]. As so, vector decay kinetics was analyzed to evaluate if the increased IP productivity was consequence of increased vector stability (Fig. 8). Medium manipulation did not improve inherent vector stability, revealed by the identical values in viral decay in neutral media among the different conditions. Cocktail medium increased vectors half-life in 2 hours. However, this stabilization effect cannot be responsible for the high titers, 6 fold improvement, in the cocktail condition since 3 hours stabilization effect also occurred for nucleoside supplemented medium with only 1.9 fold improvement in infectious vectors production.

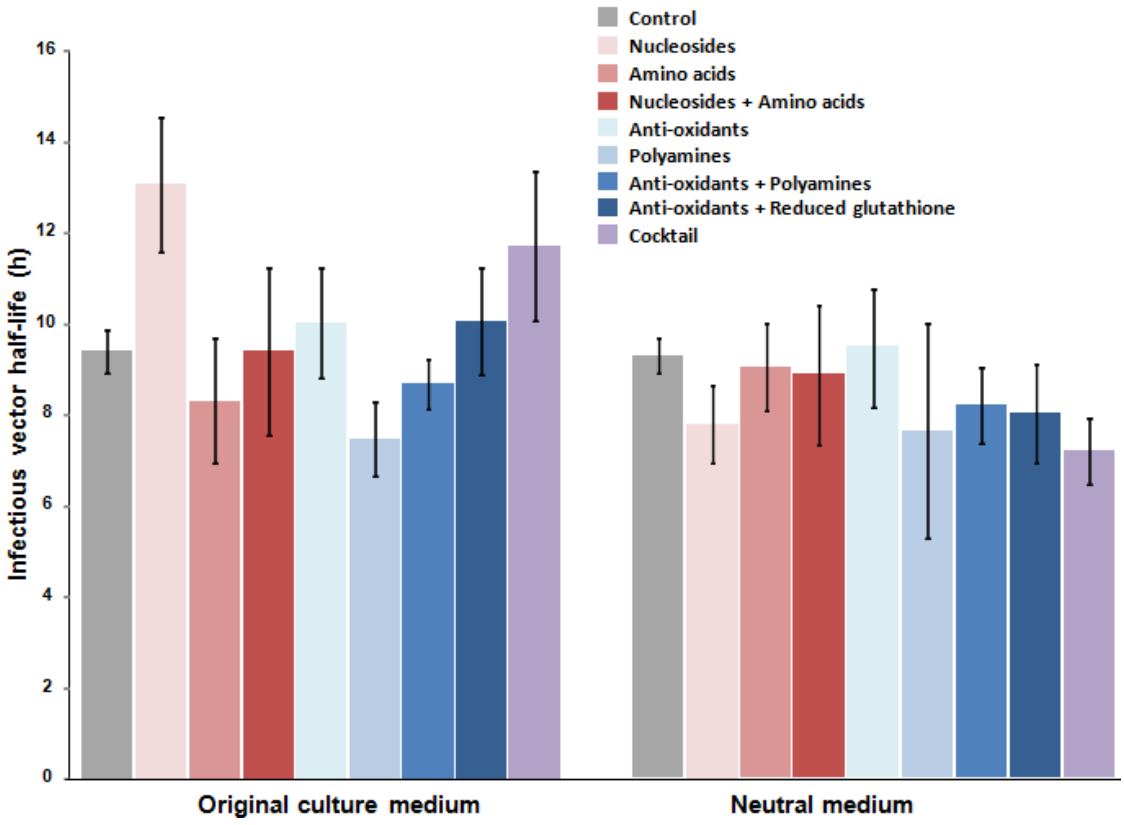

Figure 8 - Decay kinetics of retroviral vectors produced in supplementation medium. Original culture medium refers to decay kinetics evaluated in the original culture conditions; neutral medium refers to decay kinetics evaluated in non-supplemented 10% (v/v) FBS medium. Error bars represent standard deviation of 3 biological replicates.

Part II – Implementation of a single step cloning-titration protocol for high-throughput metabolic engineering of retrovirus producer cells.

Packaging cell line development is a laborious and time consuming process, especially if cell engineering is applied to improve productivity. To reduce the time of clone screening a protocol merging cloning and titration with high-throughput potential was implemented. This protocol is based on a transcomplementation assay using a split GFP, where part of it is encoded by the vector transgene and the other part stably expressed in cells to be infected (Target cells). The protocol makes use of black 96 well plates with clear bottom where retroviral vector producing clone is seeded at 1 cell/well followed by 14 incubation days for cell expansion. During this period Target cells are co-cultured in each well and polyberen (10µg/ml) is added periodically to potentiate viral infection. After 14 days the clones are selected based on the highest GFP signal with a fluorometer. GFP signal is produced by the Target cell line after GFP S10 transcomplementation with GFP S11 fragment upon infection of the retrovirus produced by the clone. Finally, the GFP signal is normalized to a growth indicator marker, mCherry, stably expressed by the retroviral vector producing clone, providing an estimative of the specific productivity of that clone. High producing clones can be isolated from the Target cells by any of the viral components selectable marker (Zeocine, Blasticidine and Neomycin) and/or by Puromycin (part of the mCherry construction). Figure 9 shows the work-flow in the implementation of this protocol.

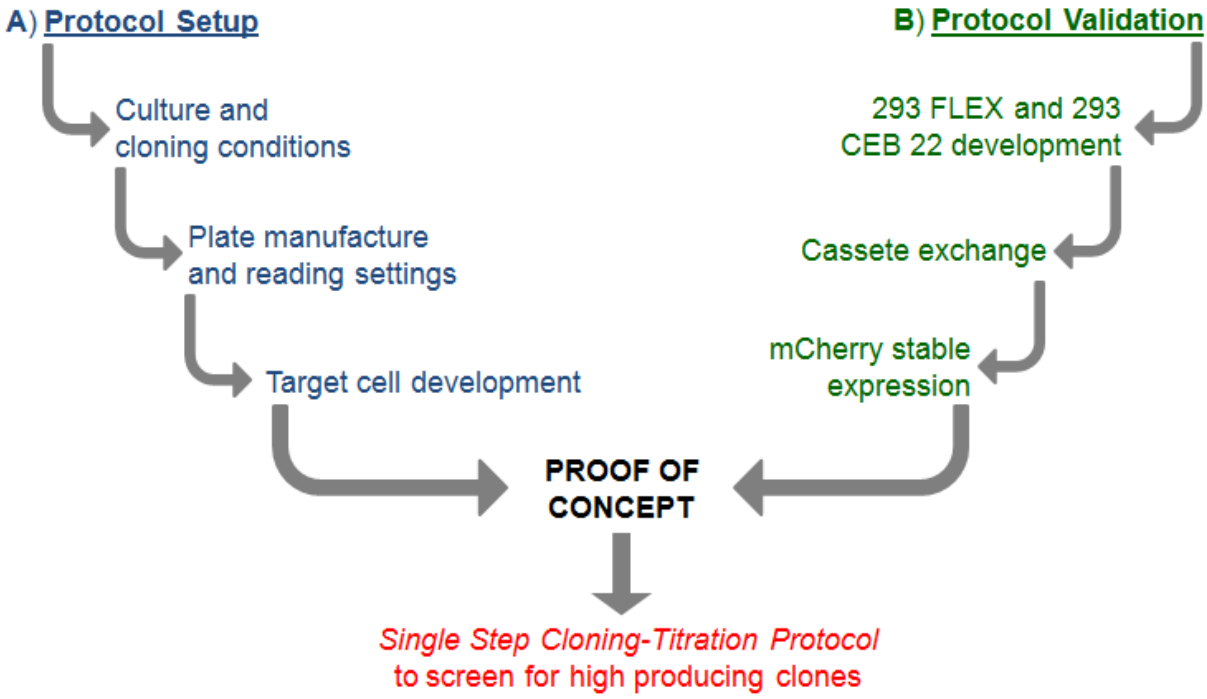

Figure 9 – Workflow of single step cloning-titration protocol implementation.

1. Culture and cloning conditions

To determine the best culture conditions for fluorometer readings a Nunc plate was prepared with combinations of: cells, culture medium, PBS and serum (Fig. 10). Medium without phenol red was used as previous work reported phenol red interference with fluorometer readings [61].

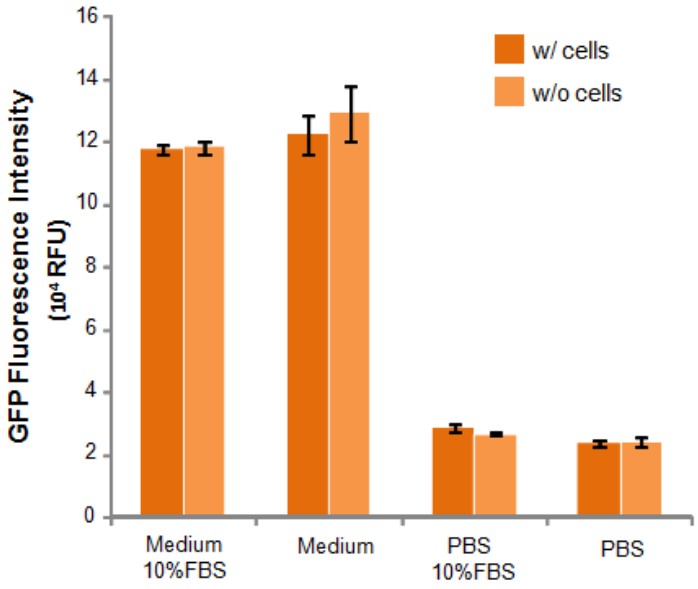

Figure 10 – Culture conditions influencing fluorometer reading for GFP signal (485/528 nm). Error bars correspond to standard deviation (n=12); RFU – Relative Fluorescence Units.

The greatest contribution for background signal was found to come from medium; cells and serum content did not show relevant interference. Thus, to minimize background without compromising cell viability, further readings were carried out by replacing medium with PBS 5% FBS prior to analysis. Serum was reduced to 5% to guarantee minimal signal interference avoiding cell damage.

The protocol requires 14 days incubation to allow clone expansion, concomitant with virus production and subsequent infection of Target cells. To optimize Target cells inoculums these were seeded at day 0 with 50, 100 and 200 cells/well, 24 hours later the cloning procedure was started by adding 1 retroviral vector producer cell per well. This strategy aimed to provide a layer of cells that would be not only the infection targets but also function as a feeder layer facilitating clone expansion. This approach, however, did not succeed as clones were unable to expand in co-culture.

In order to enable clone expansion the culture setup was redesigned and cloning procedure started at day 0 with medium supplemented with 20% (v/v) FBS. Target cells were added at 3.3 x 10^3 cells/well at day 9. After 24 h polyberen was added to a final concentration of 10 µg/ml and plates were agitated for 2 h. At day 13, polyberen was again supplied and

plates were agitated for 4 h. Fluorometer readings to assess GFP signal were performed 24h later. In this experimental protocol, cells were able to proliferate without compromising cell viability and target layer was transduced with RV produced by the clone. Although this system allowed for producing clone detection, GFP signal was poor (data not shown). In order to increase GFP expressing cells, a third set up was performed. This time, Target cells were seeded at day 6 (500 cell/well) and three infection rounds (addition of polyberen and agitation) were carried out. This way it was ensured that infected target cells were able to proliferate and therefore amplifying GFP signal. Indeed, GFP signal was enhanced and enabled detection of producing clones; thus, this was the procedure followed from herein forward. Concerns regarding clonogenicity were raised by previous works [61]; to overcome this problem we used CoStar plates (Part II, section 2), DMEM 20% (v/v) FBS supplemented with B27 (Gibco) and also compared the plate agitation effect during clone expansion (Table 2).

Table 2 – Cloning efficiency during 14 days of culture with medium supplemented with 20% (v/v) FBS.

Plate manufacture	**Control (Nunc)**	**CoStar**		
B27 (1x)	no	no	yes	yes
Periodic agitation	no	no	no	yes
% clonogenicity	48%	37%	53%	42%

CoStar plates presented lower clonogenicity efficiency (37%) compared to regular Nunc plates (48%) used for limiting dilution procedures. However, this outcome was restored upon the addition of B27 to the culture medium (53%). Additionally, the mild agitation of culture plates had a negative effect in clone survival. Therefore, agitation should only be performed in the 3rd infection round to homogenize viral particles content in the well.

2. Plate manufacture and fluorometer reading settings

When starting this work, Nunc plates commonly used in our laboratory for fluorometer readings were the first choice. Their performance (GFP signal detection; support cell culture) was then compared to two other plates: Corning CoStar and Perkin Elmer. GFP fluorescence signal detection was assessed by infection with retroviral vectors harboring a GFP transgene using different MOIs in Te671 cells, Fig. 11 A. The three plates were additionally evaluated

for their ability to provide a suitable growing surface under low inoculums conditions, to be used both for Target cells as well as for the retroviral vectors producing clone (Fig. 11 B).

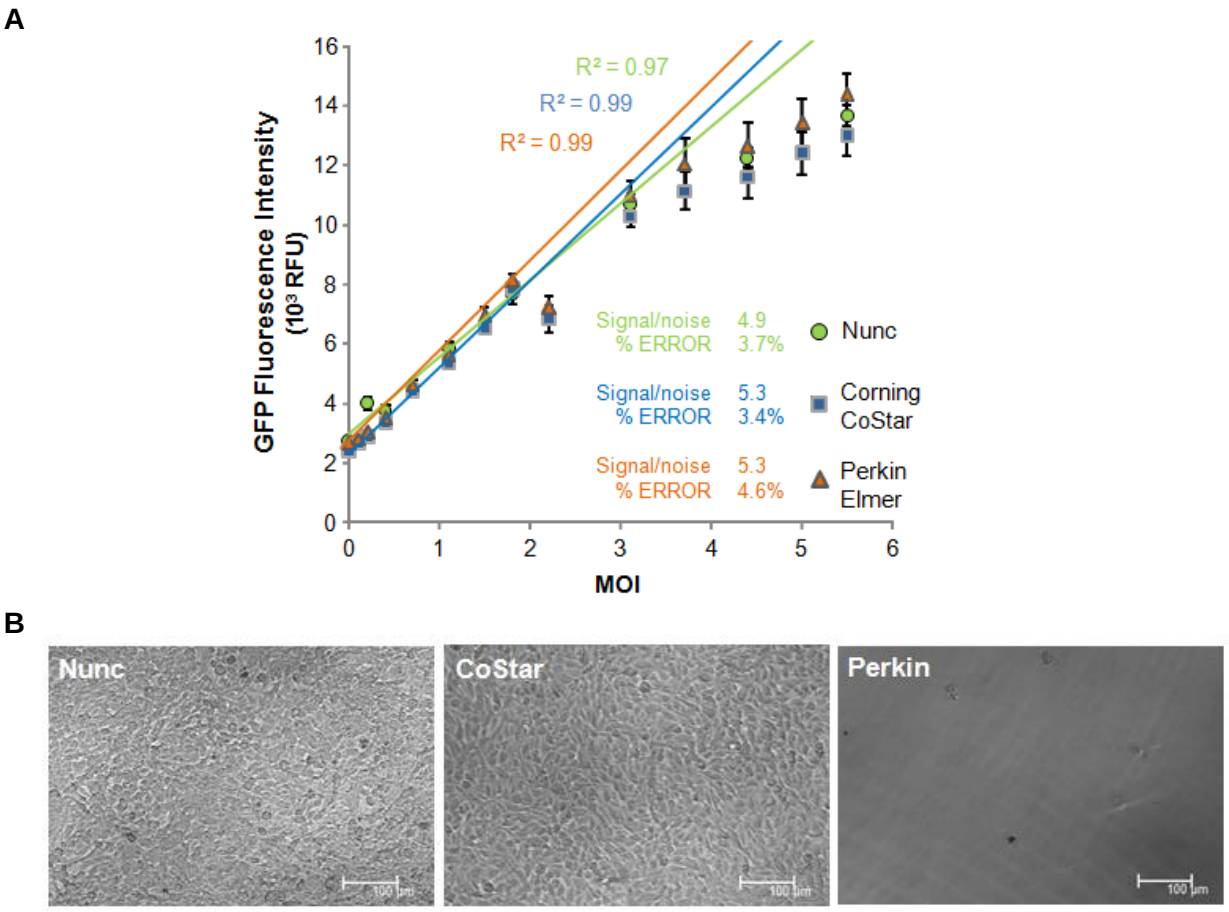

Figure 11 – GFP detection and cell growth performance of Nunc, Corning CoStar and Perkin Elmer plates. A) Fluorescence intensity (485/528 nm) of GFP-expressing cells 48 hours upon transduction with retroviral vectors containing GFP transgene at MOI ranging from 0 to 5.5. Line of best-fit for MOI ranging from 0 to 2 (Nunc R^2=0.97, Corning CoStar and Perkin Elmer R^2=0.99). Error bars represent standard deviation (n=12). B) Cell growth after 14 days culture in each plate (initial inoculums of 50 cells/well). RFU - Relative Fluorescence Units. MOI – multiplicity of infection.

GFP signal could be detected linearly until the multiplicity of infection (MOI) of 2 in all tested plates. The distinction of GFP signal from the background was similar in all plates, although Corning CoStar and Perkin Elmer manufacture showed the best signal to noise ratio (5.3). Both, Nunc and Corning CoStar plates supported good cell proliferation for 14 days, whereas in Perkin Elmer plates, cells failed to reach the confluence. Finally, the average error between wells of same condition was minimized in Corning CoStar plates (3.4%). Therefore, they were selected to establish the protocol.

mCherry was chosen as a cell growth normalization marker as contrarily to other red proteins, its excitation/emission spectra does not overlap with those of GFP [77]. To evaluate

the potential of mCherry as a future normalization marker, mCherry signal was quantified in cells infected with canine adenoviral vectors (CAV) already in use in our laboratory (Fig. 12). Transfection procedures were avoided as they would be difficult to carry in such small scale (96 well) and could lead to significant cell death capable of interfering with fluorescence readings.

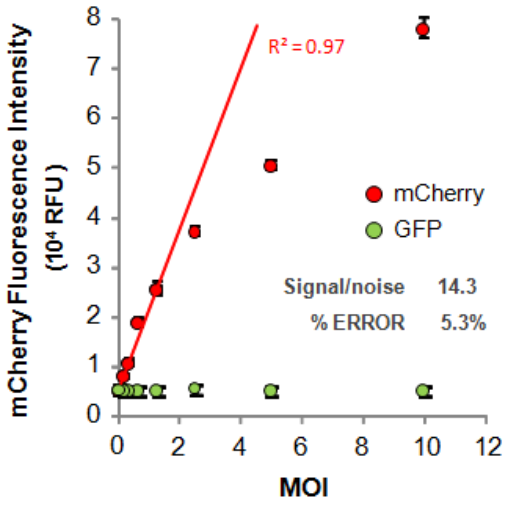

Figure 12 - Fluorescence intensity (560/645 nm) of mCherry-expressing cells transduced with CAV mCherry adenoviruses at MOI ranging from 0 to 10. Line of best-fit for MOI ranging from 0 to 1.3 (R^2=0.97). Error bars represent standard deviation (n=12). MOI – multiplicity of infection.

As shown in Fig. 12, mCherry signal could be detected above background level in all MOIs, linearly until MOI 1.3. mCherry signal was higher than that of GFP (Fig. 11 A) and therefore signal/noise ratio was higher (14.3). Altough the MOIs used in this assay were not comparable to the ones used in GFP assay, the mCherry signal detection enabled higher sensitivity and better distinction from background. In addition, this assay showed no signal interference between the excitation/emission filter sets used for GFP and mCherry detection. This parameter is extremely important to allow clone selection during protocol implementation since it enables GFP titer signal normalization to the producer cells signal.

3. Target Cells Development

To establish the Target cell line, two cell lines commonly used to titrate retroviral vectors, Te671 and 293T, were considered. Infectability was first evaluated; assays were carried out by infection with retrovirus harbouring common GFP. The results are shown in Fig. 13.

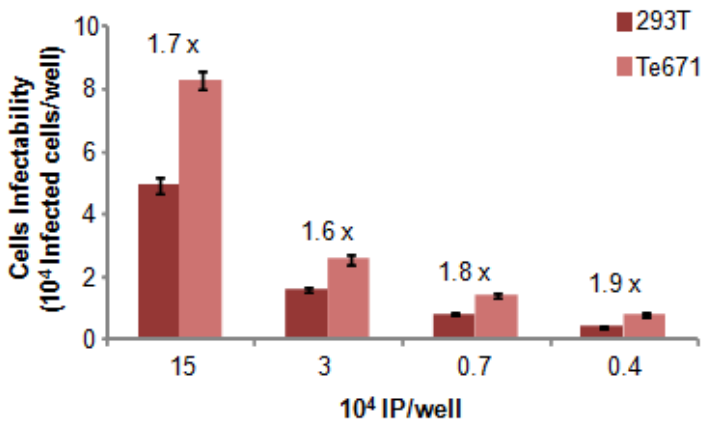

Figure 13 – Infectability of Te671 and 293T cells. The numbers on top are relative fold difference between the two cell lines, error bars correspond to standard deviation (n=2).

Te671 cells were more prone to infection than 293T cells. Considering that is important to have a good GFP signal to detect producing clones, Te671 cells showed to be a better candidate for the development of the Target cell line.

As chemical transfection with pCMV mGFP S10 failed to yield high GFP S10 expressing cells, GFP S10 was cloned in a lentiviral vector and used to infect Te671 cells at very high MOI (30). This would ensure that a high number of GFP S10 gene copies was delivered to each cell. The transduced population was amplified and infected with lentiviral vectors harboring the LacZ-S11 gene confirming a 100% transcomplementation (data not shown) and, thus, high levels of GFP S10 expression in all Te671 infected cell. From this population, 38 clones were isolated, amplified and screened for GFP transcomplementation after retroviral and lentiviral vectors containing LacZ-S11 transgene infection. After this preliminary clone characterization, 9 clones that presented the highest capacity of transcomplementation were chosen to assess signal/noise ratio of the GFP signal as cells that express GFP S10 (Te 671 S10) presented superior intrinsic fluorescence than the parental cell line. The cut line for GFP signal/noise ratio upon infection with retroviral and lentiviral vectors harboring LacZ-S11 transgene was 10 and 8, respectively (Fig. 14). From these clones, #3, #12, #27, and #48 guaranteed at least one cut line for signal/noise ratios and were selected for a more detailed study.

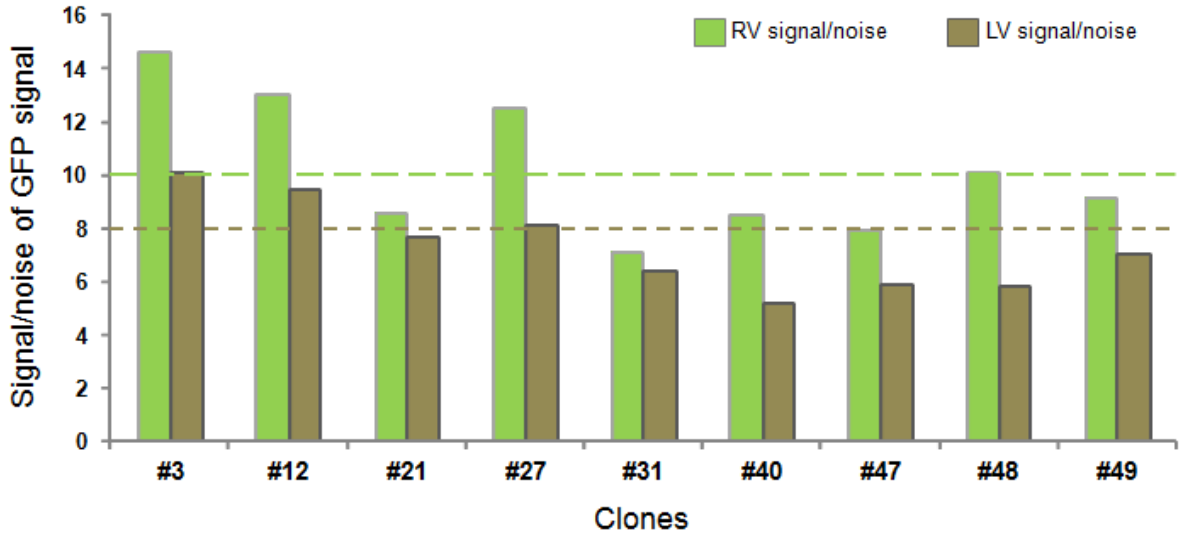

Figure 14 – Screening of Te 671 S10 clones. Ratios of GFP signal to noise of Te671 S10 clones after infection with viral vectors harboring LacZ-S11 transgene. The dashed lines are the cut line for each GFP signal depicted by the color.

The four selected clones were tested for infectability using common GFP retrovirus to evaluate if transduction efficiency was maintained after GFP 1-10 stable expression (Fig. 15). Except for clone #27, Te671 S10 clones presented lower infectability. Clone #27, with infectability similar to the control (Te 671 cells), was thus selected to analyze GFP transcomplementation titer and compare it to LacZ titer of retrovirus harboring *LacZ-S11* transgene produced with two distinct envelopes, GaLV and VSV G (Fig. 16).

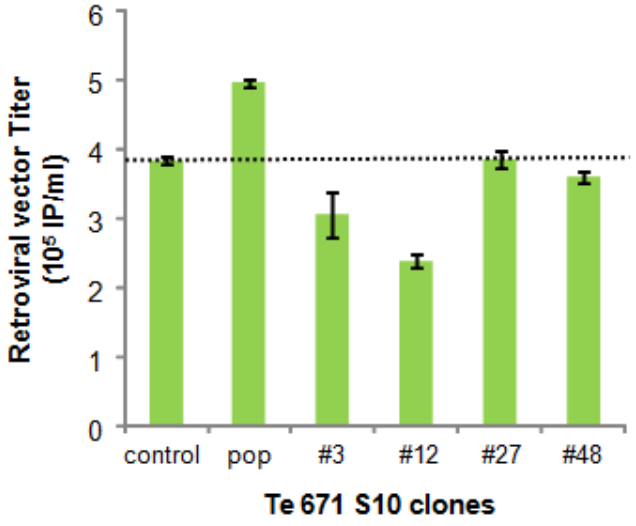

Figure 15 – GFP retroviral vectors titer calculation in Te671 S10 clones. The dashed line indicates the titer obtained with the control (parental cell line – Te 671). Error bars correspond to standard deviation (n=2). Pop – Te 671 S10 population.

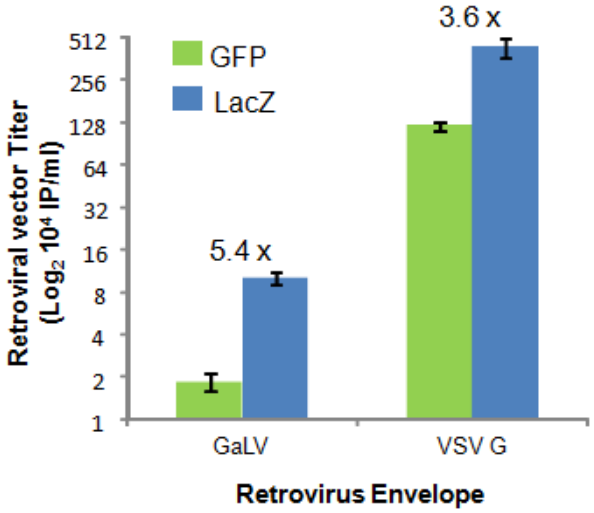

Figure 16 – Titers of the two reporter proteins encoded by *LacZ-S11* transgene. Titer was assessed in Te671 S10 #27 cells for retrovirus produced with two distinct envelopes, GaLV and VSV G. The numbers on top are relative fold difference between the titers of the two reporters. Error bars correspond to standard deviation (GFP n=2; LacZ n=3). GaLV – Gibbon Ape Leukemia Virus; VSV G – Vesicular Stomatitis Virus G Protein.

GFP titers could not be correlated with LacZ titers (Fig. 16); since GFP titers were lower than LacZ titers we hypothesis that GFP titration was affected by lack of GFP S10 expression in Te 671 S10 #27 cells. To evaluate if the GFP transcomplementation titer was compromised by GFP S10 deficiency in the clone candidate to establish the Target cell line (clone #27) was re-infected with lentivirus containing GFP S10 transgene (MOI of 30) and analyzed for GFP transcomplementation titer. The results are shown in Fig. 17.

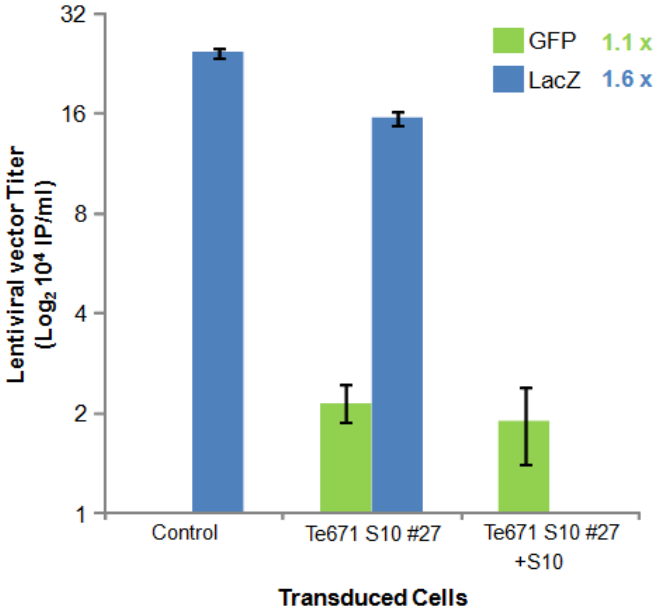

Figure 17 – Titers of the reporter proteins encoded by *LacZ-S11* transgene. Titration was performed in Te671 cells (control), Te671 S10 #27 cells and Te671 S10 #27 cells reinfected with lentiviral vectors carrying *GFP S10* transgene (MOI 30). The numbers are relative fold difference between the titers. Error bars correspond to standard deviation (GFP n=2; LacZ n=3).

The titer obtained by GFP transcomplementation did not increase when *GFP S10* gene was added to Te671 S10 #27. Moreover, LacZ titer was still lower when assessed in different S10 cells, 1.6 fold lower in clone #27 when compared to the control (Fig. 17). These results demonstrated that clone Te671 S10 #27 expresses sufficient amounts of *GFP S10* to carry GFP S11 transcomplementation in full extension. The titer differences between the two reports should be based in other mechanisms rather than insufficient *GFP S10* expression.

Fluorometer readings for GFP transcomplementation signal was finally performed, with an assay similar to the previously described, using Te 671 S10 #27 cells infected with *LacZ-S11* retroviruses with MOIs ranging from 0 to 4.2 (Fig. 18).

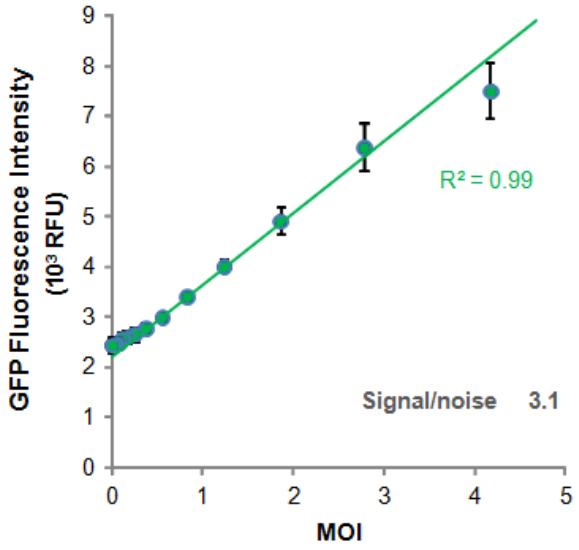

Figure 18 - Fluorescence intensity (485/528 nm) of GFP-expressing cells (Te 671 S10 #27) transduced with LacZ-S11 retroviral vectors with MOI ranging from 0 to 4.2. Line of best-fit for MOI ranging from 0 to 2.8 (R^2=0.99). Error bars represent standard deviation (n=12). MOI – multiplicity of infection.

The background provided by the Target cells was comparable to the parental cell line and GFP transcomplementation signal was detected above background level from MOI 0.4 and linearly until MOI 2.8 (R^2 of 0.99). Distinction of GFP signal from background (0.7 signal/noise MOI^{-1}) was lower than the signal provided by retrovirus carrying GFP transgene (0.9 signal/noise *per* MOI) (Fig. 11).

4. 293 FLEX 18 and 293 CEB 22 development: RMCE and mCherry insertion

The development of a cell line with LacZ-S11 fused protein as a transgene took advantage of RMCE technology to target 293 FLEX and 293 CEB 22 cell lines with pTAR LacS11. These cell lines were developed in our group and hold a single copy of the tagging construct (pIRES Galeo). The use of Flp/FRT cassette exchange allowed for transgene

exchange in a defined chromosomal locus of the packaging cell line in a single positive selection step [9]. After recombination by the use of two non-interacting FRT sites, the targeting of the pTAR LacS11 construct restored the *neo* gene by insertion of the missing ATG start codon and *hygtk* selection marker was eliminated. Upon antibiotic selection, genomic DNA extraction and purification was performed and evaluated by PCR and qRT-PCR for correct targeting of exchanged 293 CEB 22 S11 and 293 FLEX S11 (Fig. 19 and Table 3); analyzed clones were previously tested for GFP transcomplementation (data not shown).

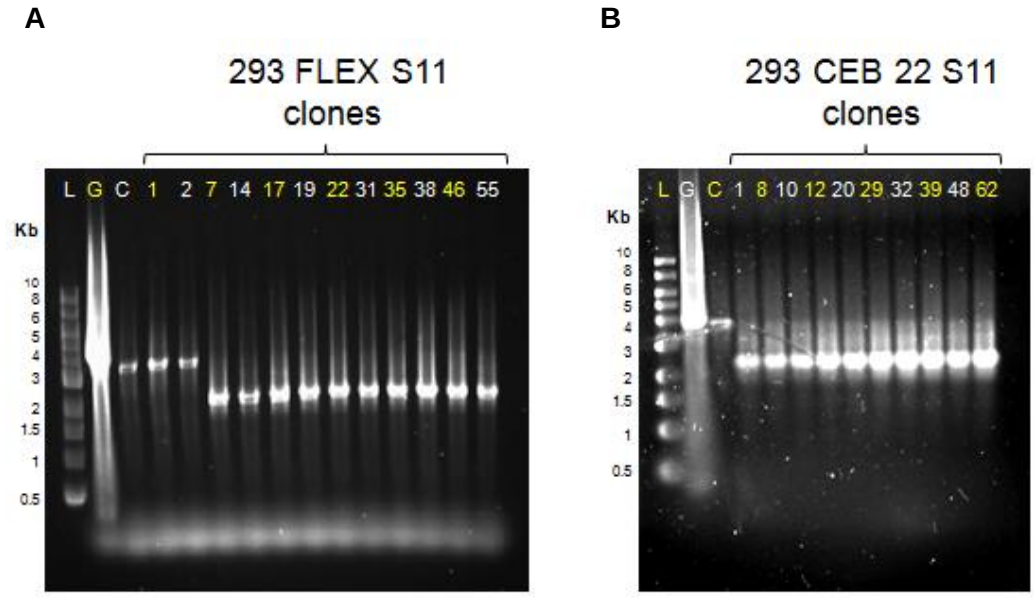

Figure 19 – Electrophoresis in 0.7% (w/v) agarose gels for the PCR reaction performed to amplify the transgene sequence of: A) 293 FLEX S11 clones; B) 293 CEB 22 S11 clone. L- ladder; G – pIRES GALEO; C – control (parental cell line); number correspond to targeted clones.

Transgene exchange was verified by the loss of approximately 2Kb in amplicon size in 10 of the 12 293 FLEX S11 clones and in all 10 clones of 293 CEB 22 S11 (Fig. 19). The results showed single copy in all 293 CEB 22 S11 clones, whereas for 293 FLEX S11, two clones presented an additional copy, considering a 1.4 cut off (Table 3).

Table 3 – Number of copies of integrated transgene *per* cell in 293 FLEX S11 and 293 CEB 22 S11 exchanged clones. Quantification of the transgene was performed by qRT-PCR for the *LacZ* gene and normalized to the respective parental cell line.

Cell Line	Clones	Copy / Cell	Cell Line	Clones	Copy / Cell
293 FLEX S11	Parental	1	**293 CEB 22 S11**	Parental	1
	7	2.0		1	0.7
	14	1.7		8	0.8
	17	1.2		10	0.8
	19	1.1		16	1.0
	22	1.1		20	0.8
	31	1.4		29	0.6
	35	1.3		32	1.1
	38	0.8		39	1.2
	46	1.3		48	1.3
	51	1.0		62	0.6

After exchange, the virus production from 293 FLEX S11 clones dropped more than two orders of magnitude and from 293 CEB 22, only 2 out of 10 clones were able to produce in 10^6 I.P./mL range (data not shown). Therefore, only 293 CEB 22 S11 clones were selected for pPURO mCherry transfection. After Puromycin selection period, the mCherry populations were sorted to select a more homogenous population for mCherry signal.

5. Cloning-titration protocol

Transgene exchanged resulted in a drop of virus production in the clones of 293 FLEX cells, which was unexpected. On the other hand 293 CEB 22 transgene exchanged enabled viral production in 2 of the 10 clones analyzed; being 293 CEB 22 the parental cells of 293 FLEX it was clear that cassette exchange was possible in the later. Thus, we postulated that 293 FLEX S11 population should contain a small number of correctly exchanged clones and used the cloning-titration protocol to find them. High Clonogenicity was ensured (50%), but only one producing clone out of 450 was detected (Fig. 20). Due to time restriction, it was not possible to analyze the isolated clone in order to determine the number of copies of integrated transgene and its specific production. If correct transgene exchange is confirmed, the mCherry cell marker will be inserted.

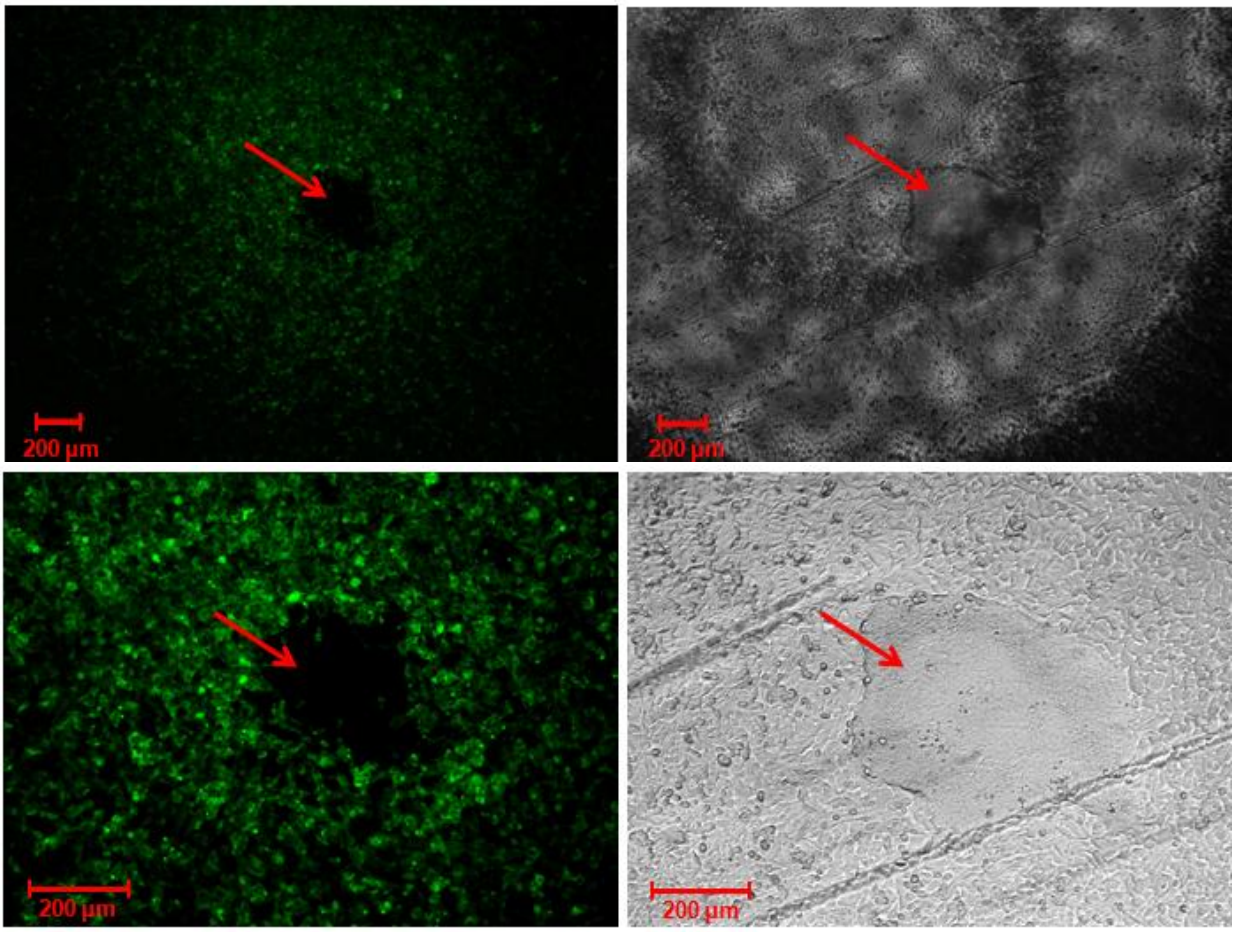

Figure 20 – 293 FLEX S11 producing clone isolated with the single-step cloning titration protocol. Arrows point to retroviral vector producing colony and GFP positive cells are infected (transcomplemented) Target cells.

The validation of the implemented protocol was carried out with a model of retroviral vector producer cell line: 293 CEB 22 S11. This cell line is the precursor of 293 FLEX cells prior to envelope insertion. Thus, the correspondent mCherry sorted population was transfected with pGaLV (envelope) in order to establish a producing population. Upon 15 days of Zeocin selection (envelope selection marker), the GaLV population was subjected to the cloning-titration protocol. This analysis is currently being performed and due to time restriction it was no possible to include those results in this thesis. However to evaluate the functionality of the assay a co-culture with GaLV selected population and Target cells was carried out (Fig. 21).

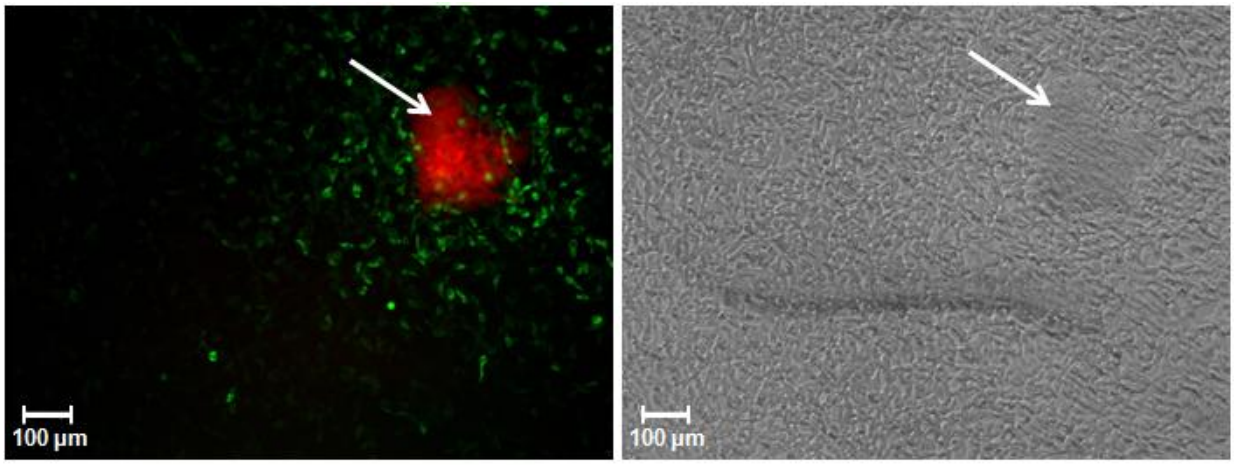

Figure 21 - 293 CEB S11 GaLV producing clone co-cultured with Te 671 S10. Arrows point to retroviral vector producing colony (in red) and GFP positive cells are infected (transcomplemented) Target cells.

Figure 21 shows Target cells (in green) infected by retrovirus harboring the LacZ-S11 transgene produced by the 293 CEB 22 S11 clone (in red).

Part III – Metabolic engineering

From the transcriptional profiling analysis [65], more than 30 genes were identified as potential manipulation candidates (for over-expression or down-regulation) towards improved virus production. Four of those genes glutathione synthase, isocitrate dehydrogenase 1 and 2 and hypoxia inducible factor 1 inhibitor were cloned in a lentiviral vector for over-expression. In the case of hypoxia inducible factor 1 inhibitor, its over-expression aims at simulating the down-regulation of hypoxia inducible factor I while avoiding the adverse effects of using knock-down tools [78] (typically, RNA interference). Given that the 293 FLEX S11 clone isolated was still under expansion, we conducted a preliminary study of metabolic engineering with these genes using 293 FLEX cells and evaluated the average viral productivity in cell populations (Table 4).

Table 4 – Increase in viral productivity of 293 FLEX cell populations after transduction with GSS, IDH1, IDH2 and HIF1AN

Single gene	***GSS***		***IDH1***	***IDH2***	***HIF1AN***	
	2.1		1.8	2.6	2.7	
Multiple gene	***IDH1***	***IDH2***	*NP*	*NP*	***IDH1***	***IDH2***
	2.3	*NC*			4.5	5.8

Values indicate fold-change increase relatively to the non-transduced cells and after mock control normalization. *NC*: no change; *NP*: not performed. Multiple gene refers to transduction with the indicated genes of the above single gene transduced population. *GSS*: glutathione synthase; *IDH*: isocitrate dehydrogenase; *HIF1AN*: inhibitor of hypoxia inducible factor 1.

For all the tested genes, average viral productivity was found to be increased. More interestingly, viral production was potentiated in *GSS* and *HIF1AN* populations after *IDH*s transduction in a relatively predictable way (Fig. 22): i) *IDH1* potentiates *GSS* effect increasing the flux through the first step of pentose phosphate pathway; ii) *HIF1AN* – activating pyruvate dehydrogenase through *HIF1* inhibition thus increasing the flux through the first step of the TCA cycle [58] – is potentiated by *IDH2* catalyzing its third step. These results strongly support the strategy of multiple gene manipulation for improved virus production. Moreover, increases in average cell population productivity are promising indicators of hyperproducing clones, isolatable when using the implemented protocol in 293 FLEX S11.

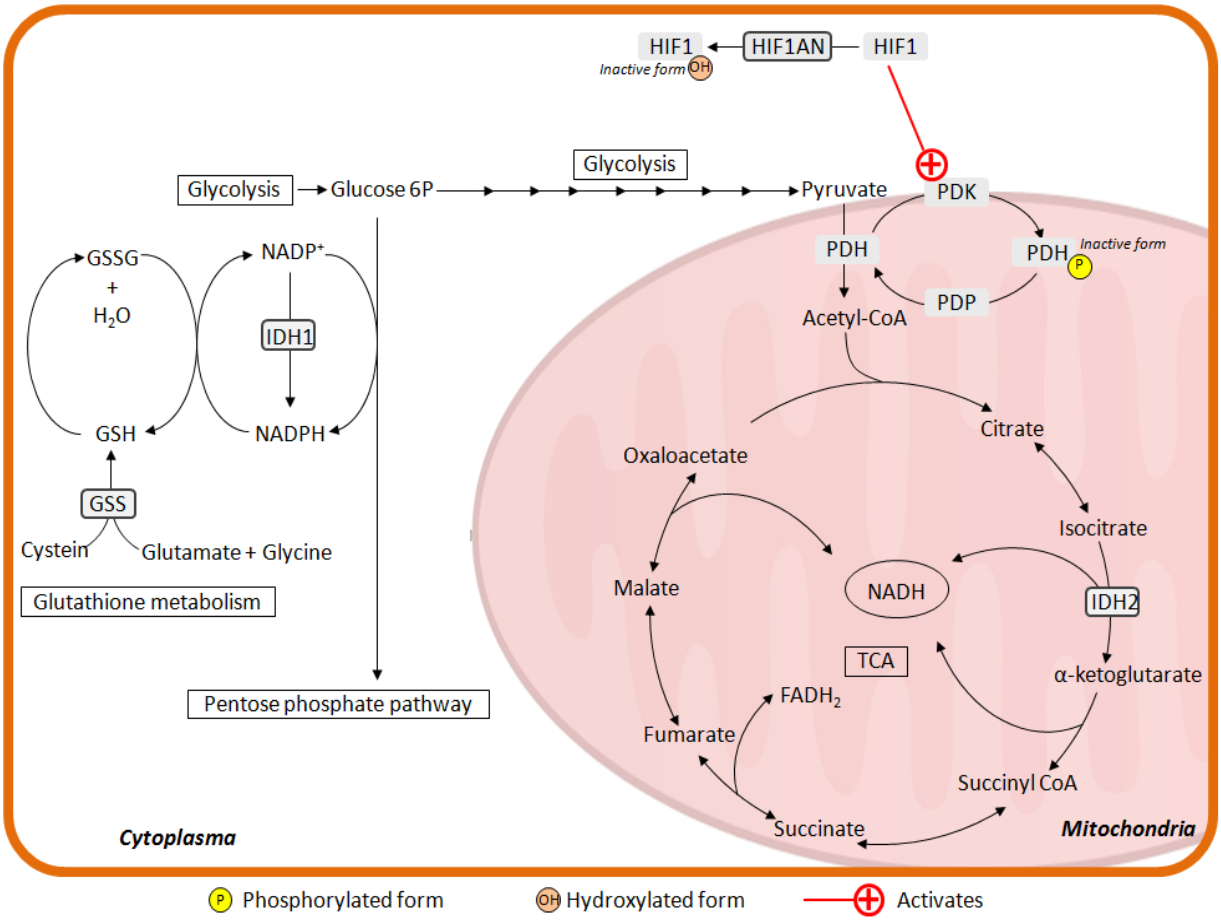

Figure 22 – Schematic representation of targeted pathways by the over-expression of GSS, IDH1, IDH2 and HIF1AN. Genes are shown in grey boxes. For a matter of simplicity, only targeted genes and directly activated/inactivated genes are shown. Targeted genes boxes are surrounded by a thick line. GSS: glutathione synthase; IDH: isocitrate dehydrogenase; HIF1AN: inhibitor of hypoxia inducible factor 1.

5. Discussion and conclusion

The increasing demand for recombinant pharmaceutical bioproducts (e.g. antibodies and virus) has placed significant pressure to develop robust high-yielding mammalian cell-based production systems. Retrovirus based vectors comprise several valuable biopharmaceuticals such as gene therapy vectors, recombinant vaccines and replicative-competent particles for oncolytic therapy [5,6]. However, the platforms used for their productions are characterized by low yield preparations and high contaminant content of non-infective virus. A transcriptional profiling analysis of relevant networks recruited when establishing a producer from its correspondent parental cell line showed that the most relevant changes occurred in metabolic pathways (Fig. 4) [65]. Considering these findings, we validated the results by targeting easy to manipulate pathways through medium supplementation (Fig. 7). This was our starting point aiming retroviral based vector production improvement.

Culture medium manipulation of easy to manipulate pathways - *Nucleic Acid Metabolism, Amino Acid Metabolism, Free Radical Scavenging* and *Polyamine Metabolism* – increased RV titer in all culture supplements (Fig. 7 A). However, the expression of viral genes (Fig. 7 B) was not significantly affected by these manipulations and particle stability did not improve significantly (Fig. 8), suggesting that titer improvement must be related with the increment of infectious particles present in the viral preparation and thus to the quality of viral supernatants. Therefore, the expression of viral components was not the bottleneck of RV production and modulating cell metabolism by medium manipulation led to increased infectious retroviral based vectors production. It was showed that by targeting metabolic pathways it is possible to increase the production capacity of 293 FLEX cells. In general, the improvement in viral preparation quality could be a consequence of improvement in cell robustness and production machinery and/or due to enhanced particle assembly and budding.

The addition of nucleosides to the culture medium increased infectious viral vectors production in 1.9 fold; this was the highest titer achieved by the addition of only one supplement. Nucleosides are the precursor of nucleotides which in turn constitute the building blocks of nucleic acids [74]. This effect may be a result of improved synthesis of vector genome and/or a general enhance of the transcription rate of the viral components.

Amino acid supplementation modestly increased infectious particles production (1.3 fold), when amino acids and nucleosides were added together the titer improved in 1.5 fold but production levels were not rescued to the ones described above. Amino acids supplementation may have an impact in translation processes enabling the producer cell to

initiate the translation of more viral proteins. Additionally, amino acids degradation is known to be a main TCA feeding source to support the energy metabolism [74]; thus a more favorable energy cell state may be potentiated which in turn affects the final viral preparation. To distinguish these two effects (carbon skeleton or energy suppliers) ammonia production in cell supernatant will be assessed; if it is identical to the control, the amino acids were likely to be consumed for the translation processes; on the other hand if it is increased, amino acids degradation to feed TCA cycle should be occurring.

Polyamines supplementation yielded 1.8 fold-improvement in viral titers. Polyamines are low molecular weight aliphatic molecules with regularly spaced positive charges. These molecules can bind to RNA and DNA and participate in DNA stabilization, modulation of chromatin structure and gene transcription events. Additionally, polyamines also have a role in signal transduction, cell growth and proliferation, membrane rigidity and possess anti-oxidant properties [79]. All this properties may have an influence in viral production increment, particularly: stabilization of viral genome leading to easier encapsidation, membrane rigidity and prevention of its lipid peroxidation can improve viral budding and envelope quality. Actually it was already reported that polyamines are important for the activity of viral RNA polymerase [80,81] and may interfere with virus assembly at the level of DNA packaging or capsid envelopment or both [82].

Down regulation of enzymes involved in oxidative stress defenses indicated that the "virus producer status" increases oxidative stress in the cells [65]. The addition of anti-oxidant supplements to culture medium may alleviate the burn of this cell status and lead to the 1.5 fold increase in infectious virus production. Additionally, this supplementation could also influence viral preparation quality by improving particle resistance do oxidizing stress; however, virus half-life was not enhanced with this supplement, which would be expected if this was the case. When complemented with polyamines or reduced glutathione supplement, producing cells gained even more oxidative protection which resulted in increased viral titers (2 and 2.1 fold, respectively). Moreover, reduced glutathione supplement can also play a role in pentose phosphate pathway once it can affect its regulation by changing the ratio of NAD^+/NADH. When the cell is converting NADPH to $NADP^+$ upon glutathione oxidation the level of $NADP^+$ rises, stimulating glucose-6-phosphate dehydrogenase and thereby increasing the flux of glucose-6-phosphate through the pentose phosphate pathway [74]. In the other hand, the pentose phosphate pathway reduces $NADP^+$ to NADPH and produces pentose phosphates. NADPH provides reducing power for biosynthetic reactions, and ribose 5-phosphate is a precursor for nucleotide and nucleic acid synthesis, which in turn may lead to enhanced cell capacity to express viral genes.

Two of the easy to manipulate pathways – *Lipid metabolism* and *Vitamin and mineral metabolism* - were not target in this work. Lipid metabolism and its implications in virus

production of 293 FLEX cells was already studied [12,13]. The results showed the need of highly active lipid synthesis machinery to support infectious vector production under serum (lipid source) deprivation, suggesting lipid biosynthesis as a promising target for improved productivities. However, under standard 10% (v/v) FBS conditions (those used in this work), transcriptional profiling results showed a general down-regulation of lipid biosynthetic machinery in response to virus production, particularly cholesterol, while activating external lipid consumption. Transcriptional profiling also suggested this phenomenon to be mediated by AMPK inhibition, a strategy that chronic diseases caused by viruses are known to rely on to benefit their own replication over cell replication [83]. However, in this work, all cultures were conducted at 10% (v/v) FBS and, as serum provides lipid nutrients in large excess, additional supplementation should not result in improved productivities. Vitamin and mineral metabolism, pyridoxal phosphate (PLP, the biological active form of B6 vitamin) and tetrahydrofolate (THF) metabolism, could also be easily manipulated by culture medium supplementation. These are important co-factors of several metabolic enzymes serving different pathways including amino acid metabolism, in both cases, gluconeogenesis, glycogenolysis and lipid metabolism, in the case of PLP, and nucleic acid metabolism, in the case of THF. As many of these pathways had already been covered, the addition of these molecules would probably only potentiate their effects.

Single medium manipulation led to a modest improvement in infectious virus production (1.3 to 1.9 fold), as well, double manipulation raised titers in approximately 2 fold, but more impressive was the cocktail medium manipulation which enabled 6 fold higher productivity. To apply this strategy at larger scale requires a fed-batch system and use of expensive supplements and thus, not attractive from the bioprocess point of view. However, these results highlighted a very important point: orchestrated rearrangements of unrelated metabolic pathways lead to superior titers, supporting multiple gene engineering of metabolic pathways as the road for cell hyperproductivity.

Metabolic engineering of a single target may take up to one year to be complete as it requires an extensive clone screening to find those with the highest infectious retrovirus production as high productivity is a rare event in a producer population. If multiple pathways were target at once, clone screening time would exponentially increase. Therefore it was it was essential to develop a protocol that enables the high producing clones at early stages of the process, such as the cloning step.

A high-throughput screening method was already described by Green and Rasco (2002) [61]; however this method needs the time to titrate clones viral supernatant upon cloning procedure and makes use of fluorescent proteins that are expressed in both producer and target cell line. For clinical therapeutic vectors the last mentioned trait it is not desirable, since the therapeutic vector would need to express a fluorescent reporter.

The method implemented in this work reduces the time and manipulation required for the generation of high-titer producing clone since ensures the titration and cloning procedures are performed in one single step. The transgene expressed by the producing cells only enables the expression of a fluorescence protein upon viral infection of Target cells and it can be exchanged to any therapeutic gene for clinical purposes. Lastly, in Green and Rasco [61] assay the authors reported low clonogenicity efficiency (10%) which reduces the chances of finding a clone candidate, whereas in this work it was possible to achieve 53% of clone survival by culturing cell in Corning CoStar black-walled 96 well plates with DMEM 20% (v/v) FBS and B27 (1x) supplement (Table 2).

The replacement of medium with PBS prior to analysis was already identified as a critical step to ensure reproducibility and reduce variance in fluorometer readings [61]. In the developed protocol PBS supplemented with 10% FBS was used without compromising background signal interference. Cell viability and sterility is maintained during analysis and only producing cells have selection markers; this way we ensure that clones can be further cultured after the assay and isolated from Target cell. Moreover, it is of outmost importance to ensure single colony in the selected wells, for that, single cell deposition by cell sorting will be used in the future. The use of two florescence proteins in the established protocol did not compromise their detection since the excitation/emission spectra do not overlap [77].

Our main challenge was to establish the Target cell line. Te 671 cell line was the best candidate since they demonstrated higher infectability (Fig.13). However, it was not possible to insert GFP S10 gene by chemical transfection of pCMV m GFP S10 plasmid. Its delivery was only possible upon lentiviral infection at saturating MOI. From all the analyzed clones, Te 671 S10 #27 was selected to establish the Target cell line because it showed the best GFP transcomplementation signal and similar infectability compared to parental cell line (Fig. 14 and 15). Nevertheless, GFP titers could not be correlated with LacZ titers (Fig. 16) although lower GFP titers were not due to lack of GFP S10 expression in Target cells (Fig. 17). LacZ titer was reduced when titration was performed in Te 671 S10 cell compared to the parental cell line (Fig. 17), which may indicate that upon GFP transcomplementation LacZ loses enzymatic activity. Therefore, upon transcomplementation, GFP fluorescence might be also be compromised by LacZ fused protein interactions, leading to the different titer obtained with the two reporter proteins. However, this should not be a problem since GFP signal (although under estimating the real titer) is directly proportional to the viral titer (Fig.18). In fact, it can turn out to be an advantage since signal saturation will only occur for very high titer clones, the ones to be detected with this method.

Targeted 293 FLEX clones lost retrovirus production capacity; this was not expected since RMCE technology enables transgene exchange, maintaining a predictable vector production [9]. 293 CEB 22 cells were properly exchanged, thus, due to time restriction we

used 293 CEB 22 S11 (clone 32, Fig. 19, Table 3) for mCherry insertion and mCherry sorted population selected (Zeocin) for GaLV expression, to validate the protocol. Nevertheless, since only 2 producing clones from 293 CEB 22 (precursor cell line of 293 FLEX) cassette exchanged population were isolated, we postulated that in general cassette exchange procedure was inefficient, which in turn lead to rare properly exchanged events. Therefore, we used the implemented protocol to extensive screen 293 FLEX S11 population. Indeed, we were able to isolate a producing 293 FLEX S11 clone out of 450, confirming the scarcity of producing clones in targeted population. Additionally, these results highlighted the screening potential of the implemented protocol since only 1 clone out of 450 was detected has a virus producer. However, further analysis needs to be carried to ensure single copy of the transgene and to assess specific productivity. Moreover, additional screening to find more 293 FLEX S11 producing clones will be performed.

Optimizing vector titer has important implications for both clinical and research-based gene transfer. The cloning-titration protocol developed allows high-throughput screening to overcome the limitations imposed by the current labor intensive and time-consuming flow cytometric and antibiotic selection methods by maximizing the chance of identifying clones with rare hyperproductivity. This protocol should find other valuable applications as shortening the time-frame of establishing new virus producer cells or rapidly screen alternative cell substrates (cell lines) for virus production. If the analysis could be performed by using robotics much greater throughput will be achievable. Additionally, this work allowed extending the knowledge on viral production and gave insights for further metabolic engineering of 293 FLEX S11 cells. This cell engineering will only be feasible with the proper tools for clone screening; the established cloning-titration protocol will significantly contribute for that future work. Multiple metabolic engineering towards the delivery of an improved mammalian cell platform for the production of RV retrovirus can be later transformed into healthcare therapeutic services and products. The preliminary results on 293 FLEX metabolic gene manipulation (Table 4) strongly encourage the continuation of this work in a multiple gene approach.

The huge potential of HEK293 derived production systems for biotechnological and pharmaceutical applications renders this master project not only scientifically but also technologically relevant. Moreover, retroviral vectors constitute an excellent representative of similarly enveloped virus, potentiating the broadening of the generated knowledge to other viral vectors (e.g lentivirus), viral vaccines (e.g. influenza) and VLPs (e.g. retroviral VLPs). Thus, this project contributes greatly to the advancement of virus based biopharmaceutical production platforms in biotechnology.

6. Ongoing and future work

The results obtained during this master project suggest that the low retroviral vectors production yields from 293 FLEX cells are mainly due to a metabolic constrain. The hypothesis that metabolic engineering of this platform may lead to a more efficient metabolism and consequently higher RV production will be confirmed by combinatorial gene manipulations of the metabolic pathways previously identified and validated in this work. To follow this work line, the 293 FLEX S11 isolated clone will be analyzed for single copy of the transgene and, depending on the results, mCherry will be inserted and further metabolic engineering will be performed. For this purpose, lentivirus library harboring different metabolic genes will be produced with the constructed plasmids of section 3.1 and used to infect 293 FLEX S11. Depending on the results other genes can also be tested.

The final validation of the implemented protocol is being carried with 293 CEB 22 S11 population (GaLV clones) in a total of 600 clones. Each well will be analyzed for the GFP/mCherry signal allowing for determining the productivity profile distribution of this population. The highest GFP/mCherry yielding clones will be isolated, quantified for specific productivity and compared to 293 FLEX titers.

The knowledge gathered can also be used in future work for the improvement of production of other virus based biopharmaceuticals.

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8. Annexes

Table S1: Cloning sites, primers and templates for plasmids construction.

Final Construct	Insert			Vector		Cloning site
	gene	Source	Primers	Parental	Primers	
pPURO mCherry	mCherry	pRSET B mCherry	F – CTAGCTAGCCCACCATGGTGAGCAAGGGCGAGGAG	pSELECT Puro	-	NheI
			R – CTAGCTAGCTTACTTGTACAGCTCGTCC			
pCMV mGFP LacZ S11	LacZ	pIRES GALEO	F – ATCCTACCGGTATCCAGCCCTCACTCCTTCTC	pCMV mGFP S11	-	AgeI
			R – CGACTACCGGTTGACACCAGACCAACTGGTAAT			
pTAR LacS11	LacZ-S11	pCMV mGFP LacZ-S11	F LacZ S11 – GCGTGTACGGTGGGAGGTCTAT	pEM MFG	F – GCGATCACATGGTCCTGCTGGAGTTCGTGA	NotI
			R – GGGGAGGTGTGGGAGGTTTT		R – AGTTGTTCTTGGTAGCGGCCGCCCAGAGGTCT	
pRRLsin S10	GFP 1-10	pCMV mGFP S10	F – GTGAACCGTCAGATCCGCTAGCCG	pRRLsin eGFP	F1 – CACTCTCGGCTAGCACGAGCTGTACAAGTAAAGCG	NheI PciI
			R – GGCTGGCTAGCATCAGTTATCTAGATCCG		R1 – GCGAGCGGTATCAGCTCACTCAAAGGCGGT	
pRRsin LacZ S11	LacZ-S11	pCMV mGFP LacZ-S11	F LacZ S11		F2 – TCGGGTTTCGCCACCTCTGACTTGAGCGTC	
			R – GTTTTGCTAGCCAAGTAAAACCTCTACA		R2 – GGTGGAGCTAGCTGGGGAGAGAGGTCGGTGAT	
pRRLsin S11 GSS	GSS	HsCD00045255 *	F pDONR22 – CAATGCACCGGTATAATGCCAACTTTGTAC	pRRLsin LacZ S11	-	AgeI
			R – GCTGGGACCGGTACAGGGTATGGGTTGTC			
pRRLsin S11 HIF1 inihibitor	HIF1 inhibitor	HsCD00044819 *	F pDONR22			
			R – CTGGGACCGGTTTGTATCGGCCCTTG			
pRRLsin S11 IDH1	IDH1	HsCD00043452 *	F pDONR22			
			R – GCTGGGACCGGTAGTTTGGCCTGAGCTAG			
pRRLsin S11 IDH2	IDH2	HsCD00044869 *	F pDONR22			
			R – CTGGGACCGGTTGCCTGCCCAGGGCTCT			
pRRLsin MOCK	-					

* DNASU clone ID

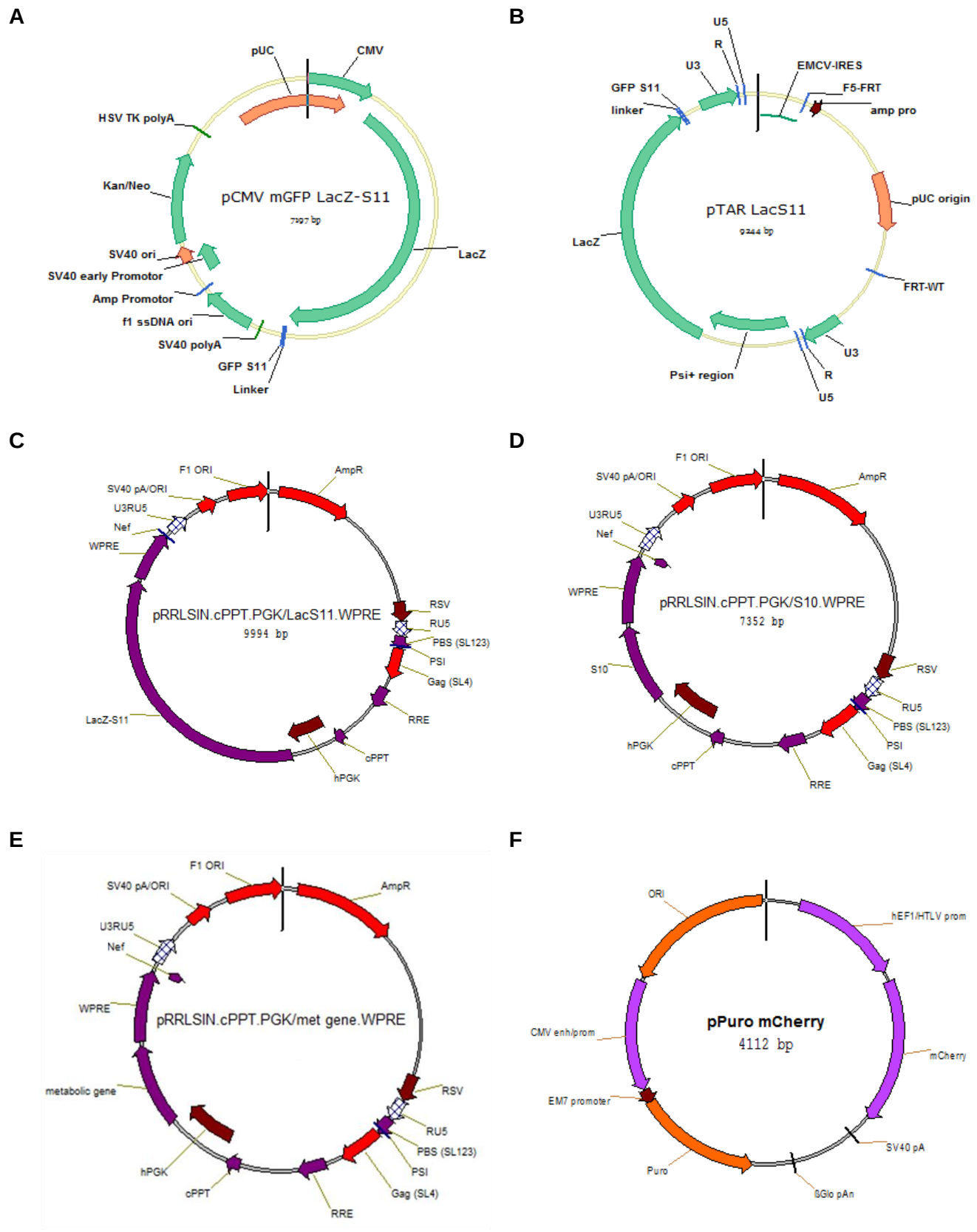

Figure S1 – Final plasmid constructs. A) pCMV mGFP LacZ-S11; B) pTAR LacS11; C) pRRLsin LacS11; D) pRRLsin S10; E) pRRLsin with metabolic gene (i.e. GSS, HIF1 inhibitor; IDH1 or IDH2); F) pPuro mCherry.

Table S2 – Final composition of supplemented culture media.

		Base composition	NC	AAs	AO	POLY	GLUT
Culture Condition	1	Advanced DMEM 10% (v/v) FBS, 4mM glutamine	-	-	-	-	-
	2	Advanced DMEM 10% (v/v) FBS, 4mM glutamine	5X	-	-	-	-
	3	Advanced DMEM 10% (v/v) FBS, 4mM glutamine	-	✓	-	-	-
	4	Advanced DMEM 10% (v/v) FBS, 4mM glutamine	5X	✓	-	-	-
	5	Advanced DMEM 10% (v/v) FBS, 4mM glutamine	-	-	1X	-	-
	6	Advanced DMEM 10% (v/v) FBS, 4mM glutamine	-	-	-	1X	-
	7	Advanced DMEM 10% (v/v) FBS, 4mM glutamine	-	-	1X	1X	-
	8	Advanced DMEM 10% (v/v) FBS, 4mM glutamine	-	-	1X	-	2 mM
	9	Advanced DMEM 10% (v/v) FBS, 4mM glutamine	5x	✓	1X	1X	2mM

NC, nucleosides supplement; AAs, amino acids supplement; AO, anti-oxidant supplement, POLY, polyamine supplement, GLUT, reduced glutathione. Final concentrations of NC, AO, POLY and GLUT were chose based on a previous screening, as those yielding the highest infectious vector productivities. AAs supplement was prepared in Advanced DMEM as a concentrated stock of the following individual amino acids (final concentration in the culture medium): serine (400 mM), asparagines (400 mM), arginine (100 mM), methionine (100 mM), phenylalanine (100 mM), leucine (300 mM), valine (300 mM) and isoleucine (300 mM); AAs were chosen based on previous consumption study by HPLC, as those highly consumed and/or rapidly depleted from the culture medium.

Table S3 – Primers used for RT PCR

	Forward primer (5’ to 3’)	Reverse primer (5’ to 3’)
RPL22	CTGCCAATTTTGAGCAGTTT	CTTTGCTGTTAGCAACTACGC
LacZ	ACTATCCCGACCGCCTTACT	TAGCGGCTGATGTTGAACTG
Gag-Pol	GTCCACTATCGCCAGTTGCT	CTGGGTCCTCAGGGTCATAA
Envelope	GGACCAAAATAGCGAATGGA	GGTGAACTGTACGCCTGGAT
Neomycin	-	AGCCATGATGGATACTTTCTCG

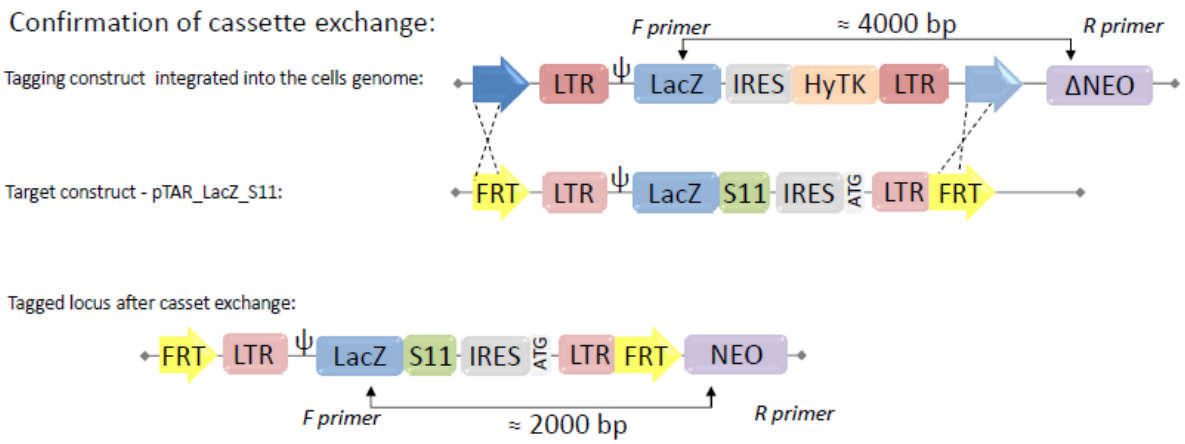

Figure S2 – Scheme of cassette exchange predictability.

Table S4 – Outcome of PCR and qRT PCR from genomic DNA of cells after RMCE.

Possible outputs		Cassette exchanged		Cassette did not exchange		Cassette excision	
	Additional integration	no	yes	no	yes	no	yes
Analysis method	PCR product	2000 bp	2000 bp	4000 bp $^{\Delta}$	4000 bp *	#	No PCR product
	qRT PCR	Clone copy number = Parental cell line copy number	Clone copy number ≠ Parental cell line copy number	—	—	—	—

Δ Excluded possibility since only GFP S10^{+} clones proceeded to analysis;
* Additional copy not detectable;
# Excluded possibility since only LacZ^{+} clones proceeded to analysis.