UNIVERSITAT AUTÒNOMA DE BARCELONA
ESCOLA D'ENGINYERIA
DEPARTAMENT D'ENGINYERIA QUÍMICA
GRUP D'ENGINYERIA CEL·LULAR I BIOPROCESSOS

Study and characterisation of human HEK293 cell line as a platform for recombinant protein production

A dissertation submitted in partial fullfillment of the requirements
for the degree of Doctor of Philosophy in Biotechnology at
Universitat Autònoma de Barcelona

Leticia Liste Calleja

July 2015

CHAPTER 7: GENERAL CONCLUSIONS AND FUTURE WORK

In this work, the study of three different approaches based on gene engineering of HEK293 cell line for the production of the recombinant protein CapPCV2 as a model protein has been conducted. The driving force behind the study was the increment on volumetric productivity towards the development of a robust, efficient and scalable bioprocess. Therefore, HEK293 cell culture has also been characterized and optimized from the bioprocess engineering perspective and HEK293 phenotype and metabolism has been well characterised in order to establish metabolic engineering tools for bioprocess optimization.

From the work developed and the obtained results it can be concluded that:

- Cell media and cell culture strategy study are key points in process development in order to increase viable cell densities. In particular, after that study it was possible to increase more than 10-fold HEK293 cell density in culture. After these good results, it would have been promising moving towards perfusion cell culture, as it is extensively reported the higher viable cell densities achieved with this culture mode in comparison to batch or fed-batch strategy. Also, higher product titres are achieved in perfusion cell cultures if the product of interest is labile or can be degraded along the cell culture period. Moreover, due to the election of stable cell lines for recombinant protein production as the best strategy (further explained some points below), the increment of cell densities may be directly related to higher protein volumetric productivities. The key parameters that should be studied in particularly detail in case that perfusion culture strategy is addressed in the future, are: cell retention systems[1], the avoidance of O_2 limitations and CO_2 excessive accumulation [1,2], determination of the optimal perfusion rate[3,4] and the implementation of control strategies for automated perfusion such as cell specific perfusion rate[1].

- Although adenoviruses are a powerful tool, they have become inefficient for the production of the selected model protein (i.e. r-CapPCV2). Nevertheless, it is proposed to further study this strategy for protein production as it came up with good results when GFP was the protein produced. In future projects it would be worth to study different infection parameters with an adenovirus expressing the model protein and a reporter protein (e.g. r-CapPCV2 fused to a fluorescence protein). It is proposed that the outcomes of this study will be more similar to the results obtained with the model protein expressed solely. The study

may be designed as the one in this thesis and enlarge it to a full-fractional experiment. Besides, the study of the best parameters for infection resulted in the assessment of a significant drop on bioprocess productivity when infected at cell densities over $1x10^6$ cell/mL. Other authors have already reported this phenomenon and it is well known as the "cell density effect"[5]. Some authors correlate the "cell density effect" with nutrient limitation and/or with toxic co-metabolites (mainly ammonia) accumulation in media. Hence, working in perfusion mode or bolus feeding after infection has been studied as methods for overcoming the limitation on cell density infection[5,6]. Other authors propose to infect at very low MOI in order to reinfect the cells during cell culture expansion. This strategy has also resulted in significantly increment adenoviral titres obtained at higher cell densities[7]. In this work, we have explored the media replacement strategy as an alternative for avoiding the "cell density effect". This strategy resulted in a significant increment of both protein production and adenoviral vector. Nevertheless, the hypothesis elucidated from the results suggest that the limitation on infecting cells at high TOI is more related to the lactate metabolic switch observed in HEK293 cells rather than to a limitation on media nutritional components. Nevertheless, this hypothesis should be further studied and the implementation of flux balances analysis for metabolic engineering (also carried in this work and explained on the last point of this conclusions chapter) may help to understand how these metabolic changes are affecting viral replication.

- The generation of stable cell lines for protein production lead to higher recombinant protein titres in comparison to protein production upon adenovirus infection. Particularly, the site directed integration of the gene of interest into HEK293 genome's loci pre-characterized as "high-transcription-sites" resulted in the best results regarding protein volumetric productivity. Nevertheless, some comments should be addressed to these results towards possible future work on generating stable cell lines. On one hand, alternative strategies for illegitimate gene insertion into the host cell genome might be studied. Based on recent literature, three methodologies are proposed: (1) lentiviral transfection (2) *sleeping beauty* system and (3) *piggy Bac* system. Lentiviral transfection results in high rates of positive transfection and also in high titers of the recombinant protein expressed[8,9]. Moreover, optimization of protocols for lentiviral vector production has been recently addressed for suspension cell lines[10]. The other two proposed systems are based on integration of foreign DNA into the host cell's genomes using transposase enzyme. Both systems have reported high protein production rates either in constitutively expression systems or inducible

expression systems[11–13]. The exploration of these strategies with HEK239-3F6 cell line used in the present work might result in high titres of the protein of interest.

- Nevertheless, as also stated in previous point, the best results obtained in this work in terms of protein production were achieved after the site-specific integration of the gene of interest by RCME technology. However, the adaption to grow in suspension of the adherent cell line obtained after RMCE technology resulted in a drop on specific production which was not completely compensated by the higher cell densities reach in suspension cell culture. Therefore, as an improvement of the bioprocess studied in this Thesis, the following strategy is proposed:
    1. Select a suspension cell line that can achieve high viable cell densities.
    2. Tag this cell line with the tagging vector encoding for the reporter gene.
    3. Perform cell sorting or end-point dilution in order to obtain individual clones.
    4. Select the best producer clones (i.e. the most fluorescent) and perform characterisation for unique integration site. This characterization will allow establishing the new master cell lines.
    5. Target the master cell line with the targeting vector encoding the gene of interest.

  It must be pointed out that the characterisation of unique integration site is highly valuable in order to generate more stable cell producer platforms which will directly results in drastic reduction of new producer cell lines when a new protein should be expressed[14].

- Recently, there has been an increasing interest on studying techniques for gene editing based on nucleases. Among them, zinc fingers nuclease (ZFNs), Transcription activator-like effector nucleases (TALENs) and Clustered regularly interspaced short palindromic repeats (CRISPRs) are the most studied and implemented. Although this techniques are not useful for the integration of complete genes within the cell's genome, they have shown to be highly efficient tools to make specific changes at targeted genomic sites enabling the change on the phenotype of the host cell (e.g. knockdown or overexpression of an specific gene). This tools, together with high-throughoutput screening tools could be used in future work to increase the productivity of the master cell lines generated by RCME.

- Also, regarding the optimization of the process under the gene engineering perspective, it must be mentioned the increment on specific productivity achieved after the optimization of codon usage of the DNA sequence encoding for the gene of interest. This fact might be born

in mind for other proteins that might be expressed in the HEK293 cell line production platform established in this work and perform codon optimisation at initial stages of the bioprocess development. Finally, the study of other secretion signal peptides to be cloned into the expression vector should be addressed in future research projects. From the author's knowledge, it would be worth cloning the secretion signal of *Gaussia Luciferasa* based on the results published in recent literature[15,16]. In fact, this is an on-going work within the research group and some promising results have already been achieved. Besides, some rational tools for the optimization of genetic sequences for the increment on protein secretion are commercially available and it might be worth to use them in future projects[17].

- The phenotypic studies addressed under different conditions of initial pH (pH_0) and lactate concentration ($[lac]_0$), arose the hypothesis that HEK293 cells can detect the unfavourable acidic environmental conditions and trigger the co-transport of H+ and lactate to the intracellular space as a mechanism of pH detoxification. The influx of lactate into the cytosol is concomitant to glucose consumption of media and both metabolites are used as carbon source for the cell. Even more important was the possibility of triggering the co-transport of H^+ and lactate from the beginning of cell culture when pH_0 was set at 6.6 and $[lac]_0$ was in the range of 4-12mM, avoiding the secretion and consequent accumulation of lactate in cell culture broth. The metabolic study through Parsimonius Flux Balance Analyisi (pFBA) mechanistically supports the proposed hypothesis. Moreover, pFBA application to HEK293 cells under different environmental situations showed how, when co-consumption of glucose and lactate occurs, cells present a more efficient and balanced metabolism. More efficient in terms that glucose consumption rates drastically drop in comparsion to the glucose unique consumption, without affecting the fluxes into the TCA cycle and, hence, getting the same amount of energy available for the cell. The "more balanced" metabolism means that when glucose and lactate are consumed together, the fluxes through the glycolytic pathway decreased and become comparable to the fluxes on the fluxes on the TCA cycle and the malate shuttle fluxes. Hence, pyruvate is not accumulated in the cytosol and $NADH+H^+$ can be regenerated, resulting in no lactate generation from pyruvate and avoiding lactate secretion. Upon the metabolic model generated in this PhD thesis, it will be able to in silico study the upregulation or downregulation of target genes in order to obtain engineered cell lines presenting an optimized metabolism, which would result in higher cell densities and higher protein titres. Besides, further effort can be directed for the obtention of more informative metabolic models. The current one still deals with the limitation of

dealing with spatial heterogeneity caused by the gradient of nutrients or signals across the different compartments within the cell[18]. In this direction, moving towards the application of dynamic metabolic fluxes would provide a practical alternative for incorporating intracellular structure. Moreover, dynamic flux balance offers the additional possibility of formulating substrate uptake kinetics and hence, partially overcoming the limitation of the current FBA when incorporating cellular regulation[19].

7.1. REFERENCES

1. Kompala, D. S. & Ozturk, S. S. in *Cell culture technology for pharmaceutical and cell-based therapies* (eds. Oztruk, S. & Hu, W.-S.) 387–416 (CRC Press, 2005).

2. Matanguihan, R. *et al.* in *Animal Cell Technology: From Target to Market SE - 95* (eds. Lindner-Olsson, E., Chatzissavidou, N. & Lüllau, E.) **1,** 399–402 (Springer Netherlands, 2001).

3. Seamans, T. & HU, Q. Kinetics of growth and antibody production by a hybridoma cell line in a perfusion culture. *J. Ferment. Bioeng.* **4,** 357–364 (1990).

4. Galvez, J. Desenvolupament i anàlisi d'un bioprocés per a l'obtenció de vectors adenovirals. (Universitat Autònoma de Barcelona, 2010).

5. Kamen, A. & Henry, O. Development and optimization of an adenovirus production process. *J. Gene Med.* **6,** S184–S192 (2004).

6. Ferreira, T. B., Ferreira, a L., Carrondo, M. J. T. & Alves, P. M. Effect of re-feed strategies and non-ammoniagenic medium on adenovirus production at high cell densities. *J. Biotechnol.* **119,** 272–80 (2005).

7. Yamada, K. *et al.* Adenovirus vector production using low-multiplicity infection of 293 cells. *Cytotechnology* **59,** 153–160 (2009).

8. Cockrell, A. & Kafri, T. Gene delivery by lentivirus vectors. *Mol Biotechnol* **3,** 184–204 (2007).

9. Mao, Y. *et al.* Lentiviral Vectors Mediate Long-Term and High Efficiency Transgene Expression in HEK 293T cells. **12,** (2015).

10. Ansorge, S. *et al.* Development of a sclable process for high-yield lentiviral vector production by transient transfection of HEK293 suspension cultures. *J. Gene Med.* 868–876 (2009). doi:10.1002/jgm

11. Saha, S. & Wilson, M. H. Transposons for Non-Viral Gene Transfer. *Gene Ther. - Tools Potential Appl.* 754 (pp.269–285) (2013). doi:10.5772/52527

12. Kowarz, E., Löscher, D. & Marschalek, R. Optimized Sleeping Beauty transposons rapidly generate stable transgenic cell lines. *Biotechnol. J.* **10,** 647–653 (2015).

13. Davidson, A. E., Gratsch, T. E., Morell, M. H., O'Shea, K. S. & Krull, C. E. Use of the sleeping beauty transposon system for stable gene expression in mouse embryonic stem cells. *Cold Spring Harb. Protoc.* **4,** 1018–1024 (2009).

14. Schucht, R., Wirth, D. & May, T. Precise regulation of transgene expression level and control of cell physiology. *Cell Biol. Toxicol.* **26,** 29–42 (2010).

15. Stern, B., Olsen, L. C., Tröße, C., Ravneberg, H. & Pryme, I. F. Improving mammalian cell factories : The selection of signal peptide has a major impact on recombinant protein synthesis and secretion in mammalian cells. *Trends Cell Mol. Biol.* **2,** 1–17 (2007).

16. Knappskog, S. *et al.* The level of synthesis and secretion of Gaussia princeps luciferase in transfected CHO cells is heavily dependent on the choice of signal peptide. *J. Biotechnol.* **128,** 705–715 (2007).

17. Stern, B. *et al.* Enhanced protein synthesis and secretion using a rational signal-peptide library approach as a tailored tool. *BMC Proc.* **5,** O13 (2011).

18. Sims, J., Manteiga, S. & Kyongum, L. Towards High resolution analysis of metabolic flux in cells and Tissues. *Curr Opin. Biotechnol.* **5,** 933–939 (2013).

19. Hjersted, J. L. & Henson, M. A. in *Methods in Bioengineering: systems analysis of biological networks* (eds. Jayaraman, A. & Hahn, J.) 149–178 (Artech House, 2009).

CHAPTER 8. MATERIALS AND METHODS

8.1. BIOLOGICAL MATERIAL

8.1.1. MAMMALIAN CELL LINES

8.1.1.1. SUSPENSION CELL LINES

Four different cell suspension lines were used in this work: one parental cell line (HEK293SF-3F6) and three producer cell lines (HEK293SF-3F6-GFP, HEK293SF-3F6-SS-IL2-opCapPCV2 and 293T17+_SS-IL2-opCapPCV2)

8.1.1.1.1. HEK293SF-3F6

The parental cell line was kindly provided by Dr.Amine Kamen (National Research Council of Canada). They are derived from 293S cells after adaptation to growth in low-calcium serum-free medium (LC-SFM). The 293SF-3F6 subclone was obtained after two consecutive cloning steps by endpoint dilution of 293SF cells with LC-SFM in 96-well plates [1]

8.1.1.1.2. HEK293SF-3F6-GFP

This clone was obtained by random insertion integration of the Green Fluorescence Protein reporter gene (GFP) after transfection of pIRESPuro2GFP vector. After 5 passages in puromycin-containing selective media, a pool of puromycin resistant cells which grew at the same growth rate as the parental cell line was selected and scaled up.

8.1.1.1.3. HEK293SF-3F6- SS-IL2-opCapPCV2

This clone was obtained by random insertion integration of the Interleukin-2 secretion signal (SS-IL2) upstream of the PCV2 capside gene (optimized sequence for mammalian cell expression). The genetic construct was encoded in pIRESPuro3_SSIL2-op*Cap*PCV2 vector. After transfection of this plasmid into HEK293-3F6 parental cell line, selection in puromycin-containing media was carried out. After 5 passages in selective media a pool of puromycin resistant cells which grew at the same growth rate as the parental cell line was selected and scaled up.

8.1.1.1.4. 293T17+_SS-IL2-opCapPCV2

From the different clones obtained by RMCE with the plasmid pIRESPuro3_SSIL2-op*Cap*PCV2 one was adapted to grow in suspension. Adaptation to grow in suspension was performed b complete

media replacement from adherent cell line media to suspension cell line media *(see next section*). It must be pointed out that CB5 was initially removed and it was progressively added.

8.1.1.2. ADHERENT CELL LINES

8.1.1.2.1. AD293

The cell line used for the obtention of rAdV-CapPCV2 viral stocks is Stratagene's AD293 cell line and was purchased from Agillent Technologies (Cat #240085). This cell line is a derivative of the commonly used HEK293, with improved cell adherence and plaque formation properties.

8.1.1.2.2. 293 MZs

Master cell lines were kindly provided by Dr R.Schucht who obtained them after electroporation of tagging vector and further characterization as unique-integration site for recombination.

8.1.1.2.3. 293T MZ

293T 17+ Master Cell used in the present work was obtained after infection at low MOI of 293T/17 cell line with a recombinant retrovirus expressing GFP (S. Spencer et al, data not published). 293T/17 cell line was purchased from ATCC (ATCC CRL-11268). This cell line is a derivative of the 293T (293tsA1609neo), which is a highly transfectable derivative of the 293 cell line into which the temperature sensitive gene for SV40 T-antigen was inserted. 293T cells were cloned and the clones tested with the pBND and pZAP vectors to obtain a line capable of producing high titers of infectious retrovirus, which was named 293T/17.

8.1.2. *E.Coli* STRAINS

8.1.2.1. DH5α

This strain was developed by D. Hanahan and it is derived from K-12 E.coli strain. DH5α is a strain that enables high-efficiency transformations. From these mutations, it should be pointed out *endA1* and *recA1* mutations. The *endA1* mutation greatly improves the quality of plasmid miniprep DNA, and the *recA* mutation helps ensure insert stability. increase insert stability and improve the quality of plasmid DNA prepared from minipreps, in addition to supporting blue/white screening. In the work here presented, this strain was the one used for mainly all the transformations and for positive

clone glycerinate stocks preparation. The corresponding phenotype is specified below (genes indicated in italics signify that the bacterium carries a mutant allele):

F- 80dlacZ M15 (lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) pho AsupE44 -thi-1 gyrA96 relA1

This strain was also the one carrying pOPING plasmid (*see section 8.5.1.4*) which was diposited at Addgene database by Dr. Ray Owens (Oxford Protein Production Facility UK, Oxford University) and purchased from Addgene.

8.1.2.2. BJ5183-AD-1 ELECTROCOMPETENT CELLS

BJ5183-AD-1, is the BJ5183 strain pre-transformed with pAdEasy-1 vector (Agillent Technologies Cat. 200157). BJ5183-AD-1 strain is *recA* proficient and supplies the machinery necessary to execute the homologous recombination event between the shuttle vector and the pAdEasy-1 vector. The genotype of this strain is as follows:

*end*A1 *sbc*BC *rec*BC *gal*K *met thi*-1 *bio*T *hsd*R (Str^R) pAdEasy-1 (Amp^R) [2]

The efficiency of recovery of recombinant vectors is greatly improved by using the BJ5183-AD-1 strain: up to 80–90% of kanamycin resistant colonies are recombinants using this single transformation system, compared to 20% recombinants using the traditional cotransformation procedure.

8.1.2.3.XL10-Gold QUIMIOCOMPETENT CELLS

XL10-Gold ultracompetent cells, were used to amplify the vector encoding plasmid encoding for Adenovirus genome and the gene of interest (which was obtained after recombination of pShuttle and pAdEasy-1). The cells were provided within AdEasy XL Adenoviral system and they can be purchased from Agillent Technologies (Cat. 200314). This strain is both endonuclease deficient (*endA1*) and recombination deficient (*recA*). The genotype of the strain is detailed below:

Tet^R Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F′ *proAB* $lacI^qZ\Delta M15$ Tn*10* (Tet^R) Amy Cam^R] [3,4].

8.1.2.4. DH10B

As DH5α strain, DH10B is derived from *E.coli* K-12. In this work, DH10B (also known as TOP10) were used for pTargeting plasmid obtenition and amplification. Main genomic features of the strain are as follows:

F- endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lacZΔM15 ara D139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ-

8.2. CELL CULTURE MEDIA

8.2.1. MEDIA FOR SUSPENSION MAMMALIAN CELL CULTURE.

8.2.1.1. BASAL CELL MEDIA

Different cell culture media have been tested in this work (Table 8.1). All of them have been specially designed for HEK293 cell culture and manufactured under cGMP. Their composition is company proprietary but all of them are chemically-defined and animal derived component free.

Table 8.1. Basal cell media initially used for cell culture of HEK293-3F6 suspension cell line.

Cell media	Format	Manufacturer	Reference
CDM4 HEK293™	Liquid	HyClone	SH30858.02
SFM4HEK293	Liquid	HyClone	SH30521.02
SFM4Transfx-293™	Liquid	HyClone	SH30860.02

8.2.1.2. SUPPLEMENTS

CDM4 HEK293™ and SFM4Transfx-293™ were always supplemented with GlutaMAX (200mM, Gibco) to a final concentration of 4mM (i.e. 20mL per 1L of media). This supplementation was not necessary for SFM4HEK293 media as it is L-glutamine containing media.

Whenever was necessary, media were supplemented with different nutritional supplements and/or antibiotic. Preparation of stock solution of the supplements and antibiotics for suspension cell media is specified in the Table 8.2.

Table 8.2. Suplements added to cell media used for suspension cell line culture.

Supplement	Manufacturer	Stock Solution	Final concentration	Reference
FBS	Sigma Aldrich	--	5% (v/v)	F9665
CB1	HyClone	40 g/L	10% (v/v)	SH30584.01
CB5	HyClone	80 g/L	10% (v/v)	SH30865.01
CB6	HyClone	40 g/L	10% (v/v)	SH30866.01
Pluronic F-68	Sigma Adrich	10% (w/v)	50ppm	P1300
Antifoam C	Sigma Aldrich	1% (w/v)	0.2% (v/v)	A8011
Puromycin	Sigma Aldrich	1mg/mL	1-1.25 µg/mL	P8833

FBS

Fetal bovine serum (FBS) is the sterile liquid that is obtained from the clotted blood of the bovine fetus and it contains numerous factors that are needed for the survival and propagation of mammalian cells in culture [5]. FBS used in this work was purchased from Sigma Aldrich (lot Number: 043M3397) is heat-inactivated, South American origin serum. Heat inactivation (incubation at 56ºC for 30minuts) is necessary for complement's inactivation. The complement system consists on a number of small proteins found in the blood, which act as unspecific elements of immune system and that can trigger cell lysis. FBS was stored at -30ºC. In order to avoid extensive freezing-thawing cycles of stock solutions, sterile aliquots of 50mL were routinely prepared from 500mL FBS bottles.

Cell Boosts

Cell Boosts (CBs) are supplements designed to increase cell productivity in a variety of cell lines. They are designed to provide nutrients such as amino acids, vitamins, lipids, colesterol, growth factors and glucose. These supplements aid in fed-batch culture applications by providing concentrated nutrient solutions. CB1, CB5 and CB6 were purchased in powder format. They were dissolved in MilliQ water under continuous stirring and pH was adjusted to 7.2 adding NaOH (50M) drop-by-drop. The solutions were sterile filtered using 0.22 µm filter units with filling bell (Millipore, Cat SVGVB1010). Solutions were stored at 4ºC for a maximum of 6 monts.

Supplements for bioreactor culture (Pluronic and Antifoam C)

Pluronic F-68 is a non-ionic detergent that protects cells from hydrodynamic damage and Antifoam C is a 30% solution of Polydimethylsiloxane (PDMS) which supress the formation of foams. Therefore, both supplements were added in bioreactor cell cultures, where mechanical agitation is performed.

Pluronic F-68 and Antifoam C were disolved and diluted (respectively) in MilliQ water and heat sterilized in the autoclave (120ºC, 100kPa, 20 minutes). Solutions could be stored at 4ºC for a maximum of 6months.

Antibiotic for selective media (Puromycin)

Puromycin is an aminonucleoside antibiotic produced by the bacterium *Streptomyces alboniger*. It inhibits protein synthesis by disrupting peptide transfer on ribosomes causing premature chain termination during translation. Puromycin stock solution was prepared adding 1mL of sterile water to the vial containing 10mg of the antibiotic in powder format provided by the manufacter. The entire amount of antibiotic was gently resuspended by pipetting until complete dissolution was observed. Then, the milliliter was recovered and diluted into 9mL of sterile water to achieve a final concentraction of 1mg/mL. Finally, the solution was filtered through a 0.22µm membrane (Sterivex-GP filters, Millipore) and was dispensed into eppendorfs as aliquots of 0.5mL in order to avoid extensive freezing-thawing cycles. The aliquots were stored at -20ºC. At this temperature the antibiotic remains stable at least two years.

In Table 8.3 final cell media compositions for the suspension cell lines specified in section 8.1.1.1. are summarized.

Table 8.3. Final composition of media used for suspension cell culture.

Cell line	Cell media	Supplements
HEK293SF-3F6	SFMTransFx293™	4mMGlutaMAX 5% (v/v) FBS 10% (v/v) CB5 (80g/L)
HEK293SF-3F6-GFP	SFMTransFx293™	4mMGlutaMAX 5% (v/v) FBS 10% (v/v) CB5 (80g/L)
HEK293SF-3F6-SS-IL2-opCapPCV2	SFMTransFx293™	4mMGlutaMAX 5% (v/v) FBS 10% (v/v) CB5 (80g/L) 1 µg/mL puromycin
293T17+_SS-IL2-opCapPCV2	SFMTransFx293™	4mMGlutaMAX 5% (v/v) FBS 5% (v/v) CB5 (80g/L) 1.25µg/mL puromycin

8.2.1.3. FED-BATCH MEDIA

Cell media compositions specified in Table 8.3 were used for batch cell cultures. For the fed-batch strategies explained in Chapter 3 (section 3.4.2) the feeding media was in-home formulated and its composition is specified in Table 8.4

Table 8.4. Composition of fed-batch media

	Manufacter	Stock solution	Final conc. (% v/v)
CB5	HyClone	80g/L	10
Glucose	Sigma-Aldrich	200g/L	25% (=277.5mM)
FBS	Sigma-Aldrich	--	5%
Amino acids MEM	Gibco	50x	9%
Vitamin MEM	Gibco	100x	9%
GlutaMAX	Gibco	100x	8%
Antifoam C	Sigma-Aldrich	10%(v/v)	2% (=200ppm)
MilliQ H_2O	--	--	--

8.2.2. MEDIA FOR ADHERENT MAMMALIAN CELL CULTURE

8.2.2.1. BASAL CELL MEDIA

Two different cell media were used in this work for adherent cell lines culture (Table 8.5). For each cell line different supplementation was performed. These supplements are specified in the Table 8.6 and the stock solution preparation is explained below (except for those supplements which were purchased ready to use). Final composition of each cell media is summarized in the Table 8.7.

Table 8.5. Basal cell media used for cell culture of adherent cell lines.

Cell media	Format	Manufacturer	Reference
DMEM	Powder	Sigma Aldrich	D-7777
DMEM	Liquid	Gibco	31966-021
RPMI	Powder	Gibco	51800-035
RPMI	Liquid	Gibco	61870-044

8.2.2.2. SUPPLEMENTS

When media in powder format were used, 100mL of Hepes (1M) and 16.8g or 37.5g of $NaHCO_3$ (RPMI and DMEM respectively) were added to 10L of media and pH was adjusted to 6.9-7.1. Cell media were finally sterile filtered through a 0.22µm membrane and stored at 4ºC for no more than 3 months.

Table 8.6. Suplements added to cell media used for adherent cell line culture.

Supplement	Manufacturer	Stock Solution	Final concentration
FBS	Sigma Aldrich	--	5% (v/v)
Glutamine	Serva	0.2M	2mM
GlutaMAX	Gibco	200mM	4mM
Penn-strep	Sigma-Serva	17mM-13mM	170µM-13µM
Geneticin	Gibco	100mg/mL	1mg/mL
Ganciclovir	Gibco	10mM	10µM
Puromycin	Sigma Aldrich	1mg/mL	1.25 µg/mL

Glutamine

Glutamine is a conditionally essential aminoacid, meaning that their synthesis can be limited under special pathophysiological conditions, such as prematurity in the infant or individuals in severe catabolic distress. The stock solution was obtained dissolving 29.24g of glutamine into 0.8L of MilliQ water under continuous stirring. After complete dissolution, MilliQ water was added until 1L using a volumetric flask. Finally, the solution was filterered through a 0.22µm membrane and aliquots of 50mL were prepared and frozen at -20ºC.

Antibiotics

Penn-Strep

Penn-Strep is a solution of two antibiotics (ampicillin and streptomycin), which is active against bacterial infection. 6.06g of the former antibiotic and 10.00g of the latter were dissolved in 0.8L under continuous stirring. pH was corrected adding NaOH (1M) to a final value of 7.2. Finally, MilliQ water was added to a volume of 1L, the solution was filtered through a 0.22µm membrane and aliquots of 50mL were prepared and frozen at -20ºC.

Geneticin

Geneticin is an analog of neomycin sulfate produced by *Micromonospora rhodorangea* and it interferes with the function of 80S ribosomes and protein synthesis in eukaryotic cells. Resistance to G418 is conferred by the Neomycin resistance gene (neo**)** from Tn5. 10g of geneticin sulfat were disolved under continuous stirring in a final volume of 100mL of MilliQ water. After complete solution, it was sterile filtered through a a 0.22µm membrane and aliquots of 10mL were prepared and frozen at -20ºC.

Puromycin

Preparation of this antibiotic is explained in the previous section.

In Table 8.7 final cell media compositions for the adherent cell lines specified in section 8.1.1.2. are summarized.

Table 8.7. Final composition of media used for adherent cell culture.

Cell line	Cell media	Supplements
AD293	DMEM	4mMGlutaMAX 10% (v/v) FBS
293 MZs (parental cell line)	RPMI	1%(v/v) glutamine (if powder RPMI is used) 10% (v/v) FBS
293T MZs (parental cell line)	DMEM	1%(v/v) glutamine (if powder DMEM) or 4mM GLutaMAX 10% (v/v) FBS 1% (v/v) penn-strep
293 clones (after RMCE)	RPMI	1%(v/v) glutamine (if powder RPMI) 10% (v/v) FBS

		1% (v/v) penn-strep 1 mg/mL Geneticin 10µM Ganciclovir
293T clones (after RMCE)	DMEM	1%(v/v) glutamine (if powder DMEM is used) or 4mM GLutaMAX 10% (v/v) FBS 1% (v/v) penn-strep 1.25µg/mL puromycin

8.2.3. MEDIA FOR BACTERIAL CELL CULTURE

8.2.3.1. LYSOGENY BROTH OR LURIA BROTH (LB MEDIUM)

LB medium is a nutritionally rich medium and it is primarily used for the growth of bacteria. There are several common formulations for LB. The one used in this project is detailed in the Table 8.8.

Table 8.8. Luria Broth media composition.

Component	Concentration (g/L)	Provider
Peptone	10	Oxoid
Yeast extract	5	Oxoid
NaCl	10	Sigma Aldrich

MilliQ water was used for the dissolution of the components of LB media under continuous magnetic stirring. After complete dissolution, pH was adjusted to 7.3 by NaOH (10M) addition. Due to the high nutritional content of the media, it must be sterilized after preparation. Sterilization was performed by autoclave (1 cycle 30min 121ºC). LB media is stored at room temperature for a maximum of 6 months. The addition of antibiotics is done at the onset of cell culture.

8.2.3.2. LURIA AGAR MEDIUM (LA MEDIUM)

Preparation of solid LB medium for making culture plates was done by the addition of 20g of Agar (Oxoid, Ref. LP0011) to 1L of LB media prior to volume and pH adjustment. Then, medium is autoclaved and let to cool down until approximately 50ºC before the addition of the antibiotics. In order to ensure homogeneus mixture of antibiotic with the medium but avoiding bubble formation, bottle containing LA medium should be rolled inside the flux chamber. For making the plates, gently pour the media into the Petri plates inside the flux chamber and wait until agar has solidified completely. Store the plates at 4ºC until a maximum of 3 months.

It is also possible to store LA medium at room temperature for a maximum of 6 months. In this case,

agar must be dissolved by heating media either in a microwave or in a water bath. When it is complete dissolved, let medium cool down to 50ºC and add the antibiotics before making the plates.

8.2.3.3. SUPER OPTIMAL BROTH WITH CATABOLITE REPRESSION (SOC MEDIUM)

SOC medium is a nutrient-rich bacterial growth medium used for growth of E. coli after transformation. Using this medium instead of LB results in higher transformation efficiencies of plasmids. Composition of SOC medium is exactly the same as LB medium but the components specified in Table 8.9 must be added prior to use.

Table 8.9. Specific components of SOC medium

Component	Stock solution conc. (M)	Volume added to 1L of LB (mL)	Final Conc. (mM)	Provider
$MgCl_2$	1	10	10	Sigma-Aldrich (M2670)
$MgSO_4$	1	10	10	Panreac (141404)
Glucose	2	10	20	Panreac

All components must be sterilised before their addition to LB medium (which must be already sterile). $MgCl_2$ and $MgSO_4$ are sterilised by filtration through a 0.22µm membrane and glucose is sterilized by autoclave. All the stock solution must be stored at 4ºC for a maximum of 6 months.

8.2.3.4. ANTIBIOTICS

Antibiotics are added to cell culture media for ensuring that only microorganism carrying the plasmid(s) of interest can grow in cell culture. Along this project different E.coli clones should be selected by the addition of different antibiotics. Stock and final concentration of them is detailed in Table 8.10 and the protocol for their preparation is explained below:

Table 8.10. Antibiotics used for E.coli clones selection

Antibiotic	Stock solution concentration (mg/mL)	Working Concentracion (µg/mL)	Provider
Ampicillin	100	100	Sigma Aldrich (A0166)
Kanamycin	50	50	Sigma Aldrich (K4000)
Chloramphenicol	25	25	Sigma-Aldrich (C0378)

Ampicillin

Ampicillin is a semi-synthetic derivative of penicillin that interferes with peptidoglycan cross-linking and thus inhibits cell wall synthesis. It is a broad-spectrum antibiotic and it can be inactivated by β-lactamases [6].

The desired amount for a final concentration of 100mg/mL is dissolved in MilliQ water under continuous stirring. The solution is sterile filtered through a 0.22µm membrane, dispensed in 1mL aliquots and stored at -20ºC. It is also possible to store the solution at 2-8ºC up to three weeks. In cell culture (37ºC, pH≅7) ampicillin is stable up to three days.

Kanamycin

Kanamycin sulfate is a broad spectrum aminoglycoside-antibiotic derived from *Streptomyces kanamyceticus.* The product acts by binding to the 70S ribosomal subunit, inhibiting translocation and eliciting miscoding. This antibiotic can be altered by aminoglycoside-modifying enzymes (including acetyltransferase, phosphotransferase, nucleotidyltransferase) preventing its interaction with ribosomes [7].

Kanamycin is disolved in MilliQ water under continuous stirring. The solution is sterile filtered through a 0.22µm membrane, dispensed in 1mL aliquots and stored at -20ºC. In cell culture (37ºC, pH≅7) kanamycin is stable up to five days.

Chloramphenicol

Chloramphenicol is a synthetic broad spectrum antibiotic, isolated from strains of *Streptomyces venezuelae.* This antibiotic inhibits bacterial protein synthesis by blocking the peptidyl transferase step by binding to the 50S ribosomal subunit and preventing attachment of aminoacyl tRNA to the ribosome. It also inhibits mitochondrial and chloroplast protein synthesis and ribosomal formation of (p)ppGpp and de-pressing rRNA transcription. Chloramphenicol is disolved in EtOH under continuous stirring. The solution is sterile filtered through a 0.22µm membrane, dispensed in 1mL aliquots and stored at -20ºC.

8.3. CELL LINE MAINTENANCE

8.3.1. MAMMALIAN CELL LINE MAINTENANCE

8.3.1.1. THAWING

All cell lines described in section 9.1.1 were kept frozen in sterile criovials (Nunc 377267) inside a liquid N_2 container at -196ºC. In order to ensure an optimal recovery of the cells after thawing (i.e. high viability, low aggregation and short growth lag phase-if any-), it is mandatory to perform the protocol detailed below as fast as possible.

1. Dispense 9 and 10mL of cell media in two sterile centrifuge tubes of 15ml and warm them up to 37ºC in a water bath.
2. Take out the criovial from the liquid N_2 container and immediately put it into a water bath previously warmed at 37ºC. It is important to avoid contact of the water with the cap of the criovial. When thawing starts, shake the criovial until only a little piece of ice remains in the criovial.
3. Inside the laminar flow cabinet, pipette once the liquid in the criovial to ensure complete thawing. Immediately, take all the volume in the criovial and dispense it into the falcon with 9 mL of media.
4. Centrifuge at 300g for 5 minutes. Discard supernatant in order to remove DMSO containing media and gently resuspend the pellet by manual tapping.
5. Add 10mL of pre-warmed cell media and culture cells in a 75cm^2 t-flask without shaking.
6. In case of suspension cell lines, change from static culture to shaking culture is performed 48 hours post thawing.

8.3.1.2. FREEZING

Cells to be frozen must be in the middle of their exponential growth phase and with viability higher than 90%. Cell media used for freezing is the same media of cell culture for each cell line with the addition of DMSO (Sigma, Ref. D2438). It must be mentioned that for 293T cell line, FBS concentration was increased to 15% in the freezing media.

1. Prepare freezing media by adding DMSO to culture media to a final concentration of 15% (v/v). It is recommended to prepare freezing media just prior to cell freezing.

2. Dispense 0.5mL of freezing media into the criovials.
3. Centrifuge cells to be frozen at 200g for 8 minutes. Discard the supernatant and resuspend them by gently tapping the falcon. Add cell culture media in order to get a final concentration of 5-8x10^6 cell/mL.
4. Dispense 0.5mL of cell culture into the criovials, which contain the freezing media. By doing so, final concentration of DMSO will be 7.5% (v/v).
5. Turn the criovials upside down once in order to homogenize freezing cell media and cell culture.
6. Immediately place the criovial into the MrFrosty™ Freezing Container (Thermo Scientific , Ref. 5100-0001) and directly place it at -80ºC freezer.
7. After 24 hours take out the criovials from MrFrosty™ Freezing Container and place them into the liquid N_2 container.

8.3.1.3. CELL CULTURE MAINTENANCE: SUBCULTURE

8.3.1.3.1. Suspension cell lines

Cells were cultured in 125ml shake flasks (Corning Inc.) shaked at 110 rpm on an orbital shaker (SSL1, Stuart) at 37ºC in a humidified atmosphere with 5% CO_2 incubator (Steri-cult 2000 Incubator, Forma Scientific). Two times a week, cell passaging was routinely performed, seeding 13 mL of culture at a cell density of 0.25x10^6 cell/mL. Cultures were grown up to 2x10^6 cell/mL and then a new passage was carried out. Steps for suspension cell line subculture are as follows:

1. Take a sample from cell culture and count cells in order to calculate cell density in culture.
2. Calculate the volume to reach the desired final concentration (0.25x10^6 cell/mL) in a final volume of 13mL. Equation 8.1 is used to work out this volume.

$$V_1 = \frac{V_2 \cdot C_2}{C_1} \qquad \text{Eq.8.1}$$

Where

V_1 is the volume of initial cell broth needed for subculture

v_2 is the final volume after cell passage (i.e. 13mL)

c_2 is the desired viable cell concentration of the new cell passage after subculture (i.e. 0.25x10^6 cell/mL)

c_1 is viable cell density before subculturing

3. Remove cell culture from the shake flask until only V_1 previously calculated remains in the shake flask.
4. Add fresh cell media previously warmed up to 37ºC to a final volume of 13mL.

8.3.1.3.2. Adherent cell lines

Adherent cell lines were subcultured in 25cm^2 t-flasks (TPP, Ref. 90026 or NUNC, Ref.156367) at 37ºC in a humidified atmosphere with 5% CO_2 incubator (Steri-cult 2000 Incubator, Forma Scientific). It is important that cells never exceed 70% confluence. In order to ensure that, twice a week cell passaging was routinely performed as follows:

1. Remove cell media from the t-flask.
2. Rinse the t-flask with 3mL of sterile PBS in order to remove any trace from FBS. It is important to take care of not disrupting cell monolayer.
3. Add 1mL of tripsin-EDTA 0.05% (v/v) (Invitrogen, 10010-031) and place the cells inside the incubator at 37ºC for 3 minutes.
4. Immediately add 4mL of culture media (which contains FBS) in order to inactivate tripsin.
5. Gently pippete the media to the bottom of t-flask in order to recover all the cells from the monolayer. Then, pipette cell culture in order to homogeneise it.
6. Take a sample of cell culture and count cells in order to calculate cell density.
7. Calculate the volume needed to have an inoculum of 4x10^4 cell/cm^2. This volume is calculated using the Equation 9.1.
8. Remove cell culture from the t-flask until only V_1 previously calculated remains in the flask.
9. Add fresh cell media previously warmed up to 37ºC until a final volume of 5mL.

8.3.2. *E.Coli* STRAINS MAINTENANCE

8.3.2.1. THAWING

8.3.2.1.1. From glycerinate stocks

E.coli strains are kept frozen in sterile criovials (Nunc 377267) at -80ºC. When it is required one criovial is thaw following the protocol below:

1. Take the selected criovial out of the freezer and immediately place it in a box filled with ice.
2. Inside a flux chamber, allow the criovial to thaw at room temperature (this should not take more than 3-5minutes).
3. During this time add the corresponding antibiotic to an erlenmeyer containing 13.5mL of pre-warmed LB medium. Volume of LB is determined in order to make 1:10 dilution of the criovial content.
4. When glycerinate thawing is almost complete, pippete once the content inside the criovial and dispense it into the erlenmeyer.
5. Grow cells overnight in an incubator at 37ºC with an agitation of 150 rpm.

8.3.2.1.2. From Cryo-beads

E.coli strains can also be kept frozen in Cryo-beads (AES Chemunex, Ref. AEB400100). This system is a little tube containing beads on which microorganisms can stick. The beads are immersed in a hyper tonic cryo-preservative solution. Once they are inoculated, the tubes are stored at -80ºC.

1. Take the tube out of the freezer and place it in a box filled with ice.
2. Inside the flux chamber, open the tube and remove one bead, using sterile tweezers.
3. Roll the bead on a surface of an agar plate.
4. Incubate the plate overnight at 37ºC in a heater.
5. The next day, pick one colony up and immerse the cells into liquid LB. Incubate at 37ºC with an agitation of 150 rpm until reaching the desired OD.

8.3.2.2. FREEZING

8.3.2.2.1. Glycerinate stocks

At the moment of freezing cells must be in the growth middle log phase and volume of cell culture must be enough to make *n* aliquots at an OD_{600}=2.5. By making this, and following the thawing protocol previously explained, cells will be in perfect conditions after thawing (i.e. OD_0=0.25 coming from a “young” cell culture) which will reduce growth lag phase. The protocol for making glycerinate stocks is as follows:

1. Grow E.coli until cell culture reaches middle log phase (OD_{600}=0.4-0.6)

2. Dispense cell broth in a sterile centrifuge tube and centrifuge it at 1500g for 10 minutes at 4ºC.
3. Remove supernatant and resuspend the pellet by manual tapping.
4. Add LB medium and sterile glycerol (80% v/v in water) in equal volume (1:1 ratio).
5. Homogeneize cell culture and dispense it into the criovials (1.5mL/criovial).

8.3.2.2.2. Cryo-beads

Optimal cell growth before freezing is the same than for glycerinate stocks. At the desired OD, the next steps should be followed:

1. Inoculate the tube with fresh and pure culture.
2. Close the tube and turn it upside down to spread the bacteria evenly.
3. With a sterile pipette, remove the maximum of solution from the tube. Then place the cap back on.
4. Store the cryo-beads tubes at -80ºC.

8.3.2.3. CELL CULTURE MAINTANCE: SUBCULTURE

Due to the fast growth kinetics of E. coli, the strains and the desired clones are kept at 4ºC on Luria Agar plates during the experiments. Cells must be replated in a fresh medium plate twice per month. For the generated clones carrying the expression vector of interest, it is important to add the antibiotic to the plates, in order to guarantee presence of the expression vector.

8.4. CELL CULTURE SYSTEMS

8.4.1. SUSPENSION MAMMALIAN CELL LINES

8.4.1.1. SHAKE FLASK

Plain bottom polycarbonate shake culture flasks (Corning Inc., Ref. 431143) were used in this work for suspension cell culture. These culture shake flask are available with standard two-position plug seal caps or filtered vent caps. The second option was the chosen one as it enables continuous gas exchange while ensuring sterility and preventing leakage. 125mL culture flasks were used for cell maintance and for the majoriy of the experiments while 1L flasks (Corning Inc., Ref. 431147) were

used for scaling up cell culture prior to the onset of the experiments or for protein production. Shake flask were placed on an orbital shaker (SSL1, Stuart) with stirring speed set at 110rpm. The shaker was kept in an incubator (Steri-cult 2000 Incubator, Forma Scientific) at 37ºC with an humidified atmosphere (95%) to avoid excessive cell media evaporation and CO_2 wa set at 5% in order to control pH of buffered cell media.

8.4.1.2. SFR-RAMOS (Kühner AG) CELL CULTURE SYSTEM

For the metabolic experiments presented on Chapter 4 a new combination of Shake Flask Reader (SFR) and Respiration Activity Monitoring System for online determination of OTR and CTR (RAMOS) was used (Kühner AG).

The SFR from PreSens (Precision Sensing GmbH) is an online measurement device that determines valuable parameters such as dissolved oxygen and pH in shake flasks in a non-invasive manner. The two main components of the device are the ready-to-use vessels, which contain pre-calibrated sensor spots (in our case, 250mL shake flasks (Corning, Inc.)) and the SFR main unit, which is responsible of exciting the luminiscence dyes embedded in the sensor spots (Figure 8.1). The resulting luminescence is read by the SFR and this signal is converted to actual pH and DO measurements inside the flask. The luminescence lifetime depends upon the dissolved oxygen concentration and the pH of the sample. The SFR is screwed directly on to the tray of Kühner incubator shaker. Therefore, cells can be cultivated at 37ºC in an humidified atmosphere (95%) and 5%CO_2.

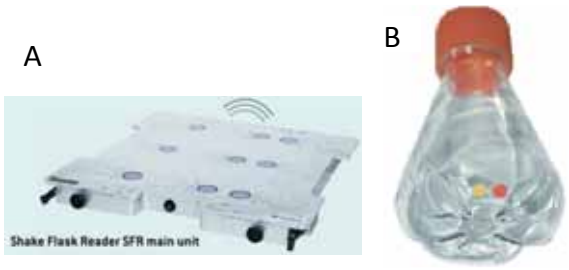


Figure 8.2. (A) Shake Flask Reader main unit. (B) 250mL shake flask with pH and pO_2 sensors fixed at the bottom .

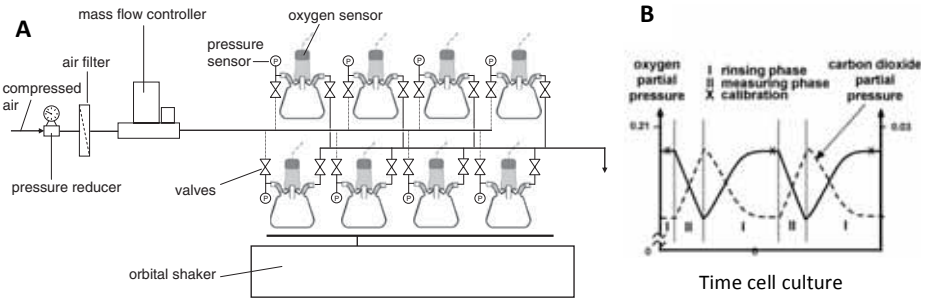


Figure 8.2.(A) General set-up of RAMOS. (B) Partial pressure of oxygen (continuous line) and carbon dioxide (dashed line) during a measuring cycle.

Figure 8.1. Set up ot the integrated SFR and RAMOS system.

RAMOS was presented by Anderlei et al. [8,9]. This system determines the oxygen transfer rate (OTR), the carbon dioxide transfer rate (CTR) and the respiratory quotient (RQ) of microbial, plant and cell cultures online. During cell culture a measuring cycle is continually repeated (Figure 8.2b). This measuring cycle is separated into a measuring and a rinsing phase. During the rinsing phase air flows through the measuring flasks. At the beginning of the measuring phase the inlet and outlet valves of the measuring flasks are closed. The sustained respiration activities of the microorganisms lead to a change in the partial pressure of oxygen and carbon dioxide in the headspace of the particular measuring flasks. At the end of the measuring phase a computer uses these changes in partial pressure to calculate the oxygen (OTR) and the carbondioxide transfer rate (CTR).

Both systems were integrated for the experiments presented in this work, resulting in the experimental set up depicted in Figure 8.3.

8.4.1.3. STIRRED TANK BIOREACTOR: BIOSTAT BPLUS

The stirred tank birreactor Biostat Bplus (Sartorius Stedim Biotech) has been designed to fulfill the widest range of R&D, process development and small-scale production demands. It is a compact system composed of two main elements: the monitoring and digital control unit (DCU) and the fermentation vessel (Figure 8.4).

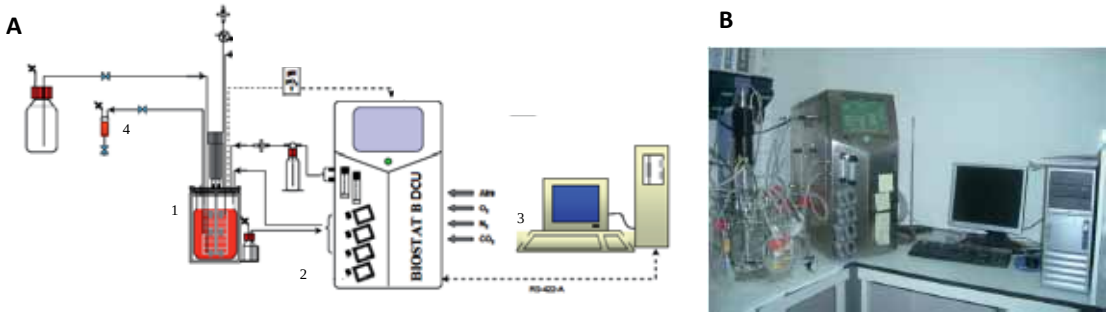


Figure 8.3. (A) Scheme of STR bioreactor operating in batch mode. (1) 2L stirred glass vessel (2) Control unit: pH, PO2 and temperature sensors; buffer addition control and gas mixture and aireation control. (3) Computer for data adquisition and parameter control (4) Sample adquistion.(B) General set-up of STR Biostat Bplus, batch mode operation.

The DCU performs the data adquisition and has the functions for sensor calibration and control loop management of basic variables. These variables are tempertaure, pH and disolved oxygen (pO_2). It can be also integrated into a hierarchical automatisation system in order to modify the pre-defined control parameteres. The software used in the present work for monitoring and control was MFCS/win 3.0 (Sartorius Stedim Biotech). Set points of the variables and how they are monitored and controlled are briefly explained below:

- Temperature (37ºC). The double-wall vessel enables the water to flow around the bioreactor in order to maintain the desired temperature. The heating element is an internal electric resistance and the cooling element is water comming from general pipeline.

- pH (6.6 or 7.1). The monitoring of this parameter was performed using a pH electrode (Hamilton, Easyfer, Plus). The acid correctors were CO_2 injected through a solenoid valvule and HCl solution (0.5M) which was added by a peristaltic pump. A solution of NaOH (1M)

was used as basic corrector and it was added also by a persiltaltic pump. It must be pointed out that for some experiments the addition of NaOH was avoided.

- pO_2 (60%): To properly monitor pO_2 in cell broth a polarographic electrode was used (Hamilton, Oxyferm FDA). The DCU has four independent gas inlet channels (air, N_2, O_2 and CO_2), a gas mix chamber and two independent gas outlet cannel each of them provided with a mass flowmeter. By the proper mixture of the gases, pO_2 is controlled.

The fermentation vessel is made from borosilicate glass and the ratio height/diameter is 2:1. The maximum capacity is 2.5L but the maximum working volume was 2L. It has a round bottom design for optimal mixing results. The stainless steel headplate is provided with 9 ports of 11mm, 2 ports of 19mm and 3 ports of 26 mm. This ports are used to connect the sampling unit, probes, gas inlet and outlet, media feeding inlet, pH-control solutions, etc.

8.4.2. ADHERENT MAMMALIAN CELL LINES

8.4.2.1. TISSUE FLASK

25cm^2 tissue flasks (TPP, Ref. 90026) were used in this work for adherent cell line maintance. These flasks were opto-mechanical treated in order to enhance cell adhesion and cell growth. The filter screw cap has a hydrophobic PTFE membrane with a pore size of 0.22 µm. This feature offers protection against contamination with optimal gas exchange at the same time. When it was required, cells were scaled up to 300cm^2 tissue flasks (TPP, Ref.90301).

8.4.2.2. CellSTACK

For protein production experiments, cell stacks (5-stack with 3180cm^2 growth area (Corning, Inc., Ref. CLS3311) were used. They have two 26 mmm diameter filling and venting ports which allow direct access to the chamber bottom. The standard 33mm threaded caps have 0.22 µm pore, hydrophobic membrane sealed directly to the caps, allowing gas exchange while minimising the contamination risk. To ensure homogeneous dsitribution of cells and media, the following steps must be followed:

1. Carefully pour the media and the inocula through one of the filling ports. Tilting the chamber slightly towards the filling port while filling will reduce foam generation.

2. Place the CellSTACK on its side and then turn the it 90º so that the filling and venting ports are up (Figure 8.5) in order to equilibrate the medium in each chamber. It is normal for the medium level in the bottom chamber section to be slightly higher.

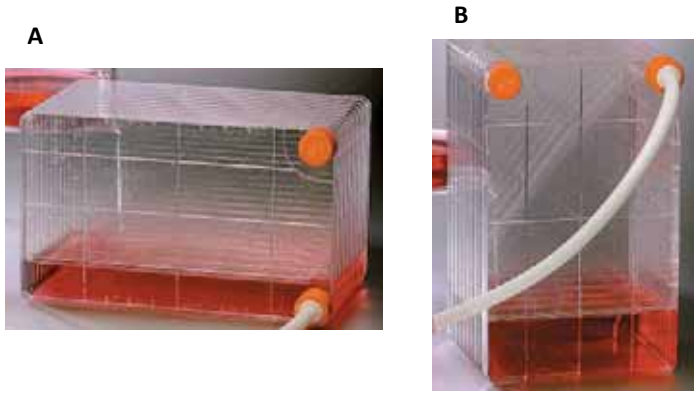


Figure 8.4. (A) Initial posiion of cell stack after filling it with cell media. (B) Coorect position of cell stack after turning it 90º for medium equilibration in each chamber.

3. Gently lower the CellSTACK to its normal horizontal incubation position.
4. Tilt the chamber back and forth until the surface of each chamber is completely covered with medium. This will ensure an even distribution of the cells across each of the growth surfaces.

8.4.3. *E.coli* CELLS

Plastic Petri dishes (Thermo Scientific) were used in this work for E.coli transformation and subculture. When necessary cells were grown in LB medium in glass erlenmeyers.

8.5. PLASMIDS

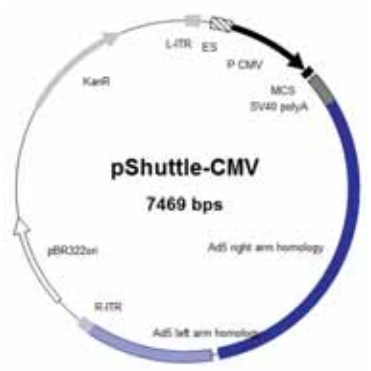
A plasmid is a small DNA molecule within a cell that is physically separated from a chromosomal DNA and can replicate independently. Commonly, they are found as small circular, double-stranded DNA molecules in bacteria. In nature, plasmids carry genes that may benefit survival of the organism (e.g. antibiotic resistance), and can be transmitted from one bacterium to another (even of diferent species) via horizontal gene transfer. While the chromosomes are big and contain all the essential information for living, plasmids usually are very small and contain additional information. Plasmids can also be used in genetic engineering in order to obtain large quantities of a gene of interest (GOI) in a short time. This gene is inserted in the artificial plasmid (also called expression vectors or vectors) and bacteria are transformed with the vector. As cell number increases, so does it the

number of plasmid copies. Another application of plasmids is conferring the capacity to an organism to express a foreign gene by introducing this gene via transformation (bacteria and yeast) or transfection (mammalian and insect cells).

8.5.1. BACKBONE VECTORS

8.5.1.1. pShuttle-CMV

The vector pShuttle-CMV contains a multiple cloning site sandwiched between the CMV promoter and the SV40 polyadenylation signal and is suitable for insertion of a large cDNA (up to 6.6 kb). The regions indicated as arms (Figure 8.6) are the stretches of sequence homology with pAdEasy-1 where the homologous recombination occurs. The R-ITR and L-ITR regions are short inverted terminal repeats (Left and Right) which have a role in replication of the viral DNA. pShuttle-CMV contains the pBR322 origin of replication and a Kanamycin resistance open reading frame.



Kpn I Not I Xho I Xba I EcoR V Hind III Sal I Bgl II

...GGTACCGCGGCCGCCTCGAGTCTAGAGATATCGAATTCAAGCTTGTCGACTCGAAGATCT...

Figure 8.5. pShuttle-CMV vector map. The sequence of the multicloning site is annotated on the right and the enzymatic restriction sites are indicated.

8.5.1.2. pAdEasy-1

The plasmid pAdEasy-1 (Figure 9.7), containing most of the human adenovirus serotype 5 (Ad5) genome, is deleted for the genes E1 and E3. The removal of these two viral genes creates space for foreign DNA and eliminates selfreplication capabilities. The E1 deletion renders the viruses defective for replication and incapable of producing infectious viral particles in target cells (if there by the host cell); the E3 region encodes proteins involved in evading host immunity and is dispensable. The deletion of both genes creates room for up to 7.5 kb of foreign DNA that can be inserted into the Ad5 genome. pAdEasy-1 vector carries the ampicillin resistance gene, which is lost after recombination with a shuttle vector.

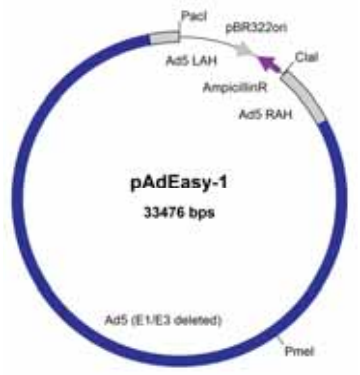


Figure 9.6. pAdEasy-1 vector map.

8.5.1.3. pIRESpuro3

pIRESpuro3 (Figure 8.8) is a bicistronic mammalian expression vector that allows to simultaneously express a protein of interest and puromycin resistance from a single, bicistronic mRNA transcript. Selective pressure exerted by puromycin on the entire expression cassette ensures that a high dose of antibiotic will select for cells expressing a high level of the gene of interest and its expression will be stable over time.

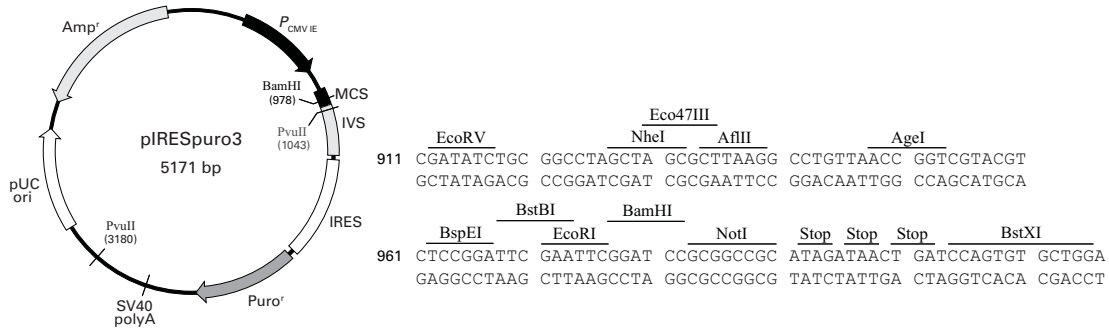


Figure 8.8. pIRESpuro3 vector map. The sequence of the multicloning site is annotated on the right and the enzymatic restriction sites are indicated.

Expression of the bicistronic transcript is driven by the constitutively active human cyto- megalovirus immediate early promoter ($P_{CMV\ IE}$), located upstream of the multiple cloning site (MCS). An encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), positioned between the MCS and the puromycin-N-acetyl-transferase gene ($Puro^R$), facilitates cap- independent translation of $Puro^R$ from an internal start site at the IRES/$Puro^R$ junction. Between the MCS and the IRES, the vector contains stop codons in all three reading frames, and a synthetic intron known to enhance the stability of the mRNA (IVS).

The vector can be replicated by E.coli as it contains pUC origin of replication. pIRESpuro3 also encodes for the β-lactamase gene, which confers ampiciline resistance. Hence, selection of positive

E.coli clones after pIRESpuro3 transformation is performed by ampicillin addition to medium.

8.5.1.4. pOPING

pOPING (Figure 8.9) is one of the vectors constructed by the OPPF using their novel high-throughput cloning process. This novel process is based on the In-Fusion™ cloning enzyme and its combination with OPPF-UK versatil suite of expression vectors. This vector in particular is derived from pTriEX-2 (Novagen) by cloning the lacZ promoter gene into the MCS (Nho-MscI). As a consequence, pOPING lacks from MCS. The clonation of GOI into pOPING vector must be performed by AgeI-PmeI restriction and subsequent ligation of plasmid and GOI. This strategy will result in the replacement of lacZ promoter gene by GOI.

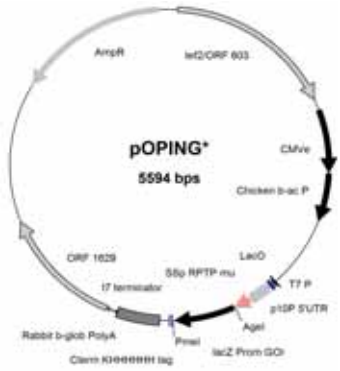


Figure 8.7. pOPING vector map.

The expression of the gene is driven by a fusion of Citomegalovirus enhancer (CMV_e) and the promoter of chicken β-actin. This promoter has been reported as more efficient for gene expression in comparison to CMV promoter [10]. Downstream of GOI there is a sequence encoding for His-tag which would ease purification process. His-tag can be cleaved either with 3C protease, SUMO protease or Carboxypeptidase A.

Replication of this vector in E.coli is posible due to the presence of pUC origin of replication. As the plasmid encodes for β-lactamase gene, selection of positive clones is made by ampicilin selection.

8.5.1.5. pTARs (pTAR-SBC, pTAR-EF1α-GCGR, pTAR-CMV-GCGRnew1)

Targeting vectors (pTAR vectors, Figure 8.10) are used to insert the gene of interest by recombinase mediated cassete exchange (RMCE) in the pre-tagged cell lines. pTAR vectors are also the responsible of restoring neoymicin resistance to the targeted cells (*see Introduction Chapter*).
Three different pTARs were used in this project, which share some basic features but differ in some

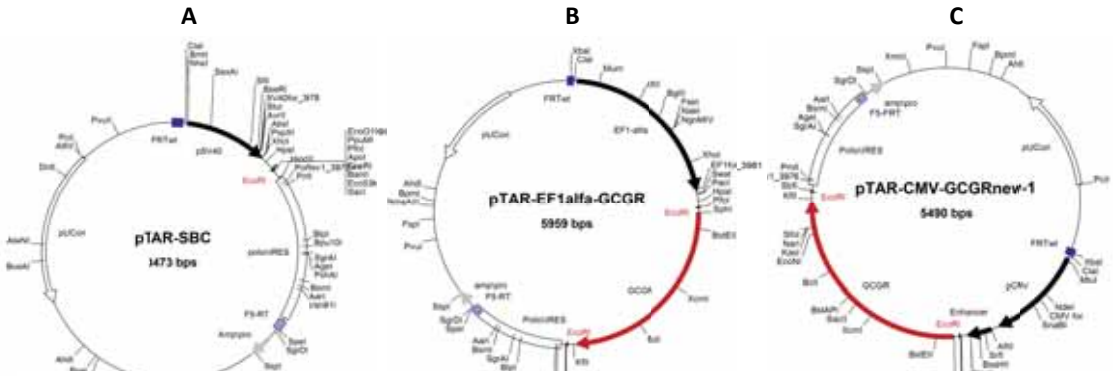


Figure 8.10. Original targeting vectors (A) pTAR-SBC (B) pTAR-EF1α-GCGR (C) pTAR-CMV-GCGRnew1. For the three vectors main features, single enzyme restriction sites and primers used for sequentiation are depicted. *EcoR*I sites –the enzyme used for GOI insertion- are also depicted and highlighted in red.

other:

Common Features

1. Two flipase recombination targets (FRTs) are present in all vectors flanking the genetic region that will remain in the genome of the tagged cells after transfection. One of the FRTs is wild-type (FRT wt) whereas the other contains a mutation (FRT F5). It is important that FRTs are not identical in order to avoid or excision of GOI ("flip-in" / "flip-out" phenomena) (Figure 8.11).

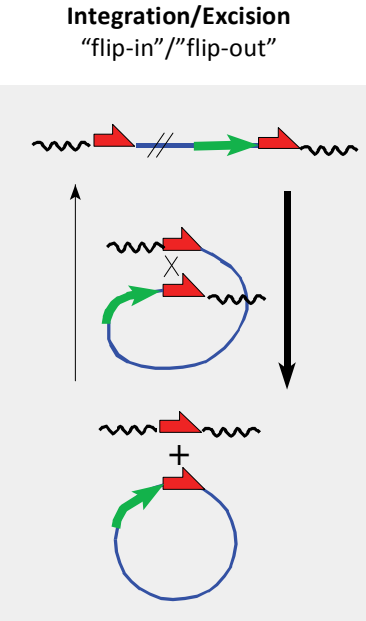


Figure 8.11. Recombination between two equi-oriented sites triggers the excision of the intervening DNA segment as a circle (center) Adapted from [11].

2. All vectors contain an IRES element. In case of pTAR-SBC this element is located downstream of MCS whereas in case of the other two pTARs it is located downstream of the glucagon receptor gene (GCGR). The presence of this element allows the simultaneously expression of the protein of interest and the neomycin resistance present in the pre-tagged cell line after RMCE has been performed.

3. Directly upstream of F5-FRT an ATG codon has been inserted in the three pTARs. This start codon is necessary for restoring neomycin resistance gene (ΔATG) of the pre-tagged cell lines after RMCE.

4. pTARs can replicate in E.coli due to the presence of pUCori in their sequence. All of them encode the β-lactamase gene and therefore, positive clones can be selected by the adition of ampicillin to cell medium after transfection.

Differential features

1. pTAR-SBC contains MCS so GOI can be inserted by cutting with any of the enzymes present in it. pTAR-EF1α-GCGR and pTAR-CMV-GCGRnew1 do not contain MCS because the glucagon receptor gene has already been cloned in it. As a consequence, GOI must replace GCGR removing the latter by EcoRI digestion.

2. Promoters driving the expression of GOI and neoymicin resistance gene (after RMCE) are different in each pTAR. pTAR-SBC encodes for Simian Vacuolating virus 40 (SV40), pTAR-EF1α-GCGR encodes

for Human elongation factor-1 alpha (EF1α) and pTAR-CMV-GCGRnew1 encodes for Cytomegalovirus (CMV). The first and the last promoters are from viral origin while the second one is from human origin.

8.5.2. OTHER VECTORS

8.5.2.1. pFlipe

The main feature of pFlipe vector (Figure 8.12) is that it encodes for the flipase gene (*Flp*). Flipase is a protein that belongs to the recombinases family. Recombinases are enzymes derived from bacteria and fungi, which catalyze directionally sensitive DNA exchange reactions between short (30–40 nucleotides) target site sequences that are specific to each recombinase. This property is used to perform site-directed recombination, used to manipulate an organism's DNA under controlled conditions *in vivo*. Specifically, *Flp* is derived from the 2µ plasmid of baker's yeast *Saccharomyces cerevisiae.* The 34bp minimal Flipase recognition target (FRT) site sequence has the sequence:

$^{5'}$GAAGTTCCTATTC*tctagaaa*G*t*ATAGGAACTTC$^{3'}$

for which the protein binds to both 13-bp 5'-GAAGTTCCTATTC-3' arms flanking the 8 bp spacer, i.e. the site-specific recombination (region of crossover) in reverse orientation. *FRT*-mediated cleavage occurs just ahead from the asymmetric 8bp core region ($^{5'}$*tctagaaa*$^{3'}$) on the top strand and behind this sequence on the bottom strand [12].

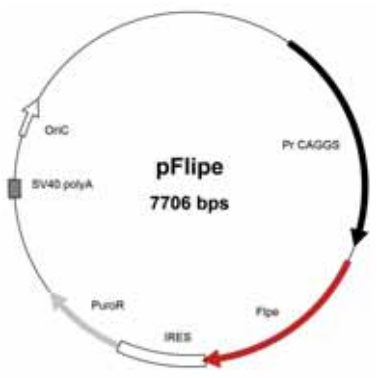


Figure 8.8 pFlipase vector map

Other features of interest of this plasmid are:

- The presence of an IRES element downstream of *FLp* gene, followed by the gene enconding for puromycin resistance. This construct would allow obtaining stable cell lines expressing the Flipase protein.
- CAGGS promoter drives the expression of the bicistron, which is a derivative of the CAG promoter. The parental promoter (CAG) is constituted from the following sequences:
  (C) the cytomegalovirus (CMV) early enhancer element
  (A) the promoter, the first exon and the first intron of chicken beta-actin gene
  (G) the splice acceptor of the rabbit beta-globin gene.
  In the case of CAGGC, two introns of the β-actin and an exon of the β-globin have been added 3' of the parental sequence. The final sequence is detailed on Annex 8.2.4.

8.6. PRIMERS

8.6.1. PRIMER DESIGN

All the primers used in this work (either for gene amplication, enzyme restriction sites addition or gene sequencing) were designed with Clone Manager software. Selection of the best pair of primers for each sequence was made on basis the constraints summarized in Table 8.11.

Table 8.11. Selected constraints for primer design with Clone Manager software.

Parameter	Value
Number base pair (bp)	20-30
Melting Temperature (ºC)	55-70
GC content (%)	50-60
Stability (kcals)	1.2
Repeats (di-nucleotide pairs)	<3
GC clamp (G's or C's at 3' end)	≥1
Absence of dimers (number of adjacent homologous bases)	<7

When it was not possible to fulfill all the criteria, number of base pair, melting temperature and absence of dimers were selected as the most important characteristics to be accomplished followed by GC content and stability. The final primer could be approved even if the GC clamp and repeats set-point values were not accomplished.

As different algorithms can be used to calculate some of the parameters in Table 9.11, the primers designed using Clone Manager were further checked with Ligation Calculator software from InSilico

online bioinformatics tools from Düsseldrof University software to ensure temperature melting, lack of dimers and false priming.

8.6.2. PRIMER LIST

Table 8.8. List of primers used in this project. The main characteristics are specified.

Name	Sequence (5'-3')	Used for	Provider
PCV2 Rev	CGCACTTCTTTCGTTTTC	CapPCV2 gene amplification	Roche
PCV2 Fw	CTTTTTTATCACTTCGTAATG		Roche
Cap2_ Rev	CAAACCTGTCCTAGATTC	CapPCV2 gene 3'end amplification	Roche
PCV2 Fw	CTTTTTTATCACTTCGTAATG		Roche
PCV2 Rev	CGCACTTCTTTCGTTTTC	CapPCV2 gene 5'end amplification	Roche
Cap2_ Fw	CGTTTTGACTGTGGTTC		Roche
Oligo ATG	ACTCGGTACCATGACGTATCCAAGG	Inserting KpnI and HindIII ERs flanking CapPCV2 gene	Roche
Oligo STOP	ACTGAAGCTTTTAAGGGTTAAGTGGG	Sequencing GOI correct insertion into pShuttle CMV (PCR1)	Roche
pShuttle Fw	GGTCTATATAAGCAGAGCTG	Sequencing GOI correct insertion into pShuttle CMV (PCR2)	Roche
pShuttle Rev	GTGGTATGGCTGATTATGATCAG		Roche
NheICapFw	AGTCGCTAGCATGACGTATCCAAGGAGGCGTTA	Insertion of CapPCV2 into pIRESpuro3	Biomers
AgeICapRV	GACTACCGGTTTAAGGGTTAAGTGGGGGGTCT		Biomers
AgeICapFw	GACTACCGGTATGACGTATCCAAGGAGGC	GOI insertion to pOPING Without Lys-HisTag and sequencing	Biomers
PmeICapRv	GCACGTTTAAACTTAAGGGTTAAGTGGGGGGTCT		Biomers
AgeICapFw	GACTACCGGTATGACGTATCCAAGGAGGC	GOI insertion to pOPING With Lys-HisTag and sequencing	Biomers
PmeICapRv2	AGCTGTTTAAACGGGTTAAGTGGGGGGTCT		Biomers
pIRESSeqFw	CTAGAGAACCCACTGCTTACTG	Sequencing GOI correct insertion into pIRESpuro3	Roche
pIRESSeqRv	CTTAGCGCAGAAGTCATGCC		Roche
SV40for	GGAGGCCTAGGCTTTTGCAA	Colony PCR and sequencing GOI correct insertion in pTAR-1	Roche
PolioRev1	GGTACAACCCCAGAGCTGTT		Roche
EF1αfor	CATTTCAGGTGTCGTGAGGA	9. Colony PCR and	Roche

PolioRev1	GGTACAACCCCAGAGCTGTT	sequencing GOI correct insertion in pTAR-2	Roche
CMV for	GCCTGGCATTATGCCCAGTACA	Colony PCR and sequencing GOI correct insertion in pTAR-3	Roche
PolioRev1	GGTACAACCCCAGAGCTGTT		Roche
Polio1for	CGTCAAGAAGGCGATAGAAGGC	Detection of postitive HEK293 clones after RCME	Roche
NeoRev	CCATGGGACGCTAGTTGTGAA		Roche

8.7. *E.coli* TRANSFORMATION

Transformation is the process of getting a circular DNA molecule in solution into E.coli cells. Basically, there are two transformation methods: heat shock transformation or electroporation of the cells. In this work, both methods were used.

8.7.1. HEAT SHOCK TRANSFORMATION

The protocol for preparation of competent cells and for transformation has been adapted from [13].

8.7.1.1. *E.coli* QUIMIOCOMPETENT CELLS PREPARATION.

First of all, E.coli cells should be treated in order to make them competent cells. This means, confer to the cells the capacity to uptake exogen DNA. In order to do it, the solutions specified in the Table 8.13 must be prepared:

Table 8.13. Composition of the solutions for quimiocompetent E.coli cells preparation

	Medium A[1]	**Storage solution B**[2]
yeast extract	2.5 g	0.5 g
tryptone	5 g	1 g
NaCl	5 g	1 g
$MgSO_4 \cdot 7H_2O$	1.23 g	0.295 g
H_2O	450 mL	64 mL
10× D (20 % w/w glucose)	5 mL	-
PEG 8000	-	12 g
Glycerol[3]	-	36 mL

[1] To avoid glucose caramelisation, 0.22 µm sterile-filtered glucose solution was added to the media A after autoclaving the other components.

[2] Medium B is sterilized by filtration through a 0.22 µm membrane (Millipore).

[3] Due to the high viscosity of glycerol it is recommended to weigh the desired quantity directly pouring glycerol into the final storage recipient.

Cells must be handled gently on ice during all processes before freezing. Transformation efficiency would decrease by 10^{-2} if the culture is centrifuged and resuspended at room temperature.

1. Inoculate a single colony or ≈5µL of glycerol stock to 5mL LB. Incubate overnight at 37ºC and 150 rpm.
2. Use 0.5mL of over-night culture to inoculate 50mL of medium A. The culture should be in the mid logarithmic phase (OD_{600}=0.6-0.8).
3. Keep cells on ice for 10 minutes.
4. Pellet cells by centrifugation at 1500g for 10 minutes at 4ºC.
5. Resuspend gently in 0.5mL of medium A precooled on ice. Add 2.5 mL of storage solution B and mix well without vortexing.
6. Aliquote cells of 100µL each in eppendorfs and store them at -80ºC until use.

8.7.1.2. *E.coli* QUIMIOCOMPETENT CELLS TRANSFORMATION.

1. Thaw the frozen cells on ice.
2. Mix competent cells with the ligation mixture (100-150ng) or purified vector (5-10 ng) and incubate on ice for 20 minutes. The purified vector transformation is used as transformation positive control. Also, it is highly recommended to include a negative transformation control (i.e. mock transformation with DNA dilution buffer).
3. Heat shock cells at 42ºC for 60 seconds.
4. Chill on ice for 1-2 minutes and add 1mL of pre-warmed SOC medium.
5. Incubate at 37ºC for 1-2 hours to allow expression of antibiotic resistance.
6. Plate 50, 100 and 200µL on agar plates containing antibiotic. Incubate at 37ºC overnight.

(When plating less than 100µl, first place a 100µl pool of LB broth on an LB-kanamycin agar plate. Pipet the transformed cells into the pool of LB broth, then use a sterile spreader to spread the mixture).

8.7.2. ELECTROPORATION

E.coli cells must also be prepared (i.e. make them electrocompetent) to undergo electroporation process. In this work, the cells were purchased already electrocompetent and therefore, this preparation was not necessary. The following transformation protocol was used for the transformation of linear pShuttle-CMV-*Cap*PCV2 recombinant vector into BJ5183-AD-1 in order to generate the recombinant adenovirus (Chapter 2).

1. Prechill five DNase-free microcentrifuge tubes and five electroporation cuvettes (0.2 cm gap) on ice.
2. Remove three aliquots of BJ5183-AD-1 electroporation competent cells from –80°C storage and thaw on ice.
3. Gently pipet 40µl of the competent cells into each of the chilled microcentrifuge tubes.
4. Into the first and second tube, pipet 5ng and 100ng of the linearized pShuttle recombined vector. This is done in order to test two DNA:cells ratio (i.e. low ratio and high ratio), which is highly recommended. Mix by tapping the tube gently and keep on ice.
5. Into the third and fourth tube, pipet 5ng and 100ng of linearized pShuttle-CMV-lacZ vector (recombination control plasmid) by tapping the tube gently and keep on ice.
6. Into the fifth tube, pipet 1µl of transformation control plasmid.
7. Set the electroporator to the following settings by referring to the instructions provided with the instrument: 200 Ω, 2.5 kV, 25 µF.
8. Transfer the contents of one microcentrifuge tube into one of the chilled electroporation cuvettes and tap the cuvette gently to settle the mixture to the bottom.
9. Slide the cuvette into the electroporation chamber until the cuvette connects with the electrical contacts.
10. Pulse the sample once, then quickly remove the cuvette. Immediately add 1mL of sterile LB broth and pipet up and down to resuspend the cells.
11. Transfer the cell suspension to a sterile 14mL BD Falcon polypropylene round-bottom tube.
12. Repeat the electroporation for the other two transformation reactions.
13. Incubate all of the transformations at 37°C for 1 hour while shaking at 225–250rpm to allow antibiotic resistance expresion.
14. For the recombination reactions (linearized DNA transformants), plate 50µl, 100µl, and 850µl of the transformed cell suspension in three LB-kanamycin. For the transformation using the transformation control plasmid, plate 10µl and 100µl of the recovered cells on LB-ampicilin agar plates.

(When plating less than 100µl, first place a 100µl pool of LB broth on an LB-kanamycin agar plate. Pipet the transformed cells into the pool of LB broth, then use a sterile spreader to spread the mixture).

15. Incubate the plates overnight at 37°C.

8.8. *E.coli* TRANSFORMANTS CHARACTERISATION

The generation of all the recombinant vectors in this thesis has the final objective of recombinant protein production in mammalian cells. The correct production of a heterologous protein is affected by a huge range of variables. Therefore, it is of high importance to ensure that the selected clone to produce the recombinant plasmid encodes the gene of interest and that this gene has not undergo any mutation along the process for its generation. With this objective in mind, after each transformation a minimum of 20 clones were selected to make a master plate and colony PCR characterisation. From the positive clones, enzymatic restriction pattern characterization of a minimum of 5 clones (in the case of cohesive ligation) or of a minimum of 10 clones (in the case of blunt end ligation) was performed. Finally, for E.coli clones encoding the final plasmid to be transfected to mammalian cells, sequencing of the gene to assess absence of mutations was carried out.

8.8.1. COLONY PCR CHARACTERISATION

Colony PCR is a commonly used method to quickly screen for plasmids containing the desired genetic sequence directly from bacterial colonies. This method eliminates the need to culture individual colonies and prepare plasmid DNA before analysis. However, the presence of bacterial cell contents and culture media in colony PCR reactions often results in polymerase inhibition. A robust polymerase is required to perform colony PCR with high efficiency in many different bacterial strains. A general protocol of this method is as follows:

1. Prepare the PCR master mix containing the corresponding primers and dispense the desired amount to x number of PCR tubes. (x=number of colonies to be characterized)
2. Use a sterile toothpick to pick up individual colonies and dip into each reaction tube. As soon as the solution looks cloudy, remove the toothpick. To create the master plate, streak the toothpick onto another agar plate containing the appropriate antibiotics and grow overnight.

3. Pick up an individual clone from the transformation positive control plate and dip it into a reaction tube. This reaction will be the negative control of the characterization.
4. Transfer PCR tubes to a PCR cycler, and perform PCR following the predefined cycling conditions.
5. Load 4-6µL of each PCR reaction directly onto an agarose gel, alongside an appropriate DNA Ladder.

8.8.2. ENZYMATIC RESTRICTION PATTERN CHARACTERISATION.

The restriction pattern is the band pattern that can be observed in an agarose gel after loading the fragments of DNA after enzymatic digestion of the original DNA molecule. This band pattern is characteristic of each DNA molecule and it will change after insertion of the sequence of interest into the original vector.
In case that a blunt-end cloning strategy is performed, enzymatic restriction pattern characterization is even more important. An incorrect direction insertion of the DNA sequence will not be detected by colony PCR, while it will be distinguished analyzing the enzymatic restriction pattern.

1. Determine the restriction(s) enzyme(s) to be used. As the most desired strategy, a single cutter of both original vector and DNA inserted sequence should be chosen. In case this is not possible, select two enzymes in order that:

    - Enzyme A is a single cutter enzyme which restriction site is present in the original vector but absent in the DNA sequence inserted.
    - Enzyme B a single cutter enzyme which restriction site is present in the DNA sequence inserted but absent in the original vector. Avoid restriction sites that are in the middle of the inserted DNA sequence in order to be able to distinguish correct/incorrect direction insertion.

    Note: When two different enzymes are used, it must be checked out if restriction buffers are compatible (one step digestion can be done) or incompatible (two step digestion would be mandatory).

2. Digest x mL of a MiniPrep (see section 8.10.2.4) from the clones to be tested. The volume depends on DNA concentration and the specificacions of restriction enzyme's manufacter.

3. Perform digestion according to manufacter's protocol.
4. Load digestion product onto an agarose gel, alongside an appropriate DNA Ladder.

8.8.3. DNA SEQUENTIATION.

Sequentiation of DNA was performed by the Genomics and Bioinformatic service of Universitat Autònoma de Barcelona using an ABI 3130XL sequentiator (Applied Biosystems). This sequentiator uses Sanger's methodology and a termostable polymerase.

8.9. MAMMALIAN CELL TRANSFECTION

Transfection is the analogous reaction of transformation, but performed in animal eukaryotic cells. There are various methods of introducing the foreign DNA into eukaryotic cells. In this work, three different methods have been used for different cell lines transformation. Regardless the transfection method, the efficiency of transfection highly depends on (a) cell concentration or cell confluence (if adherent cells are used, as is the case in this section), (b) number of cell passages and (c) DNA quality and quantity.

Cell confluence

Too few cells will cause the culture to grow poorly without cell-to cell contact. Too many cells result in contact inhibition, making cells resistant to uptake of foreign DNA. Actively dividing cells take up foreign DNA better than quiescent cells. For the obtaining of the recombinant virus, cell confluence of approximately 70% was assessed prior to AD-293 transfection.

Number of passages

Cell characteristics can change over time, and cells may not respond to the same transfection conditions after repeated passages, resulting in poor expression. The maximal number of passages before transfection was kept under 20.

DNA quality and quantity

Plasmid DNA for transfections should be free of protein, RNA and chemicals. Only vector preparations with Abs260/Abs280 ratio≥1.8 and Abs260/Abs230 ratio≥2 were used. In regards the quantity of DNA, 10µg of DNA were used per $2 \cdot 10^6$ cells (i.e. 5pg/cell ratio) in a 100mm tissue culture dish. However, this ratio should be optimized for each plasmid and cell line.

8.9.1. PEI-TRANSFECTION

Polyethylenimine (PEI) is a polymer with repeating unit composed of the amine group and two carbon aliphatic CH_2CH_2 spacer. When it is used as transfection agent, PEI condenses DNA into positively charged particles, which bind to anionic cell surface residues and are brought into the cell via endocytosis. The high efficiency of PEI-transfection is due to the "proton sponge" nature of the PEI: once inside the endosome, protonation of the amines results in an influx of counter-ions and a lowering of the osmotic potential. The additional pumping of protons into the endosome, along with the concurrent influx of chloride ions to maintain charge neutrality, increases ionic strength inside the endosome. This is then thought to cause osmotic swelling and physical rupture of the endosome, resulting in the escape of the vector from the degradative lysosomal trafficking pathway [14].

PEI transfection was applied to HEK293SF-3F6 for the obtention of HEK293SF-3F6-GFP and HEK293SF-3F6-SS-IL2-op*Cap*PCV2. The protocol was developed by Cervera et al. [15] and it is described below:

1. From a single cell culture take enough volume in order to have a final concentration of $1x10^6$cell/mL in the desired final volume (i.e. 2mL/well in a 6-well plate or 15mL/shake in 125mL shake flask). Cells must be in mid-exponential growth phase and viability should be ≥90%. Dispense the volume into a 50mL falcon tube.
2. Perform complete media replacement by centrifuging the cells for 8 minutes at 200g and withdrawing the media.
3. Resuspend the pellet by gently tapping the bottom of the falcon tube.
4. Add pre-warmed fresh media to the falcon tube and dispense the corresponding volume to the recipient where the transfection is going to be performed.
5. Prepare DNA:PEI mixture as follows:

    - Add x mL of pre-warmed fresh media and DNA (final concentration in the DNA-PEI mixture 100ng/µL) to a 50mL falcon tube. Media volume depends on the number of transfections to perform considering that DNA-PEI mixture must be 1:10 of the final volume.
    - Vortex at maximum speed for 10 seconds.
    - Add PEI to a final concentration in the DNA-PEI mixture of 1ng/mL.
    - Vortex at maximum speed for 3 seconds and rest for 3 seconds.
    - Repeat previous step two additional times.

- Incubate at room temperature for 15 minutes. The incubation time is critical for transfection efficiency.

6. Inoculate the corresponding volume to cell culture and homogeneise by manual shaking.
7. For transient transfection incubate the cells at 37ºC in a humidified atmosphere with 5% CO_2 incubator (Steri-cult 2000 Incubator, Forma Scientific) until the definied time of harvesting. For stable transfection incubate under the same conditions for 24h. After this time, completely change media to avoid extensive toxic-effect due to PEI presence.

NOTE: Cell Boost 5 must be avoided in transfection media, as it completely inhibits transfection.

8.9.2. ELECTROPORATION

Electroporation of 293-MZ for RCME was performed using Amaxa Nucleofector® technology. This technology is based on the momentary creation of small pores in cell membranes by applying an electrical pulse (electroporation). The protocol for DNA transfection is as follows:

1. Subculture 2 – 3 days before Nucleofection® (optimal confluency for Nucleofection®: 80 – 90%. Higher cell densities may cause lower efficiencies).
2. On day of transfection, prepare 6-well plates by filling appropriate number of wells with 1mL of culture media and pre-incubate plates in a humidified 37°C 5% CO_2 incubator.
3. Remove media from the cultured cells, wash cells once with PBS and harvest the cells by trypsinisation (see section 9.3.1.3.2. of this chapter).
4. Count the cells. Centrifuge the volume of cell broth in order to have a final concentration of 10^4 cell/µL in x volume. The volume is definied by the number of transfections to be performed (100µL/transfection). Centrifugation is performed at 200g for 8 minutes at room temperature.
5. Remove supernatant and resuspend cells in the x volume of Cell Line Nucleofector® Solution V. (Important: Avoid leaving the cells in Nucleofector® Solution longer than 15 minutes, as this may reduce cell viability and gene transfer efficiency). Dispense 100µL of cells to a sterile eppendorf.
6. Add 1µg of vector with the gene of interest and 2µg of pFlipe vector to the eppendorf and mix DNA with cells by gently pipetting the solution. Volume of DNA added must not exceed 12µL.
7. Transfer cell/DNA suspension into the cuvette (sample must cover the bottom of the cuvette without air bubbles) and close the cuvette with the cap.

8. Insert the cuvette with cell/DNA suspension into the Nucleofector® Cuvette Holder.
9. Select program A23 and start transfection.
10. Take the cuvette out of the holder once the program is finished.
11. Immediately add 1mL of pre-warmed culture medium to the cuvette.
12. Gently transfer the sample into the prepared 6-well plate (final volume 2mL/well). Important: Use the supplied pipettes and avoid repeated aspiration of the sample.
13. 24 hours post transfection (hpt) exchange media containing DNA to fresh media without antibiotic.
14. 48hpt exchange media to fresh media with antibiotic (neoymicin).
15. 72hpt change cells from the 6 well plate to a 10cm dish. Add ganciclovir to the media to a final concentration of 10μM.
16. Incubate cells until little colonies are detected. Then, start the scaling up of the selected clones.

8.9.3. $CaPO_4$ TRANSFECTION

The calcium phosphate transfection method for introducing DNA into mammalian cells is based on forming a calcium phosphate-DNA precipitate. Calcium phosphate facilitates the binding of the DNA to the cell surface and DNA then enters the cell by endocytosis. The $CaPO_4$ precipitate also limits the digestion of DNA by DNase associated with mammalian cells.

$CaPO_4$ transfection was used in this thesis for the co-transfection of 293T with p-Targeting vector and pFlipe for RMCE. Prior to transfection the following solutions must be prepared:

$CaCl_2$ (2.5M)

Weigh 13.87gr of $CaCl_2$ and disolve it in 50mL of MilliQ H_2O under continuous stirring. Sterile-filter the solution throuh a 0.22μm membrane.

HEBS (2x)

Weigh 818mg of NaCl, 600mg of HEPES and 10.65mg of Na_2HPO_4 and disolve them in 50mL of MilliQ H_2O under continuous stirring. Final concentrations are 0.25M, 0.05M and 1.5mM, respectively. Sterile-filter the solution throuh a 0.22μm membrane.

The following protocol is described for transfection into 10cm dish. In case that other platform is going to be used for transfection, volumes should be scaled up or down accordingly.

1. Subculture 2 – 3 days before transfection. On day of transfection, cells shoulf be 75%confluent.
2. Add 16µg of pFlipe vector and 8µg of pTargeting vector to an eppendorf (2:1 ratio).
3. Add sterile H_2O to a final volumen of 200µL and vortex at maximum speed.
4. Add 50 µL of $CaCl_2$ (2.5M).
5. Add 250 µL HEBS (2x).
6. Vortex 15 seconds at maximum speed.
7. Incubate for 25 minutes at room temperature. During this time, prepare media for transfection: add 10mL of HEPES buffer (1x) to 490mL of DMEM3+ (HEPES final concentration: 20mM). For one transfection, take 10mL of DMEM3+ with HEPES and add 10 µL of cloroquine 25mM. Cloroquine stock solution can be prepared in advance and stored at 4ºC, but the addition to the media must be performed at time of transfection.
8. Remove media from the dish containing cells for transfection and replace it with 10mL of transfection media.
9. Dispense the transfection mixture into the dish drop-by-drop in a circular motion. A fine precipitate will form. Aeration of the phosphate buffer while adding the DNA-$CaCl_2$ solution helps to ensure that this precipitate will be as fine as possible. This is important because clumped DNA will not adhere to or enter the cell as efficiently. Perform each transfection per duplicate.
10. Incubate cells for 12 hours. It is important to avoid longer incubation periods.
11. Remove media and add DMEM3+ HEPES (20mM) without antibiotic.
12. 48hpt remove media, tripsinize cells and resuspend them in cell media containing antibiotic. Change cells to a new 10cm dish and incubate until little cell colonies are detected. Then, start the scaling up of the selected clones.

8.9.4. TRANSFECTION USING ViraPACK TRANSFECTION KIT

The ViraPack Transfection Kit* (Agillent Technologies) is based on a modified $CaPO_4$ method of DNA transfection and it was used for the transfection of AD-293 cells in order to obtain the primary stocks of CapPCV2-AdV. The protocol slightly differs from the previous one and is detailed below. The main differences are underlined. The protocol is defined for 60mm tissue culture dishes.

1. Prepare the cells for transfection: r<emove the standard culture medium from the tissue culture dishes by aspiration. Wash the cells twice with phosphate-buffered saline (PBS) and add 5 ml of DMEM containing 5% solution 3 (MBS). MBS is a modified bovine serum provided with the kit. This step must be done 20-30 minutes prior to DNA addition.
2. Dilute 5µg of DNA with sterile water to a final volume of 450µL in a 5mL polystyrene round bottom tube.
3. Add 25µl Solution I ($CaCl_2$, 2.5M) and 250µl Solution II (BBS, 2x) to the tube containing the DNA. Immediately following the addition of Solutions I and II, gently mix the contents of the tube by tapping the tube.
4. Incubate the DNA suspension at room temperature for 15 minutes.
5. Gently resuspend any precipitate in the DNA suspension by pipetting the suspension up and down. The DNA suspension should appear clear to opaque.
6. Slowly add 500µL of the DNA suspension to each tissue culture dish dropwise in a circular motion to distribute the DNA suspension evenly onto each tissue culture dish**.** Make duplicates for each transfection.
7. Swirl each tissue culture dish once.
8. Incubate the dishes for 3 hours at 37°C under 5% CO_2.
9. After 3 hours, check the precipitate, which will vary in consistency from slightly perceptible to noticeably granular.
10. Remove the culture medium by aspiration and wash the tissue culture dishes three times with PBS, which should be added gently to avoid dislodging the cells. Check for residual precipitate by microscopic inspection. Add additional wash steps as required to ensure complete removal of the precipitate.
11. Add 5mL of DMEM 5%FBS 25µM cloroquine and incubate the dishes at 37°C under 5% CO_2.
12. After incubating for an additional 7 hours, remove the growth medium containing 25µM chloroquine and replace with 5mL growth medium without chloroquine.
13. Incubate the culture plates at 37°C for 7 days, replenishing the growth medium when needed (based on media color). If the cells appear to be well attached to the plate, replace the medium with 5mL of fresh medium, taking care not to dislodge the cells. Alternatively, if detached cells are observed in the growth medium, add an equal volume of fresh medium to the existing medium.

8.10. ANALYTICAL TECHNIQUES

8.10.1. MICROBIOLOGICAL TECHNIQUES

8.10.1.1. MAMMALIAN CELL NUMBER AND VIABILITY ASSESSMENT

Cell number was determined by manual counting at 100x magnification using a phase contrast microscope (Nikon eclypse, TS100) and a hemocytometer (Improved Neubauer Chamber, Brand). The hemocytometer is a thick microscope slide with two delimitated loading chambers (Figure 8.13). Each of these chambers is engraved with a laser-etched grid of perpendicular lines. The gridded area of the hemocytometer consists of nine 1 x 1 mm (1 mm^2) squares. These are subdivided in 3 directions; 0.25 x 0.25 mm (0.0625 mm^2), 0.25 x 0.20 mm (0.05 mm^2) and 0.20 x 0.20 mm (0.04 mm^2).

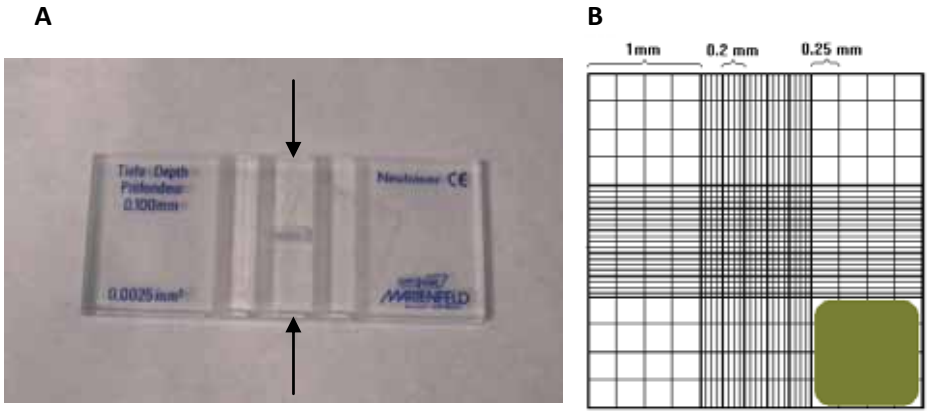


Figure 8.13 (A) Picture of a hemocytometer. Arrows indicate sites to load the sample. (B) Amplification of the loading chamber. One of the four large corner squares that are used for cell counting is indicated in green.

Viability was assessed using the Trypan blue dye exclusion method. Trypan blue dye (SigmaAldrich, T-8154) is diluted to 0.2% (v/v) in PBS. The dye will penetrate into died cells so they will be observed as blue under the microscope. By the contrary, viable cells will be observed as shiny white. The protocol to determine viable cell density is as follows:

1. A sample of 50µL from cell culture is gently mixed by pippeting with 50µL of trypan blue dye solution (0.2% v/v).
2. The lid of the hemocytometer is correctly placed onto it and sample is loaded into the two loading chambers.
3. Cells within the sixteen 0.25x0.25 mm squares are counted (i.e. four countings per chamber). For each chamber the highest and the lowest value (both for viable and dead cells) are discarded.

4. Cell density (viable cell density and dead cell density) can be calculated using the equation 8.2.

$$\left[\frac{cell}{mL}\right] = \frac{\frac{n_1+n_2}{n_s}}{d \cdot V} \cdot DF = (n_1 + n_2) \times 10^4 \qquad \text{Eq. 8.2.}$$

Where:

n_1 and n_2 are the number of counted cells within the two accepted squares.

n_s is the number of squares counted (i.e. two squares).

d is the dilution of the sample with Tripan blue dye (this value is 0.5).

V is the volume loaded in the hemocytometer chamber (i.e. $4x10^{-4}$mL).

DF is the dilution factor in case that any dilution of the sample has been done.

5. The viability percentage of cell culture can be calculated with the equation 8.3.

$$Viability(\%) = \frac{\left[{}^{cell}/_{mL}\right]_{viable}}{\left[{}^{cell}/_{mL}\right]_{total}} \times 100 \qquad \text{Eq. 8.3.}$$

8.10.1.2. ADENOVIRUS QUANTIFICATION

Virus quantification involves the determination of the number of viruses in a specific volume to determine the virus concentration. Methods for measuring adenovirus vector concentration can be divided into biological methods and physical methods. Biological methods rely on infection of cells in culture followed by subsequent events required for detection which depend on some aspect of the biological functionality of the vector. Physical assays, in contrast, measure the virion concentration by methods which are independent of biological functionality [16]. Also, it must be taken into account that most of the viral particles produced in a given biprocess appear to be noninfectious. For instance, in Chapter 4 of the present work it was reported the high influence of buffer for viral storage on AdV infectivity and that it is preferable to store AdV in Viral Storage Buffer (PBS1x+0.5mM $MgCl_2$+0.9mM $CaCl_2$+10% glicerol). The reported bioactivity of a viral stock may vary from as little as 1% to up to 50%, depending on the method and conditions of the quantification [16]. Since a vector destined for gene delivery and subsequent heterologous protein expression –as in the case ot this thesis- must be highly infected, it was necessary to select a viral method quantification that could distinguished between infective an non-infective adenoviruses. Two different quantification methods were used in this research work depending on the adenovirus to titrate: flow cytometry for rAdV-GFP quantification and AdEasy Viral titer kit for rAdV-CapPCV2 quantification.

8.10.1.2.1.Viral quantification by flow cytometry.

The set up of this method is detailed in Appendix 10.3.2. Only the final protocol for rAdV-GFP titration is explained in this section. Also, the following considerations must be contemplated in order to get better results of the assay:

- All the cells that are going to be infected for titration must belong to a single parental cell culture. At the time of infection cells should be in the middle of the exponential growth phase, viability must be ≥90% and any/low aggregation of cells must be noticed.
- Samples from the same experiment must be titrated in a single assay to avoid inter-assay variability.
- Perform duplicates of all the samples to be titrated. It is preferable that the duplicates of one sample are derived from two independent dilution banks.
- A negative control (i.e. uninfected cells, to set the autoflorescence of the cells) and a positive control (i.e. cells infected at a known MOI) must be included in each titration assay.
- The protocol here described is for titrations in a 12 well palte (Orange Scientific, 5530400). Smaller wells should be avoided as in these plattforms cells can easily aggregate under continuous shaking.

Titration should be performed as follows:

1. Count the cells of the parental cell culture. Calculate the volume of cell broth needed to fill the appropriate number of wells at a cell density of $5x10^5$ cell/mL in a final volume of 1mL/well.
2. Dispense the calculated cell broth in 50mL sterile centrifuge tube(s) (Orange Scientific, 5540100). Centrifuge the tube(s) at room temperature, 200g for 8 minutes. Resuspend the cells in the appropriate volume (i.e. 1mL x number of wells) with pre-warmed media. Check correct concentration after resuspension.
3. Add 1mL of the cell suspension each well of a the plate. Place the plates in the incubator until its use.
4. For each sample prepare two banks of serial dilutions (i.e. 1/10, 1/100, 1/1000, 1/10000) in sterile eppendorf tubes. If higher volumes are nedded, 12well plates can be used to prepare the dilution bank. Dilution bank is prepared in cell media.

5. Add two different volumes (i.e. 10µL and 50µL) of each dilution of step 4 to two independent wells in the plate. With the combination of step 4 and step 5, it will be obtained 16 values of viral concentration for a single sample (Figure 8.14).

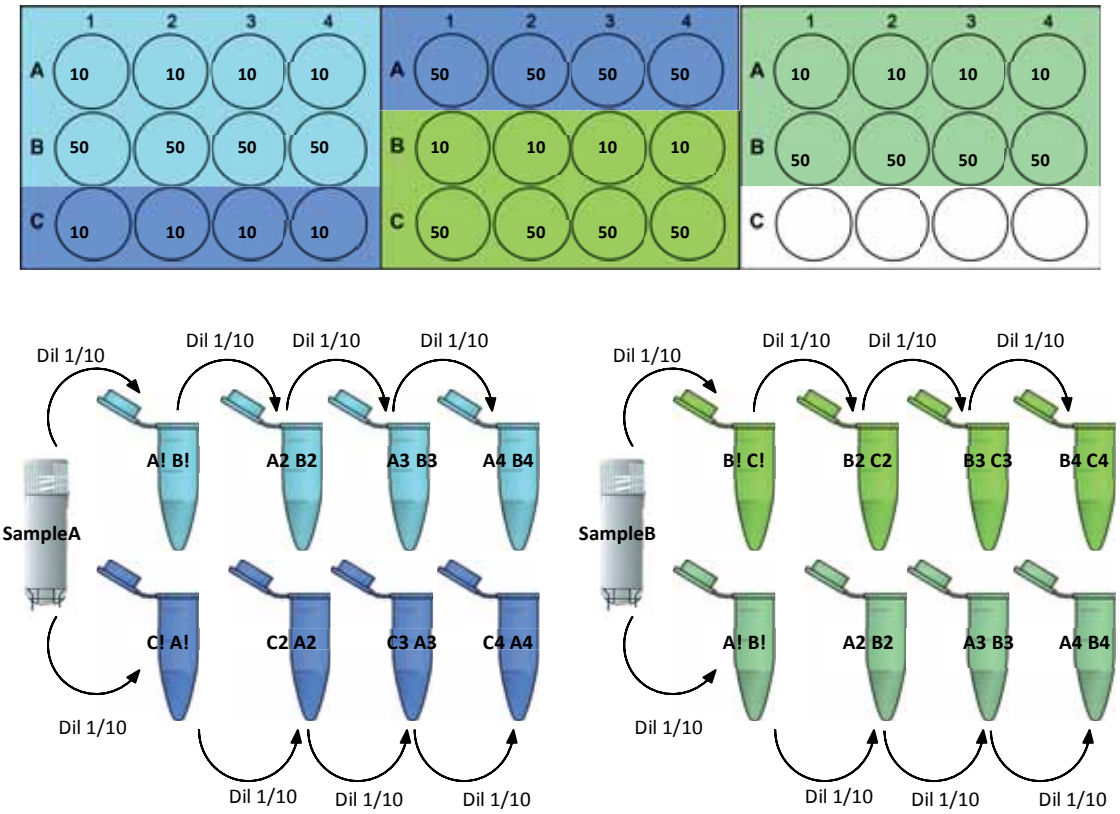


Figure 8.14. Scheme of the dilutions to be performed for the titration of two putative samples. Numbers on the plates indicate the volume to be added of the corresponding dilution of the sample. The letter-number code on each coloured eppendorf indicates the well into which the specific dilution must be inoculated.

6. Plates are incubated for 30-36 h on an orbital shaker (Stuart SSL1) at 110 rpm inside a static incubator at 37ºC and 5% CO_2.
7. After the incubation period, the volume of each well is transferred to Flow cytometry vials and kept on ice until the measurement. Avoid keeping the cell on ice longer than 15-20 minutes.
8. Alternatively, cells can be fixed for fluorescence measurement up to 72h post infection. In this case, after the incubation period the volume of each well is transferred to an eppendorf. Centrifuge the vials at 500 g for 5 minutes at room temperature. Discard the supernatant and resuspend the cells by gently tapping the bottom of the eppendorf. Add 0.5mL of a cold solution of formaldehyde 4% (v/v) in PBS to each eppendorf and homogeneize the solution by pipetting.

9. Just before appliying the sample to the flow cytometer (Becton Dickinson FACSCalibur), vortex the cytometer vial in order to completely homogeneize the samples. Otherwise, the hydraulic circuit of the equipment can be plugged with consequent damage of the equipment.
10. Infective concentration is determined using the equation 8.4:

$$[\mathrm{IPU/mL}] = \frac{(\%\ \mathrm{Positive\ GFP} - \%\mathrm{Positive\ GFP\ Neg\ Gtrl})/100}{\mathrm{Viral\ Volume}} \cdot \mathrm{TCN} \cdot \mathrm{DF} \qquad \text{Eq. 8.4}$$

Where

Neg Ctrl is an uninfected cell culture under the same conditions (cell media, cell concentration and viability) as infected cell culture.

TCN is the total cell number per well

DF is the dilution factor of the sample

8.10.1.2.1. Viral titration with AdEasy Viral titer kit.

The recombinant adenovirus rAdv_CapPCV2 obtained in this research project (*see Chapter 4*) does not have any reporter gene such as GFP, which makes easier viral quantification. Therefore, the AdEasy Viral titer kit (Agillent Technologies, Cat. 972500) was used for adenovirus titration. The kit is an enzyme-linked immunoassay that detects the adenoviral capsid protein called hexon. The most important advantage of this kit is that it can determine the titer of viral stocks in 24 to 48 hours, compared to two weeks for traditional endpoint dilution assays.

The protocol used have been adapted from the manufacter's recommendationni order to scale down the titration from 24-well plate to 96-well plate. The scaling downt of the titration enables to test more samples in the same assay while saving reagents.

1. Three days pre infection calculate the amount of cells that are going to be needed for the titration experiment. Inoculate the appropiate number of 75cm^2 t-flask at an initial cell density of $0.4 \cdot 10^6$ cell/cm^2. After 72h approximately, cells should be 75-80% confluent. Higher confluences should be avoided.
2. 12 hours pre infection harvest the cells and resuspend them in fresh media at an initial concentration of $2.5 \cdot 10^2$cell/µL. Seed 200 µL/well of the cell suspension in a 96well plate (i.e. $5 \cdot 10^4$ cell/well).
3. At the time of infection, prepare the viral dilution bank as detailed in previous section. Due

to the kit characteristic's, only the dilutions that result in 10% of positive cells will be taken into account. Therefore, the positive controls should be cells infected at MOI 0.02 (duplicate) and/or cells infected at MOI 0.05 (duplicate).

4. Carefully, aspire cell media from the 96 well plate were the cells are seed. It is very important to not disrupt the monolayer. The following considerations may help to accomplish it:
    - Never touch the bottom of the well (i.e.the cell monolayer) with the micropipette tip.
    - Dispense the volume always in the same point of the well (if disrupting part of the monolayer, the rest of it will be unaltered).
    - When dispensing volume, lean the micropipette tip onto the wall of the wells and slowly pour the volumen in the well.
5. Dispense 100µL/well of the corresponding viral solution.
6. 24 hours post infection check for correct monolayer in all the wells.
7. 48 hours post infection, aspire cell media containg the viruses and let cells dry inside the flow hood removing the tap of the plate.
8. Fix the cells by carefully adding dropwise 75µL of cold methanol to each well.
9. Incubate the plate at -20ºC for 10 minutes. (Note: it is also possible to incubate at -20ºC overnight).
10. Aspire the methanol.
11. Wash three times all the wells with the addition of 100µL of d-PBS+1% BSA (w/v). Let the third wash in the wells until the next step is performed (it is important that cells never get dry).
12. Dilute the primary antibody (Mouse anti-hexon) 1:500 in D-PBS+1%BSA (w/v).
13. Aspire the last wash and add 50µL/well of the diluted primary antibody. Incubate 1 hour at 37ºC 5%CO_2. It is also possible to perform this incubation in absence of CO_2.
14. Aspire the primary antibody.
15. Wash three times all the wells with the addition of 100µL of d-PBS+1% BSA (w/v). Let the third wash in the wells until the next step is performed.
16. Dilute the secondary antibody (Goat anti-Mouse HRP-conjugated) 1:1000 in D-PBS+1%BSA (w/v).
17. Aspire the last wash and add 50µL/well of the diluted secondary antibody. Incubate 1 hour at 37ºC 5%CO_2. It is also possible to perform this incubation in absence of CO_2.
18. Immediatly before aspiring the secondary antibody, prepare the substrate working solution.

Dilute DAB Substrate 10x provided with the kit 1:10 into Stable Peroxidase Buffer 1x (also provided with the kit). Important: Do not allow DAB Substrate 10x to reach room temperature. It will be needed 75µL/well of DAB substrate working solution.

19. Aspire the secondary antibody.
20. Wash three times all the wells with the addition of 100µL of d-PBS+1% BSA (w/v).
21. Add 75µL/well DAB working solution to each well. Incubate in darkness, at room temperature for 10 minutes. Note: the color may be intensified if the incubation time is extended overnight at 4ºC. Nevertheless, the background color will also be higher.
22. Observe the cells under the microscope using a 20x objective. Divide the 96 well into 10 fields and count the positive cells (dark brown to black) within the field. Compute the average number of positive cells per field. Only the wells with less than 10% of positive cells should be considered for counting.
23. Calculate the infectious units (IFU) per ml for each well using the equation 8.5:

$$\left({}^{IFU}/_{mL}\right) = \frac{{}^{Average\ number\ of\ cells}/_{field} \cdot {}^{field}/_{well}}{vol.of\ diluted\ virus\ used\ in\ each\ well(ml) \cdot dilution factor} \qquad \text{Eq. 8.5}$$

The number of total fields when using a 20× objective is derived as follows:

Radius of a standard 20× objective = 0.45 mm

The area per field = 0.045 cm × 0.045 cm × 3.1415 = 6.36 × 10^{-3} cm^2.

Fields/well=area per well/area fer field

8.10.2. DNA ANALYSIS TECHNIQUES

8.10.2.1.PCR AMPLIFICATION

The polymerase chain reaction (PCR) is a technology used to amplify a single or few copies of a piece of DNA, generating thousands to millions of copies of that particular sequence. It is a fast technology that relies thermal cycling for DNA melting and enzymatic replication of DNA using primers (short DNA fragments complementary to the sequence to be amplified) and a DNA polymerase.

As DNA melting temperature is highly dependent on DNA sequence, temperature and time of DNA melting cycle must be adjusted for each PCR. Moreover, the number of amplification cycles can also

be altered depending on the initial DNA concentration and the desired final concentration. Along this work, different PCRs have been performed for different purposes. The detailed programs for the reactions are detailed on Table 8.16-8.19. Moreover, two different polymerase kits have been used: Pwo Master (Roche) and BioMIx™ (Bioline). All the PCRs were carried out in the Gene Cycler thermal cycler from Bio-Rad.

In order to minimise differences among PCRs derived from experimental error, a unique Master Mix containing dNTPs, DNA polymerase, forward and reverse primers, water and other common components should be prepared. Then, the corresponding volume of the mixture is dispensed in each PCR eppendorf. Finally the DNA sequence to be amplified is added to each eppendorf. The composition of the Master Mix for Pwo Master polymerase kit and BioMix polymerase Kit are detailed in Table 8.14 and Table 8.15, respectively.

Table 8.14. Pwo Master polymerase kit Master Mix

Reagent		Volume (µL/reaction)	Final concentration
Master Mix	PwoMaster	12.5	1.25U Pwo^{SUPERYIELD} DNA Polumerase 0.2mM dNTPs 2mM $MgCl_2$
	Fw Primer	1.25	0.5µM
	Rv Primer	1.25	0.5µM
	Sterile H_2O	7.5	--
DNA template		2.5	Genomic DNA: 0.2ng/µL-20ng/µL Plasmid DNA: 4pg/µL-400pg/µL

Table 8.15. BioMIx™ polymerase kit Master Mix

Reagent		Volume (µL/reaction)	Final concentration
Master Mix	BioMIx	7.5	x U* Taq DNA Polymerase 0.2mM dNTPs 2.5mM $MgCl_2$
	Fw Primer	1.25	0.8µM
	Rv Primer	1.25	0.8µM
	DMSO	0.5	0.03%
	Sterile H_2O	3	--
DNA template		1.5	100ng/µL

* Final Taq DNA polymerase concentration is kept as trade secret (not provided).

8.10.2.2. PCR PROGRAMS

Table 8.16. PCR program for CapPCV2 gene amplification from viral DNA

Number of cicles	T (ºC)	t (min)
1	94	5
	94	0.5
30	50	1
	72	0.5
1	72	7

Table 8.9. PCR program for KpnI/HindIII ERs addition to CapPCV2 gene

Number of cicles	T (ºC)	t (min)
1	94	5
	94	0.5
3	42	1
	72	0.5
	94	0.5
30	53	1
	72	0.5
1	72	7
1	4	∞

Table 8.10. PCR program for AgeI/NheI AgeI/PmeI ERs addition to CapPCV2 gene

Number of cicles	T (ºC)	t (min)
1	94	5
	94	0.5
30	59	1
	72	0.5
1	72	7
1	4	∞

Table 8.11. PCR program for colony-PCR pTAR E.coli transformants

Nº of cycles	Temperature (ºC)	Duration
1	94	5’
1	72	7’
	95	30’’
25	55	45’’
	72	4’
1	72	7
1	16	∞

8.10.2.2. DNA LIGATION

DNA ligation is an enzymatic reaction catalyzed by DNA ligase. In nature, ligases play essential roles in DNA replication and repair. In the laboratory, this reaction is used to join double stranded DNA fragments with blunt or cohesive ends to form recombinant DNA plasmids, among other applications. In order to avoid that the digested vector could circularise again, a dephosphorilation step of the vector can be performed prior to ligation.

Rapid DNA dephosphorylation and ligation kit (Roche) was used in the present work. The protocol is detailed below and it was adapted from manufacter’s instructions:

1. Prior to dephosphorilation and ligation ensure having a high-quality insert and vector DNA. This means that 260/280 ratio must be ≈ 1.8 – lower values indicate presence of protein, phenol or other contaminants that absorb at 280nm)- and ratio 260/230 between 2.0-2.2 – lower values indicate presence of organic contaminants-. Also, they have to be enough concentrated in order to add the desired amount of DNA (see step 3.1 of this protocol) in a volume≤8 µL (this volume refers to the sum of vector volume and insert volume). In order to achieve both premises, DNA clean-up can be done using a PCR purification kit.

2. Dephosphorilation

2.1. Add the reagents specified in Table 8.20 to a vial:

Table 8.20. Reagents for dephosphorilation step

Reagent	Volume (µL)	Final quantity/concentration
Vector DNA	x	Up to 1µg
rAPid Alkaline Phosphatase Buffer (10x)	2	1x
rAPid Alkaline Phosphatase	1	1U. Reagents for dephoso
Sterile H_2O	Up to 20	

2.2. Mix thoroughly by vortexing and centrifuge briefly.

2.3. Incubate for 15min at 37ºC in a thermo block (Thermomixer Comfort, Eppendorf).

2.4. Inactivate the rAPid Alkaline Phosphatase at 75ºC for 5 minutes.

2.5. Immediately proceed to ligation step or store dephosphorylated vector at -20ºC.

3. Ligation

3.1. Add the reagents specified in Table 8.21 to a vial:

Table 8.21. Reagents for ligation step (I)

Reagent	Volume (µL)	Final quantity/concentration
Vector DNA	x	50ng*
Insert DNA	x	150ng*
DNA dilution buffer, 5x	2	1x
Sterile H_2O	Up to 10	

*DNA quantity using this ligation protocol cannot exceed 200ng. The quantity specified in the table corresponds to a molar ligation ratio of 1:3 (vector:insert), which is the standard one. Nevertheless, the optimal ratio can vary depending various parameters (i.e. size of vector and insert, DNA quality). Therefore, it is recommended to test at least two molar ratios for each ligation. Quantity of DNA was calcultated using Ligation Calculator software from InSilico online bioinformatics tools from Düsseldrof University.

3.2. Mix thoroughly by vortexing

3.3. Add the reagents specified in Table 8.22 to the vial prepared on step 3.1.

Table 8.22. Reagents for ligation step (II)

Reagent	Volume (µL)	Final quantity/concentration
T4 DNA Ligation Buffer	10	1x
T4 DNA Ligase	1	5U

3.4. Mix thoroughly by vortexing and centrifuge briefly.

3.5. Incubate at room temperature for 10minutes.

3.6. Proceed immediately to transformation. Alternatively, DNA ligation product can be stored at 4ºC for 1-2 hours. For longer periods, store it at -20ºC.

8.10.2.3. ENZYMATIC RESTRICTION OR DIGESTION OF DNA

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts DNA at or near specific recognition nucleotides sequences known as restriction sites [17,18]. Restriction enzymes are commonly classified into three types, which differ in their structure and wether they cut their DNA substrate at their recognition site, ori f the recognition and cleaveage are separated from one another. These enzymes are found in bacteria and archaea and provide a defence mechanism against invading viruses[19,20]. Inside a prokaryote, the restriction enzymes slectively cut up a foreign DNA in a process called restriction, while host DNA is protected by a modification enzyme (a methyltransferase) that modifies the prokaryotic DNA and blocks cleaveage).

From the three restriction enzyme types, Type II are the most used in biotechnology and molecular biology field. They recognise sites which are usually undivided and which are palindromic (meaning the base sequence reads the same backwards and forwards. Hence, they are symmetric). And 4-8 nucleotides in lenght. They recognize and cleave DNA at the same site and they do require Mg^{2+} as a cofactor but do not requiere ATP. For their activity. Some enzymes cleave the doubled DNA strand at the symmetry axis (leaving blunt ends at DNA molecule) and some others don't cut at the simmetry axis (leaving sticky ends at DNA molecule).

In this work, almost all the restriction enzymes were FastDigest (Fermentas). These enzymes have the advantage to share the same buffer and, consequently, multiple restrictions can be performed at the same time. Buffers are prepared as 10x and must be stored at -20ºC. The activity of the enzymes can be measured in activity units, which is defined as the necessary amount of an enzyme to completely digest 1µg of DNA in 60 minutes. Due to the glycerol present in buffer storage of the

enzyme, the added volume of the enzyme must be less than 1/10 of the final volume, as glycerol could interefere in the reation if its concentration is higher than 5%. For a standard enzymatic digestion, the components specified in the Table 8.23 should be added.

Table 8.23. Components needed for an standard enzymatic restriction of DNA.

Component	Volume (µL)
DNA sample	x*
Restriction Buffer (10x)	1
Restriction enzyme	1 (1-5 U/µg DNA)
Sterile ultrapure H_2O	Up to 10

* Volume must be adapted on basis to DNA concentration and incubation time.

It must be taken into account that the restriction condictions (basically, temperature and NaCl concentration) might differ depending on the enzyme. This might be considered in order to perform a double-digestion in parallel or sequencially. Besides, if an enzyme different from FastDigest (Fermentas) was used, the intructions of the manufacter were followed.

8.10.2.4. EXTRACTION AND PURIFICATION OF PLASMIDIC DNA

The plasmids of interest were transformed into E.coli cells in order to achieve high number of copies of the plasmid after their replication within the cells. Therefore, in order to work with the plasmids, they had to be extracted and purified from E.coli cell culture. In this project, three different kits were used for plasmid preparation, depending on the final desired concentration of the plasmid and the volume needed. If low concentrations and small volumes were needed, *QIAprep Miniprep* (QIAgen, Cat. 27104) was used, following the manufacter's recommendations. When concentration or volume needed was higher, *HiSpeed Plasmid Midiprep* or *EndoFree Plasmid Maxiprep* (QIAgen, Cat. 12643 and 12362, respectively) were used following instructions from the manufacter.

The three kits are based on a four-step procedure: (1) Growth of bacterial culture (2) Harvesting and modified alkaline lysis of bacteria (3) Binding of the plasmid to a membrane/resine in order to separate it from impurities and (4) elution or precipitation and resuspension of DNA. It is worth to mention that the initial volume of cell broth would depen on the copy number of the plasmid within the cell, which will depend on the origin of replication encoded in the plasmid. In the Table 8.24, the most common backbone vectors and their characteristics are specified.

Table 8.24. Origin of replication and copy number of the most common plasmids.

Plasmid	Origin of replication	Copy number	Classification
pUC vectors	pMB1*	500–700	High copy
pBluescript vectors	ColE1	300–500	High copy
pGEM vectors	pMB1*	300–400	High copy
pTZ vectors	pMB1*	>1000	High copy
pBR322 and derivatives	pMB1*	15–20	Low copy
pACYC and derivatives	p15A	10–12	Low copy
pSC101 and derivatives	pSC101	~5	Very low copy

*The pMB1 origin of replication is closely related to that of ColE1 and falls in the same incompatibility group. The hig-copy number plasmids listed here contain mutated versions of this origin.

8.10.2.5. EXTRACTION AND PURIFICATION OF GENOMIC DNA

For the characterisation of the stable cell lines generated in this work (*see Chapter 5*), it was necessary to isolate their genomic DNA to detect by PCR amplification the sequence of interest within it. For that purpose, the *Wizard® Genomic DNA Purification kit* (Promega, Cat. A1125) was used, following the protocol for "isolating Genomic DNA from Tissue Culture Cells and Animal Tissue".

Tis kit is based on a four-step process. In the first step cells and nuclei are lysed. An RNase digestion step may be included at this time, although it is optional for some applications. Then the cellular proteins are removed by a salt precipitation step, which precipitates the proteins but leaves the high molecular wight genomic DNA in solution. Finally, the genomic DNA is concentrated and desalted by isopropanol precipitation.

8.10.2.6. DNA SEPARATION BY ELECTROPHORESIS

Agarose gel electrophoresis is a method used or the separation of a mixed population of DNA in a matrix of agarose by applying and electric field to move the DNA molecules or DNA fragments. As DNA is negatively charged, the electric field will move DNA fragments towards the positive pole of the chamber while the polymerised agarose will hinder their migration. The electrophoretic mobility rate is inversely proportional to the $\log_{10}$ of the number of base pairs. Hence, the smaller the fragment would be, the fastest it will migrate through the gel. The electrophoretic mobility depends on other factors, though. This factors are the agarose concentration, the voltage applied, the ionic

strength of the buffer used, the concentration of the intercalating dye and the conformation of the DNA molecule. Therefore, these variables should be defined and use the same values for DNA samples of similar characteristics.

When an intercalating dye such as Syber Green (Invitrogen, Ref.S-7567) is applied to DNA mixture, it is possible to observe DNA bands under an UV lamp. The length (i.e. number of base pairs) of each band obtained can be established by comparing its migration on the gel with the band migration of a DNA marker. The DNA marker is a mixture of DNA fragments of a known length and, sometimes, known concentration.

For DNA electrophoresis, the following solutions are needed:

- TAE electrophoresis buffer (1x) 4mM Tris (Panreac) , 20mM acetic acid (Panreac), 2mM EDTA (Panreac)
- Loading buffer (6x) 10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glicerol, 60 mM EDTA (Fermentas)
- Syber Green solution (x10.000) (Invitrogen)
- Agarose (Boehringer Mannheim)
- Molecular weight markers: GeneRuler^{TM} 1kb DNA adder (SM0313 Fermentas), GeneRuler^{TM} DNA Ladder Mix (SM0333 Fermentas) Lambda DNA/HindIII Marker (SM0103 Fermentas) HyperLadder^{TM} 1kb (BIO-33053 Bioline)

TAE buffer was prepared initially to 50x and stored at room temperature. When electrophoresis was performed, the buffer was diluted 50 times with ultrapure water. It is important to prepare the agarose gel in the same buffer that is going to be used for filling the electrophoretic chamber. The following procedure was followed for gel preparation and DNA separation:

1. Weigh the corresponding amount of agarose powder and add the corresponding volume of TAE buffer (1x). Agarose concentration will depend on the size of the expected DNA bands. The larger the band, the lower the agarose percentage. Normally, 1% (w/v) agarose gels were prepared.
2. Melt the agarose in a microwave until any solid particle remains in suspension. It is very important to avoid boiling agarose solution. Hence, short periods of warming in the microwave should be applied followed by vigorous mixture of the solution.

3. Allow to cool to 50-55 ° C .
4. Add Syber Green to agarose solution (dilution 1:10.000).
5. Prepare the moldplate for agarose gel sealing it with tape. Alternatively, a gel caster (BioRad) can be used. Place the comb so it fits firmly on the gel box. Be sure to leave 0.5-1cm between the comb and the upper side of the gel. Also, ensure that there is a space between the moldplate and the bottom of the comb.
6. Pour agarose solution into the gelbox and let it cool down until agarose solidifies.
7. Take out the comb carefully and remove the tape from the gelbox. Immerse the gelbox with the agarose gel into the DNA electrophoresis apparatus containing electrophoresis buffer. The electrophoresis buffer should cover the entire surface of the gel.
8. Add loading dye (6x) to DNA sample. As a standard procedure, prepare the samples in order to reach DNA final concentration of 15ng/µL.
9. Load DNA samples into the gel. The volume of DNA to be loaded in the gel will depend on its concentration and on the size of the lanes performed in the gel. As a standard procedure load 10µL of the previously prepared DNA/loadind dye mixture to each lane. In regards the DNA markers, load 5µL to the gel.
10. Start the electrophoresis considering that the constant voltage applied should not exceed 5V/cm.
11. Run electrophoresis until the dye of the loading buffer reaches 2/3 of the gel. In case that the main interest of the electrophoresis is separate large bands, it is possible to let the electrophoresis run until the dye reaches the end of the gel.
12. DNA bands are visualized using Gel Doc EZ Imager (Bio Rad).

8.10.2.7. PURIFICATION OF DNA FROM AGAROSE GEL

For the recovery of DNA fragments after their loading onto agarose gels, two different kits were used. Both of them rely on the adsorption of DNA to silica in hight salt solution [21]. Both kits are based on a four step procedure: (1) Melting agarose gel containing the DNA of interest in an appropiatte buffer (2) capture of DNA onto silica material (3) removal of impurities such as agarose, proteins, salt and ethidium bromide (or other DNA dyes) and (4) recovery of DNA. One of the kits (*QIAquick Gel extraction Kit*, QIAgen Cat.28704) attaches the DNA into a membrane placed inside a little column, whereas the other (*QIAEX II*, QIAgen Cat. 20021) adsorbs the DNA onto silica particles. QIAquick Gel extraction kit is recommended for DNA fragments from 70bp to 40kb and up to 10µg of DNA can be recovered. QIAEXII is used for DNA fragments from 40bp to 50kband the maximal DNA

to be recovered is 5 µg. Therefore, the first kit was routenly used whereas the latter was used for the recovery of the recombinant adenovirus genome generated in this project (*see chapter 4*).
The conditions for gel extraction were as thery are indicated on the manufacter's instructions.

8.10.2.8. SOUTHERN BLOT PROTOCOL

Solutions needed:

	Working Solution	Stock Solution	Dilution of Storage solution	Storage
Incubation solution	0.4M NaOH	5M NaOH	Water	RT
Neutralization Solution	2xSSC	20xSSC*	Water	RT
Pre-Hybridization Solution		--	--	-20ºC
TE Buffer‡	1x			
Washing Solution	2xSSC 0.5% SDS	20xSSC* 10%SDS	Water	RT

* 1L: NaCl (173.5g); Tri-natriumcitrate*2H_2O (100.5g). Adjust pH to 7-7.5 with Citric Acid. Filter the solution.
‡ 1L: Tris(1.21g); EDTA (0.39g). Adjust pH to 7.5 with HCl.

Day 1. Running DNA- Electrophoresis gel & Blotting

Perform electrophoresis as explained in section 8.10.2.6

Blotting

1. Incubate the gel in incubation solution 10' RTº.
2. Cut a piece of membrane (Hybond-XL GE Healthcare) of the same size of the agarose gel. Incubate the membrane in incubation solution approx 10' RTº.
3. During the incubation time, cut 5 pieces of Whatmann Paper) of the same of the gel and an extra one to make the bridge (=same size of the gel +3cm per side) and start to build the blotting cassette (Figure 8.15):

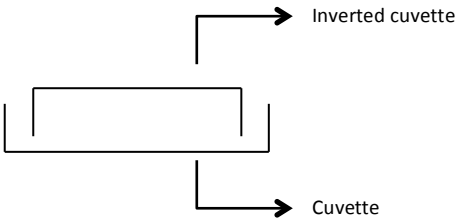


Figure 8.15. Blotting cassette initialization

4. Wet one Whatmann paper in incubation solution and lay it on the inverted cuvette (Figure 8.15). Remove the bubbles by rolling a pipet glass onto it (x2).
5. Wet the bridge paper in incubation solution and lay it on the inverted cuvette (Figure1). Remove the bubbles by rolling a pipet glass onto it.
6. Turn down the gel and put it onto the bridge (this is to have the DNA samples at the same level and hence they are at the same distance to the membrane).
7. Lay the membrane onto the gel (does not matter the direction) and remove the bubbles.
8. Wet one Whatmann paper in incubation solution and lay it on the inverted cuvette (Figure1). Remove the bubbles by rolling a pipet glas sonto it (x2)
9. Lay one **DRY** Whatmann paper onto the two previous ones.
10. Divide a block of Green absorbent paper into two blocks and lay them on the last Whatmann paper (Figure 8.16)
11. Put something heavy (i.e. 1L water bottle) on it (Figure 8.16).
12. Displace two glass pipette on the sides of the cassete to avoid that the Green absorbent paper get in contact with the bridge (hence, avoid that the flowthrough of incubation solution does not go completely vertical)
13. Fill the cuvette (Figure 8.15) with incubation solution. Let the blotting o/n

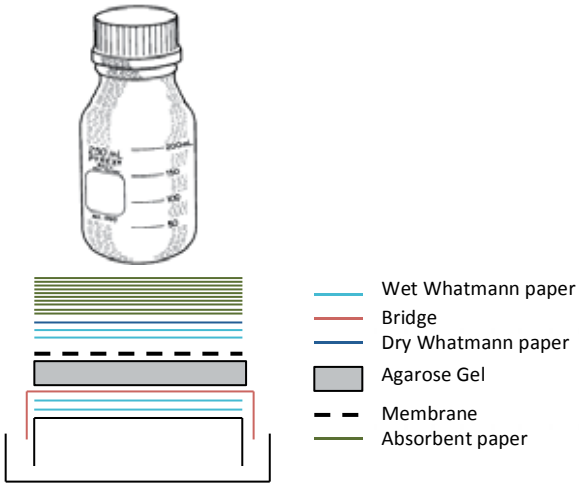


Figure 8.16. Schematic representation of the proper material disposition for o/n incubation.

Day 2. DNA Cross-linking, unspecific DNA-coating of the membrane, probe Labelling and hybridization

DNA cross-linking

1. Remove the membrane and cut it into two pieces (easy to handle).
2. Label the side that was NOT in contact to the DNA.
3. Incubate the membrane in the neutralization solution during 30'-60' RT on a rocking shaker. During this time pre-warm the oven (80ºC).
4. Remove the membranes and wrap them into Whattmann Paper and close it with metal clips.
5. Incubate during 2h at 80ºC (to allow DNA cross-linking).

------ (After cross linking the membrane can be stored or you can proceed to next step) ------

Unspecific DNA coating

1. Pre-warm the water bath (2.47.lab) and the hybridazation oven (rolling oven in the radio lab) at 65ºC.
2. Thaw the pre-hybridization solution that was stored at -20ªC. 20 ml of this solution are needed for each hybridization tube (= for each membrane).
3. During this time
   - Add water to the boiler (in the radio lab) and make it boil.

- Clean the hybridization tubes with water. Fill them with water and pre-warm them at 65 ºC (water bath at 2.47 lab)

4. Boil the pre-hybridization solution (in the boiler at the radio lab) during 10 min. Afterwards, cool the solution to 65ºC by putting the falcon tubes in the water bath.
5. Pour water in a metalbox and rehydrate the membrane.
6. Remove the water from the hybrydization tubes.
7. Roll the membrane onto a glass pipette in a way that the part that has been in contact with DNA touches the pipette.
8. Introduce the pipette into the hybridization tube and roll it against the walls of the tube in order to adhere the membrane to the tube (part that has been in contact with DNA is now facing the inner part of the tube).
9. Add 20mL of pre-hybridization solutions to each tube. Introduce the tubes into the hybridization oven. Is it better to balance the rotor of the oven (i.e. one tube at the opposite site of the other).
10. Let the unspecific-DNA coating perform minimum for 30' (no maximal time specified).

Probe labelling

Labelling kit: Amersham RediPrime II Random Primer Labelling System (RPN1633 GE Healthcare).
Radioactivity used: dCTP, [α-32P]- 3000Ci/mmol 10mCi/ml Lead, 100 µCi (Perkin Elmer NEG013H100UC)

1. Add 2.5uL of the purified probe (**the purified probe must be ≈10ng/uL)**[*]· into an eppi and fill it up to 45uL with 1xTE buffer (onwards TE-probe). Prepare 1 eppi with the probe for each hybridization tube.
2. Pick up all the material needed to the radiolab:
   - Micropipettes
   - Tips
   - 1 vial Amersham RediPrime II/hybridization tube
   - Empty eppendorf (1/hybridization tube)
3. Boil TE-probe in the boiler at the radiolab for 5' in order to separate the DNA strands of the hexamers.

4. **Immediately after** place the eppendorf in ice in order to prevent DNA strands come together again.
5. Heat the thermoblock to 37ºC.
6. Pipet the TE-probe into the Amersham vial.
7. Add 5uL of radioactivity to each vial.
8. Mix by pipetting up and down until blue colour is homogeneus.
9. Incubate at 37ºC (thermoblock) for 30'.
10. After this time, stop the radioactivity-labelling reaction by adding 2.5uL of 0.2M EDTA to each vial.
11. Purify the radiolabelled-probe by size-exclusion chromatography (illustra Microspin G-50column; 27-5330-02 GE Healthcare):
    - Mix the resin by vortexing 5-10''
    - Remove the lower and thinner part of the column.

    - Open the lid a little bit in order to allow air pass through the Sephadex resin when centrifuging.
    - Centrifuge 1' (x1000) rpm to remove the buffer and dry the column resin.
    - Remove the eppendorf and place the column into a new one.
    - Load the radiolabelled mixture to the column. Close the column tightly with the lid.
    - Centrifuge 2' (x1000) rpm to purify the probe. **Important:** place the column in the same position as on previous centrifugation step.
    - After centrifugation the coloured material must remain in the column while the eluted material (i.e. labelled probe) must be at the bottom of the eppi with clear appearance.
    - Check radioactivity of the eppendorf containing radiolabelled probe (60.000 IPS is OK).
    - Heat the boiler to 100ºC.
    - Boil the probes for 5'.
    - Immediately after place the eppendorfs in ice in order to prevent DNA strands come together again.
    - Pippete up and down the probe sample and load it to the hybridization tube (NOT directly to the membrane, but to the pre-hybridization solution).
    - Fast, place the hybridization tubes into the hybridization oven and incubate o/n.

Day 3. Washing of blot and prepare blot for developing

Washing of Blot

1. Dump the hybridization solution in the radioactive waste container being careful not to drip.
2. Use a paper towel to catch the drips off the lip of the tube.
3. Add 30 ml of 2x SSC to hybridization tube.
4. Roll in hybridization tube for 10 minutes at room temperature.
5. Dump solution in radioactive waste.
6. Wash with 30 ml of 2x SSC at room temperature for 10 minutes.
7. Dump solution in radioactive waste.
8. Check radioactivity of the blot with the Geiger counter. The counts should be around 10 to 50 counts per second and there should be bands of irradiation peaks.
9. If the counts are higher than 50 count/second or no bands can be heard, more washing is needed follow the following list (Remember that it is important to go slow so not to completely wash off all the probe):
    - 30 ml of 2x SSC + 1% SDS at 65O C for 15 minutes (measure radioactivity)
    - 30 ml of 2x SSC + 1% SDS at 65O C for 15 minutes (measure radioactivity)
    - 30 ml of 2x SSC + 0.1% SDS at 65O C for 10 minutes (measure radioactivity)

Wrapping and Preparing of the Blot for Developing

Place a new Saran wrap down on cart.

Place blot face down and make sure there is no bubbles.

Carefully wrap the blot trimming excess from the edges.

Tape blot to the filter paper (the size of the film cassette - 8 x 10 inches).

Take to darkroom and place film on blot making sure to crease the top right corner of the film for reference.

Place cassette with film and blot into the -70º C freezer overnight.

Develop Film

-Take film and develop.

-Mark on the film the samples/enzyme/date/time of the exposure.

-If needed, place another sheet of film down on the blot and return it to the freezer and let expose over the weekend.

8.10.3. PROTEIN ANALYSIS TECHNIQUES

8.10.3.1. PROTEIN SEPARATION BY SDS-PAGE

Similarly to DNA separation previously explained, proteins can also be separated by electrophoresis. In an electric field, proteins move toward the electrode of opposite charge. The rate at which they move (migration rate, in units of $cm^2/Vsec$) is governed by a complex relationship between the physical characteristics of both the electrophoresis system and the proteins. Factors affecting protein electrophoresis include the strength of the electric field, the temperature of the system, the pH, ion type, and concentration of the buffer as well as the size, shape, and charge of the proteins.

In this thesis, Polyacrylamide Gel Electrophoresis (PAGE) in presence of SDS (SDS-PAGE) were performed using precast Bio-Rad's Mini-PROTEAN® gels. This system is a discontinuous buffer system, meaning that it uses a gel separated into two sections (a large-pore stacking gel on top of a small-pore resolving gel) and different buffers in the gels and electrode solutions.

By the addition of SDS to the samples, proteins become fully denatured and dissociate from each other. In addition, SDS binds noncovalently to proteins in a manner that imparts:

- An overall negative charge on the proteins. Since SDS is negatively charged, it masks the intrinsic charge of the protein it binds.
- A similar charge-to-mass ratio for all proteins in a mixture, since SDS binds at a consistent rate of 1.4g of SDS per 1g protein (a stoichiometry of about one SDS molecule per two amino acids).
- A long, rod-like shape on the proteins instead of a complex tertiary conformation.

As a result, the rate at which SDS-bound protein migrates in a gel depends primarily on its size, enabling molecular weight estimation.

The following procedure was followed for protein separation:

1. Running buffer is prepared 10x and diluted into MilliQ H_2O prior to its use. In order to prepare 1L of running buffer (10x) add the following components to a 1L bottle:

Tris base	30.30g
Glycine	144.10g
SDS	10g
MilliQ H_2O	Up to 1L

2. Prepare enough loading buffer for all the samples in a single eppendorf and dispense x µL of the buffer into each vial containing the same volume of the sample. The volume of the sample depends on the concentration of the protein of interest, taking into account that:
   - The maximal volume to be loaded in the precast Bio-Rad's Mini-PROTEAN® gel is 30µL
   - A minimum of 300ng, 10ng or 1ng of protein are needed to detect a band using Comassie staining, Colloidal Comassie staining and silver staining, respectively.

   For a single sample, loading buffer is as follows:

Laemmli buffer (2x)	4.75 µL
B-mercaptoetanol	0.25 µL

3. Heat the samples at 95ºC for 5 minutes in a termoblock.
4. Immediately place samples on ice for 2 minutes.
5. Pulse centrifuge the vials to recover all the sample in the bottom of the vial.
6. Remove the sealing tape on the bottom of the precast Bio-Rad's Mini-PROTEAN® gel.
7. Place the gel on the electrode module. Put the module on dry paper and pour running buffer (1x) in it. Be sure that any leaking is observed.
8. Place the electrode module into the tank of te Mini-Protean Cell. Carefully, take out the comb and fill the inside of the electrode module with running buffer until it completely covers all the lanes of the gel.
9. Pour running buffer into the tank until half part of the volume. It is very important that the same running buffer is used for filling the electrode module and the tank .
10. Homogenise sample by pipetting up and down and load up to 30 µL into each lane of the gel. Into the first and last lane load 5 µL of protein standard (Precision Plus protein standard All Blue, 161-0373 BioRad).
11. Run electrophoresis for 40 minutes at fixed voltage (200V).
12. After this time, remove the gel cassette and open it according to the manufacturer's instructions. Sometimes it is helpful to lift one edge of the gel with a spatula.

8.10.3.2. PROTEIN DETECTION BY COLLOIDAL COMASSIE STAINING

Proteins separated by gel electrophoresis can be visualized using different staining procedures. In this work, a colloidal comassie blue staining adapted from Candiano et al. [22] was used.
For the preparation of the colloidal comassie dye the following steps should be followed:

1. Add 100mL of MilliQ H_2O into a 1L beaker.
2. Add 100mL of phosphoric acid into the beaker.
3. Weigh 100g of ammonium sulfate and slowly dissolved it into de solution inside the beaker under continuous stirring.
4. When ammonium sulfate is completely dissolved, add 1.2gr of comassie blue G250 (Merck, Ref. 15444).
5. Once it is completely dissolved, add MilliQ H_2O until 800mL.
6. Ad 200mL of anhydrous methanol.
7. Pour the solution into a stained bottle to protect the dye from light.

To stain the gel, put it into a glass dish and cover it completely with colloidal comassie blue dye. Incubate overnight at room temperature under continuous soft agitation using a rocker.

8.10.3.3. CapPCV2 PROTEIN QUANTIFICATION

For the detection of the protein of interest (CapPCV2) a commercial ELISA sandwich test was chosen as the most suitable technique. The most important advantages of this method are (1) its high specificity. Since two antibodies are used, the antigen/analyte is specifically captured and detected and (2) it is suitable for complex samples, since the antigen does not require purification prior to measurement.

The ELISA test used in this work was PCV2 DAS (Ingenasa, Ref. 11CIR.K2)), which is definied as a semi-quantitative ELISA method. In order to change this test to a quantitative test the quantification of the antigen in the commercial anti-PCV2 vaccine Porcilis PCV2 (Intervet) was carried out. Thereafter, the corresponding dilutions of the vaccine will be added in each protein quantification assay in order to correlate OD_{450}nm signal with CapPCV2 protein concentration (i.e. calibration curve of the assay). In Figure 8.17, the results of the SDS-PAGE of the vaccine are presented. As the molecular weight of CapPCV2 is known (28kDa) and no other proteins of this size are present in Porcilis PCV2, it was possible to quantify the concentration of antigen in the sample comparing the

signal intensity of [BSA]=20µL/mL with the band corresponding to CapPCV2 in Porcilis PCV2 sample. The antigen concentration of the vaccine was determined at 10.63 µL/mL, which was in good correlation with bibliography [23].

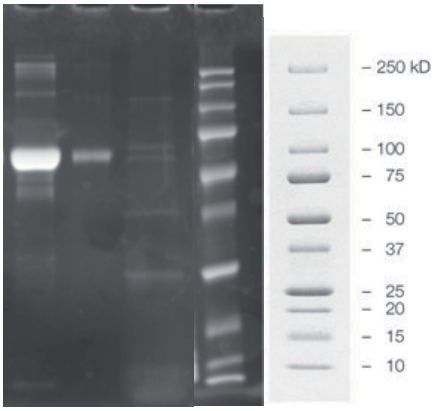


Figure 8.17. SDS-PAGE for Porcilis PCV2 quantification.

Table 8.25. Identification of the samples loaded in the gel depicted in Figure 8.17.

Lane	ID	Vol (µL)
1	Albumin standard $[BSA]_f$=500µL/mL	10
2	Albumin standard $[BSA]_f$=20 uL/mL	20
3	Aquous phase Porcilis	10
4	Ladder Precision Plus Bio Rad	5

Testing the saturation limit of the ELISA test, the appropriated dilutions of Porcilis PCV2 for the calibration curve performance were assessed. As it is shown in Figure 8.18, OD_{450}nm signal was saturated for dilutions lower than 1/8.

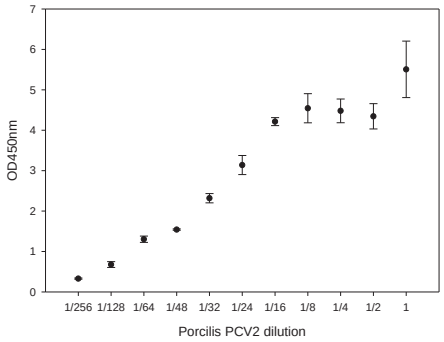


Figure 8.9 Correlation between Porcilis PCV2 dilution and OD_{450}nm signal.

Procedure:

Two hours before starting the test, take the following reagents out from fridge in order to bring them to room temperature (20-25ºC): ELISA plate, concentrated diluent (5x), concentrated washing solution (25x), MilliQ H_2O, positive control and negative control of the test.

One hour before starting the test, take the following reagent out from fridge in order to bring it to room temperature (20-25ºC): secondary antibody (mouse monoclonal anti-CapPCV2 HRP conjugated).

If the sample to be tested is stable at room temperature, bring it to room temperature two hours before starting the test. In our case, the stability of the samples at room temperature was unknown. Hence, samples were stored at 4ºC until their use.

1. Dilute the concentrated washing solution to a final concentration 1x with MilliQ H_2O.
2. Dilute the concentrated diluent solution to a final concentration 1x with MilliQ H_2O.
3. Dilute Porcilis PCV2 1/8 with diluent solution (1x).
4. Perform two-fold dilution and thre-fold dilution of Porcilis PCV2 (1/8) in the second and third well of the calibration curve column. Then, perform two-fold dilutions in order to obtain the following dilutions: 1/32, 1/48, 1/64, 1/96, 1/128. Hence, final dilutions in the calibration curve calibration curve will be: 1/8, 1/16, 1/24, 1/32, 1/48, 1/64, 1/96, 1/128
5. Samples and controls: add to the respective wells 100µL of positive and negative controls in duplicate. Add 100µL of the samples in the corresponding well. In case that dilution of the sample is performed, do it direcly appliying the sample to the well with the corresponging volume of diluent solution (1x) in it. Whenever is possible, test the samples in duplicate.
6. Seal the plate and incubate 1 hour at room temperature. When starting this incubation take the rest of the components of the kit (i.e. TMB substrate (1x) and stop solution (1x)) out from the fridge and bring them to room temperature.
7. Wash 5 times by pipetting. After the last step of washing shake the plate turned over an absorbent filter paper.
8. Add 100µL of conjugate to each well. Seal the plate and incubate 30minutes at room temperature.
9. Wash 5 times by pipetting. After the last step of washing shake the plate turned over an absorbent filter paper.
10. Add 100µL of substrate to each well and incubate for 15 minutes at room temperature and in darkness conditions (count the time since the first well had been filled. Let the controls wells be the last to be filled).
11. Add 100µL of stop solution to each well. The stop solution must be dispensed in the same order in which the substrate was added.
12. Read the OD of each well at 450nm.

8.10.4. METABOLITES ANALYSIS

8.10.4.1.GLUCOSE AND LACTATE MEASUREMENT

Glucose and lactate are measured in an YSI automatic glucose and lactate analyzer (Yellow Springs Instrument, 2700 Select).

YSI membranes contain three layers. The first layer, porous polycarbonate, limits the diffusion of the substrate into the second layer (in which the enzyme is retained) preventing the reaction from becoming enzyme-limited. The secon layer, is the immobilized enzyme specific for the substrate of interest (glucose oxidase or L-lactate oxidase). The substrate is oxidized as it enters the enzyme layer, producing hydrogen peroxide, which passes through the third later. This layer, made from cellulose acetate, permits only small molecules (such as hydrogen peroxide) to reach the platinum electrode, eliminating many electrochemically-active compounds that could interfere with the measurement. At the electrode, the hydrogen peroxide is oxidized and the resulting current is proportional to the concentration of the substrate. The analyzer uses 25µL of the sample and provides the values of glucose and lactate concentration (g/L), with an error of ± 0.1 g/L . The instrument range is 0.05-20.0 g/L for glucose and 0.05-2.00 g/L for lactate. When the samples are too concentrated it is necessary to dilute them with MilliQ H_2O until the measure fits into the range of measurement.

8.10.4.2. AMINOACIDS MEASUREMENT

Analysis were carried out at Scientific amd Technological Centers of the University of Barcelona (CCIT-UB).

Chromatographic separation by cation-exchange chromatography followed by post-column derivatization with ninhydrin and UV detection [24]

- **Amino Acid Analyzer:** Brand: Biochrom; Model: Biochrom 30
- **Column:** PEEK manufactured column with cation-exchange resin (Ultropac, polystyrene/divinylbenzene sulfonate) 5 µm, 200 x 4 mm (Biochrom Ltd.). Column Temperature controlled by Peltier system (34-80 ºC)
- **Sample Injection:** Samples are resuspended or diluted in Lithium Citrate Loading Buffer pH 2.20 (Biochrom Ltd.) and loaded after being filtered. Injection volume: 20-100 ul
- **Elution:** Gradient elution with Biochrom Amino Acid Reagents consisting of five lithium citrate buffers of increasing pH (2.80 – 3.55) and I (0.2 M- 1.65 M) along the chromatogram.

- Column regeneration with litium hydroxide 0.3 M.
- **Detection:** Column effluent reacts with ninhydrin in a reaction coil with temperature control at 135 ºC. Derivatized amino acids are detected colorimetrically at 570 nm and 440 nm wavelenghts.
- **Identification:** Amino acid peaks are identified according to the retention times of corresponding standards.
- **Quantitation:** Amount of amino acids is calculated by internal standard (IS) method. A known amount of norleucine, as IS, is added to the sample and analyte amount is calculated using area responses of analytes and IS.
- **Detection limit:** Approx. 15 pmol.
- **Reproducibility:** RSD 1.5 % (10 nmol)
- **Standards**: Amino acid standard A6282, A6407, A2908 (Sigma Aldrich) are used.
- Internal standard solutions are prepared using DL-Norleucine (Sima-Aldrich).
- **Data acquisition and treatment**: EZChrom-Elite software.

8.10.5. MITHOCONDRIA ISOLATION AND RESPIROMETRY METHODOLOGY

8.10.5.1 MITOCHONDRIA ISOLATION

The Mitochondrial isolation kit for cultured cells (Thermo Scientific) was used for the isolation of mitochondria from HEK293 cells. From the two possible protocols, the isolation using reagent-based method was applied following the manufacter's instructions. Mitochondria were isolated from a HEK293 cell culture in HyQ SFMTransFx-293+FBS (5% v/v) at Xv=4x10^6 cell/mL (inocula cell density was of 0.25x10^6 cell/mL). At the time of harvesting, cells were centrifuged at 850g for 2 minutes and then Thermo Fisher protocol was followed. It is highly important that all the procedure is performed at 4ºC in pre-cooled centrifuge tubes. The incubation periods must be carried out in ice. After the last step indicated by the manufacter, the pellet must be resuspended in 150 µL of incubation buffer (IB) and must be stored at 4ºC until the respirometry assay. The incubation buffer composition is detailed in the Table 8.26. The respirometry assay must be performed before 4 hours from the last centrifugation of the mitochondrial isolation protocol. Before respirometry, protein quantification was done by Bradford or Lowry method. Protein concentration must be within the range 40-80 mg/mL.

Table 8.26. Isolation buffer compostition

Component	Quantity for 100 mL	Final concentration
Manitol	4,10 g	225 mM
Sacarose	2,57 g	75 mM
EDTA	29,22 mg	1 mM
HEPES	119,15 mg	5 mM
BSA	100 mg	1 mg/ml
KCl	c.s.p pH 7,4	
Milli-Q water	c.s.p 100 ml	

8.10.5.2. RESPIROMETRY ASSAY

Prepare the mithocondrial pool (which is in the isolation buffer) by diluting the sample to approximately 200-500 µg/mL in Respiration buffer. This buffer composition is detailed in Table 8.27.

Add the desired molecule (i.e. lactate, pyruvate or blocking agent) to the respiration buffer containing mitochondria pool.

Oxygen was measured with the oxygen probe of Waveport DCU hermetically joint to the tap of the sample container.

Table 8.27. Respiration buffer compostition

Component	Quantity for 100 ml	Concentration
Manitol	218,60 mg	12 mM
Sacarose	1,54 g	45 mM
EDTA	29,22 mg	7 mM
Tris-HCl	30,29 mg	25 mM
$MgCl_2$	47,61 mg	5 mM
K_2HPO_4	261,30 mg	15 mM
KH_2PO_4	204,13 mg	15 mM
KCl	111,83 mg	15 mM
BSA	0,2 mg	0,2 % (p/v)
ADP	0,05 µg	5 nM
Milli-Q water	c.s.p 100 ml	

8.11. METABOLIC FLUX ANALYSIS

8.11.1. ADAPTION OF THE METABOLIC FLUX MODEL

The metabolic network used in this study was derived from the Homo sapiens Recon 2 genome-scale metabolic network reconstruction[25]. Genome-scale metabolic network reconstructions represent biochemical, genetic, and genomic (BiGG) knowledge bases for a target organism. Thus, they correspond to two-dimensional genome annotations: that is, they contain all nodes and links that comprise a biochemical reaction network defined by the genome[26]. These reconstructions allow the conversion of biological knowledge into a mathematical format and subsequent computation of physiological properties. Therefore they enable the formulation of a mechanistic genotype–phenotype relationship for metabolic functions in the target organism[27].

Recon 2 is a community-driven expansion integrating metabolic information from five different resources:

- Recon 1, a global human metabolic reconstruction [28]
- EHMEN, Edingurgh Human Metabolic Network [29]
- HepatoNet1, a liver metabolic reconstruction[30]
- Ac/FAO module, an acylcarnittine/fatty acid oxidation module[31]
- A human small intestinal enterocytes reconstruction[32]

Additionally, more than 370 transport and exchange reactions were added, based on a literature review.

Much of this expansion was performed at reconstruction 'jamboree' meetings[27], focused events at which domain experts apply their knowledge to refine and consolidate biochemical knowledge from existing reconstructions and published literature[25].

Although Recon 2 is simulation-ready, flux results obtained tend to be loose and non-specific. The high degree of redundancy in the model masks metabolic contribution of individual reactions. Hence, a reduced version of Recon 2 was used in this project to study the metabolism of HEK293 cells in culture. The curation criteria were the same as previously reported for a similar HEK293 study [33]. In addition, the metabolic reactions related to gluconeogenesis and lactate consumption in the mithocondria have been activated in the metabolic model used in this work. From the 7440 initial metabolic reactions contained in Recon 2, the curation process ended in a model of about 350 metabolic reactions. The complete list of reactions can be found in Appendix 10.5.

8.11.2. CALCULATION OF METABOLIC CONSUMPTION/PRODUCTION RATES

Measurements of cell specific nutrient uptake rates and product formation provide the basis for intracellular flux calculations using flux balance analysis (FBA) or metabolic flux analysis (MFA). From the stoichiometric coefficients of the reaction network described above, a matrix ST is constructed such that ST x f= q, with f being a vector of the fluxes through the various biochemical reactions. The vector q contains the actual measured specific rates of change of extracellular metabolites. Hence, the measured extracellular rates are critical inputs to these methods because they constrain the solution space of feasible intracellular fluxes. Therefore, accurate estimation of cell specific extracellular rates, and their associated uncertainties, is an essential task in the reconstruction of accurate metabolic flux maps.

In this work, a MATLAB software package called Extracellular Time-Course Analysis (ETA) was used for the calculation of the specific metabolic rates used as inputs for the constructed FBA. ETA software performs calculation of cell specific metabolic rates and their uncertainties using either Gaussian error propagation or Monte Carlo analysis, and assessment of the goodness-of-fit of the exponential (or linear) growth mode. For further information of this software development, can be found in the original paper[34]. The results of calculated rates are compiled in Appendix 10.5.

8.11.3. CALCULATION OF METABOLIC FLUXES

Optflux software[35] (was used for the determination of the metabolic fluxes presented in Chapter 6 of this doctoral thesis. Breifly, the software contains four functional modules, which accommodate tools and algorithms for the manipulation of metabolic models (Figure 8.19):

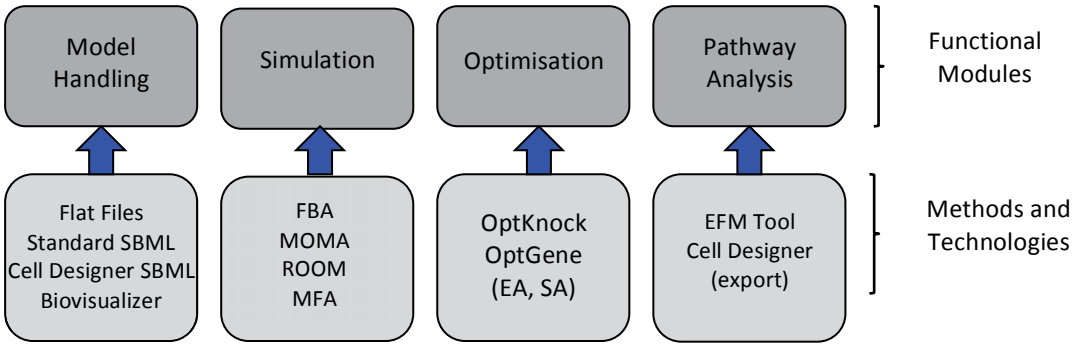


Figure 8.19. Functional modules of Optflux software. Adapted from[34]

Among the available tools within the software, the variant of flux balance analysis (FBA), referred to as Parsimonious enzyme usage FBA (pFBA) was used in this work[36]. The pFBA, method relies on the

underlying assumption that, under growth pressure, cells have been optimized to be as efficient as possible using the minimum amount of enzyme. These assumptions are approximated by employing FBA to optimize a certaing objective function, followed by minimizing the net metabolic flux through all gene-associated reactions in the network. Therefore, pFBA finds the subset of genes and proteins that most efficiently contribute to metabolic network performance under the given growth conditions.

8.11.4. METABOLIC FLUX REPRESENTATION.

Omix editor and modeling tool[37] was used for the representation of metabolic fluxes presented in Chapter 6.

8.12. REFERENCES

1. Côté, J., Garnier, A., Massie, B. & Kamen, A. Serum-free production of recombinant proteins and adenoviral vectors by 293SF-3F6 cells. *Biotechnol. Bioeng.* **59,** 567–575 (1998).

2. Hanahan, D. & Harbor, C. S. Studies o n T r a n s f o r m a t i o n o f Escherichia coli with P l a s m i d s Department of Biochemistry and Molec ~ dar Biology. **0,** 557–580 (1983).

3. Bullock, W. O., Fernandez, J. M. & Short, J. M. XL1-Blue: A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. *Biotechniques* **5,** 376–379 (1987).

4. Jerpseth, B., Callahan, M. & A, G. An efficient random mutagenesis technique using an E.coli mutator strain. *Strategies* **10,** 37–38 (1997).

5. Marazzi, J., Kleyer, J., Manuel, J., Paredes, V. & Gertsch, J. Endocannabinoid content in fetal bovine sera — Unexpected effects on mononuclear cells and osteoclastogenesis. *J. Immunol. Methods* **373,** 219–228 (2011).

6. *Martindale: The Extra Pharmacopeia*. (The Pharmaceutical Press, 1996).

7. D A A, M., P G H, B. & I, E. No Title. *Arch Leb.* **29,** 121–127 (1978).

8. Anderlei, T. & Büchs, J. Device for sterile online measurement of the oxygen transfer rate in shaking flasks. **7,** 157–162 (2001).

9. Anderlei, T., Zang, W., Papaspyrou, M. & Büchs, J. Online respiration activity measurement (OTR , CTR , RQ) in shake flasks. **17,** 187–194 (2004).

10. H, N., K, Y. & J, M. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **2,** 193–199 (1991).

11. Turan, S. *et al.* Recombinase-Mediated Cassette Exchange (RMCE): Traditional Concepts and Current Challenges. *J. Mol. Biol.* **407,** 193–221 (2011).

12. Zhu, X. & Sadowski, P. D. Cleavage-dependent Ligation by the FLP Recombinase. **270,** 23044–23054 (1995).

13. Nishimura, A., Moritai, M., Nishimura, Y. & Sugino, Y. A rapid and highly efficient method for preparation of competent Escherichia coli cells. **18,** 6169 (1990).

14. Akinc, A. & Langer, R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. 657–663 (2005). doi:10.1002/jgm.696

15. Cervera, L. *et al.* Generation of HIV-1 Gag VLPs by transient transfection of HEK 293 suspension cell cultures using an optimized animal-derived component free medium. *J. Biotechnol.* **166,** 152–165 (2013).

16. Mittereder, N., March, K. L. & Trapnell, B. C. Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy. *J. Virol.* **70,** 7498–7509 (1996).

17. RJ, R. Restriction endonucleases. *Rviews Biochem.* **2,** 123–164 (1976).

18. Kessler C, M. V. Specifity of restriction endonucleases and DNA modification methyltransferases a review. *Gene* **2,** 1–248 (1990).

19. S, A. W. L. DNA modifications and restriction. *Annu. Rev. Biochem.* 467–500 (1969).

20. H, K. D. & A, B. T. Bacteriophage survival: multiple mechanisms for avoiding the deoxyribonucleic acid restriction systems of their hosts. *Microbiol. Rev.* **3,** 345–360 (1983).

21. B, V. & D, G. Preparative and analytical purification of DNA from agarose. *Proc. Natl. Acad. Sci.* 615–619 (1979).

22. Candiano, G. *et al.* Blue silver : A very sensitive colloidal Coomassie G-250 staining for proteome analysis. 1327–1333 (2004). doi:10.1002/elps.200305844

23. Roerink, F. & Woensel, P. Van. PCV-2 VACCINE. (2009).

24. Spackman, D. H., Stein, W. H. & Moore, S. Automatic Recording Apparatus for Use in Chromatography of Amino Acids. *Anal. Chem.* **30,** 1190–1206 (1958).

25. Dennis, M. K. *et al.* A community-driven global reconstruction of human metabolism. *Nat Biotechnol* **5,** 358–366 (2013).

26. Palsson, B. Two-dimensional annotation of genomes. *Nat Biotechnol* 1218–1219 (2004).

27. Thiele, I. & Palsson, B. Ø. Reconstruction annotation jamborees: a community approach to systems biology. *Mol. Syst. Biol.* **6,** 361 (2010).

28. Duarte, N. C. *et al.* Global reconstruction of the human metabolic network based on genomic and bibliomic data. **104,** (2007).

29. Hao, T., Ma, H., Zhao, X. & Goryanin, I. Compartmentalization of the Edinburgh Human Metabolic Network. (2010).

30. Gille, C. *et al.* HepatoNet1 : a comprehensive metabolic reconstruction of the human hepatocyte for the analysis of liver physiology. *Mol. Syst. Biol.* **6,** (2010).

31. Sahoo, S., Franzson, L., Jonsson, J. J. & Thiele, I. A compendium of inborn errors of metabolism mapped onto the human metabolic network. *Mol. Biosyst.* **8,** 2545–2558 (2012).

32. Sahoo, S. & Thiele, I. Predicting the impact of diet and enzymopathies on human small intestinal epithelial cells. **22,** 2705–2722 (2013).

33. Quek, L. E. *et al.* Reducing Recon 2 for steady-state flux analysis of HEK cell culture. *J. Biotechnol.* **184,** 172–178 (2014).

34. Murphy, T. A. & Young, J. D. ETA: robust software for determination of cell specific rates from extracellular time courses. *Biotechnol. Bioeng.* **6,** 997–1003 (2013).

35. Rocha, I. *et al.* OptFlux: an open-source software platform for in silico metabolic engineering. *BMC Syst. Biol.* **4,** 45 (2010).

36. Lewis, N. E. *et al.* Omic data from evolved E. coli are consistent with computed optimal growth from genome-scale models. *Mol. Syst. Biol.* **6,** 390 (2010).

37. Droste, P., Miebach, S., Niedenführ, S., Wiechert, W. & Nöh, K. Visualizing multi-omics data in metabolic networks with the software Omix-A case study. *BioSystems* **105,** 154–161 (2011).

CHAPTER 9. AUTHOR'S CONTRIBUTIONS TO EACH RESULTS CHAPTER

CHAPTER 3. RESULTS (I)

Shake flask studies: experimental design, experimental work, data analysis and chapter sections writing. Research supervision from Dr. Jordi Joan Cairó and Dr. Martí Lecina.

Bioreactor studies: experimental design and experimental work of batch fermentations carried out together with Dr. Martí Lecina. Fed-batch strategy was implemented by PhD student Jonatan López-Repullo as part of his Master thesis (. Data analysis for all cell cultures strategies studied was made by the author as well as section writing. Research supervision and discussion of experimental work form Dr.Jordi Joan Cairó and Martí Lecina.

CHAPTER 4. RESULTS (II)

Recombinant adenovirus generation and set up of titration protocols: experimental design, experimental work, data analysis and chapter section writing. Research supervision from Dr. Jordi Joan Cairó and Dr.Martí Lecina. Also, Dr. Miguel Chillón provide valuable knowledgment on the field.

Screening of best conditions for infection: experimental design and its discussion was carried out by the author together with Dr.Jordi Joan Cairo and Dr. Martí Lecina. Experimental work was carried out by the author and Mrs. Mercè Farràs who did this experimental work as her Final degree project ("Methodology for vaccine production based on adenoviral vectors. Universitat Autònoma de Barcelona, November 2012). Data analysis and section chapter writing performed by the author.

Evaluation of r-CapPCV2 after infection with r-AdV-*Cap*PCV2: experimental design, experimental work, data analysis and chapter section writing. Research supervision from Dr. Jordi Joan Cairó and Dr.Martí Lecina.

CHAPTER 5. RESULTS (III)

Author's contribution: Experimental work, experimental design, data analysis and chapter writting. Research supervision from Dr. Jordi Joan Cairo, Dr. Martí Lecina, Prof. Hansjörg Hauser, Dra. Dagmar Wirth and Dr. Roland Schucht.

CHAPTER 6. RESULTS (IV)

Physiology studies: experimental work was performed by the author and Mr. Adrià Urbano as part of his Master thesis (" Estudi del metabolisme de la glucose I l'àcid lactic en cèl·lules HEK293", Univeristat Autònoma de Barcelona, July 2014). Experimental analysis was performed by the author and research supervision from Dr. Jordi Joan Cairó and Dr.Martí Lecina.

Metabolic studies: Construction of metabolic model and implementation of flux balance analysis to that model was performed by Dr J.Albiol and Dr C Solà. Prelimina experiments of isolated mitochondria metabolism were carried out by PhD student Jonatan López-Repullo. Data analysis and discussion was mainly performed by the author, Dr. Jordi Joan Cairó, Dr.Martí Lecina and Dr J. Albiol. Chapter writing was performed by the author.

10. APPENDIX

10. 1. COMPILATION OF HEK 293 CELL CULTURE STUDIES

Cell culture Strategy	Cell culture plattform	Cell line	Media	Maximal Cell Density ($x10^6$cell/mL)	μ_{max} ($x10^{-2}h^{-1}$)	Time to X_{max} (h)	Xv_0 (x106cell/mL)	Application	Reference
Batch	1.5L Bioreactor (microcarriers??)	HEK/EBNA-SF	Episerf medium+0.1%Pluronic F-68	2.25	ND	168	0.2	Transient transfection	2011_Jbiotech_Fliedl-Kaisermayer_(Transient gene expression in HEK293 and vero cells immobilised on microcarriers)
Batch	2L Bioreactor	HEK293	ND (provided by Life Technologies)	1.7-2.3	ND	144-168	0.5	Adenovirus production	1996_Cytotechnology_Iyer-Vacnate (Comparison of manufacturing techniques for adenovirus production)
Batch	2-L bioreactor (B. Braun, Melsungen, Germany) (wv=2L)	293H from GibcoBRL, Life Technologies, MD	293 SFM II	2.5	2.5	100	0.3	Cell media study and adenovirus production	2003_BiotechProgress_Lee-Wong (Low-Glutamine Fed-Batch Cultures of 293-HEK Serum-Free suspension cells for adenovirus production)
			DMEM/F12	1.5	2.7	60	0.3		
Batch	125mL shake flask (wv=25mL)	293SF	NSFM13	3.2	ND	ND	0.25	Adenovirus production	2010_BiotechnoloProg_Shen-Kamen (Reassessing Culture Media and Critical Metabolites that Affect Adenovirus Production)

continue next page

Cell culture Strategy	Cell culture plattform	Cell line	Media	Maximal Cell Density ($x10^6$cell/mL)	μ_{max} ($x10^{-2}h^{-1}$)	Time to X_{max} (h)	Xv_0 (x106cell/mL)	Application	Reference
Batch	2L Biroeactor (wv=0.5L)	HEK293-IFN (293-D9)	Freestyle F17 expression medium (Invitrogen) +4mM glutamine+0.1% anti-clumping agent+ 1% Pluronic F-68+1% penicillin–streptomycin (Invitrogen)	3.3	3.0	120	0.2	Metabolism study	2014_Jbiotech_Vallé-Henry (Exploiting the metabolism of PYC expressing HEK293 cells in fed-batch cultures)
Batch	125mL shake flask (wv=40mL)	293 (ATCC-CRL-1573)	CD293 (Invitrogen)	4	1.9	192	0.25	Adenovirus production	2005_Biotchnology Letters_Ferreria-Alves (Two different serum-free media and osmolality effect upon human 293 cell growth and adenovirus production)
			EX-Cell (JRH)	7.4	1.8	216	0.25		
Batch	1L (Biostat Q, B. Braun, Germany)	293SFE	LC-SFM (invitrogen)+0.1%Lipid mixture(1000xSigma)+ 0.5% (w/v) of Gelatin Peptone N3 (OrganoTechnie S.A.)	4	ND	144	0.28	Transient transfection	2003_Biotech&Bioeng_Pham-Durocher (Large-Scale Transient Transfection of Serum-Free Suspension-Growing HEK293 EBNA1 Cells: Peptone Additives Improve Cell Growth and Transfection Efficiency)
Batch	2L Bioreactor	293 (ATCC-CRL_1573)	CD293 (Invitrogen)+4mM Glutamine	5.5	1.8	ND	ND	Metabolism study and adenovirus production	2004_Jbiotech_Ferreira-Alves

continue next page

Cell culture Strategy	Cell culture plattform	Cell line	Media	Maximal Cell Density ($x10^6$cell/mL)	μ_{max} ($x10^{-2}h^{-1}$)	Time to X_{max} (h)	Xv_0 (x106cell/mL)	Application	Reference
Batch	3 L-bioreactor (Applikon) (wv=1.5L)	Hek293F (invitrogen)	Freestyle Expression Medium (Invitrogen) supplemented with 0.5 mM glutamine, 3 mM Glutamax, 100 mg/mL dextran sulfate (Mw = 5000 Da) and 4 mL/L Pluronic-F12	5	2.8	80	0.6	Metabolism study	2012_PlosOne_Dietmair-Nielsen (A Multi-Omics Analysis of Recombinant Protein Production in Hek293 Cells)
Batch	125mL shake flask (wv=25mL)	HEK293SF	50% LC-SFM+ 50% CD293(Invitrogen) + 0.1% BSA	6	2.9	120	0.25	Adenovirus production	2012_Jbiotech_Shen-Kamen (Hyperosmotic pressure on HEK 293 cells during the growth phase, but not the production phase, improves adenovirus production)
Batch	1L square bottle (wv=0.3L)	HEK-293 EBNA	Ex-Cell 293	6-7	ND	120	0-35	Study of cell culture conditions	2004_Biotech&Bioeng_Muler-Wurm (Orbital Shaker Technology for the Cultivation of Mammalian Cells in suspension)
Batch	3L Bioreactor Chemap type SG bioreactor (Mannedorf, Switzerland) (wv=3L)	HEK293SF	SFM4Transfx-293TM (HyQ)	8.3	2.1	192	0.25	Cell ,etabolism study and Influenza virus production	2011_BMC Biotechnology_Petiot-Kamen (Metabolic and Kinetic analyses of influenza production in perfusion HEK293 cell culture)
Batch	2L shake flask (wv=0.6L) 3-L Chemap CF-2000 bioreactor (wv=2.8L)	293SF 3F6	Low calcium H-SFM (GIBCO)+0.1 (w/v) BSA (sigma)+1%(v/v)chemically defined lipids (GIBCO)	6.7 9.7	2.9 2.9	144 216	0.2 0.2	Adenovirus infection	1998_Biotech&Bioeng_Cote-Kamen (Serum-Free Production of Recombinant Proteins and Adenoviral Vectors by 293SF-3F6 Cells)

Cell culture Strategy	Cell culture plattform	Cell line	Media	Maximal Cell Density ($x10^6$cell/mL)	μ_{max} ($x10^{-2}h^{-1}$)	Time to X_{max} (h)	Xv_0 (x106cell/mL)	Application	Reference
Batch	ND	293SF 3F6	SFM4Transfx-293TM (HyQ)	10 (data not shown)	ND	<120	1	Influenza virus production	2010_Vaccine_Le Ru-Kamen (Scalable production of influenza virus in HEK-293 cells for efficient vaccine manufacturing)
Batch	125mL shake flask	293SF 3F6	SFM4Transfx-293TM (HyQ)+4mM GlutaMax+5%FBS+10%Cb5	17	2.37	216	0.25	Adenovirus production	2013_Jbiosc&Bioeng_L Liste-Calleja, M Lecina, J Cairo (HEK293 cell culture media study towards bioprocess optimization: Animal derived component free and animal derived component containing platforms)
Fed-batch	Spinner flask(wv=70%of recommend) Feed rate: daily	293(EBNA)	HL medium (in house defined media)+heparin (30u/mL) Feed media: mixture of sugar, amino acids and vitamins	3-5	ND	ND	ND	Transient transfection	1999_Cytotechnology_Schlaeger-Chriestensen (Transient gene expression in mammalian cells grown in serum-free suspension culture)
Punctual Feeding	1L bioreactor (Biostat Q, B. Braun, Germany) (wv=0.6L)	Human embryonic kidney 293 cell line stably ex-pressing Epstein-Barr virus Nuclear Antigen-1 (293E)	LC-SFM medium enriched with 1% BCS (v/v) + sinngle feeding 10%(v/v) of 5% (w/v)casein peptone tryptone	1,62	1.9	120	0.25	Transient Transfection and protein production optimization	2005_Biotechnol&Bioeng_Pham-durocher (Transient Gene Expressionin HEK293 cells: peptone addtion postransfection improves recombinant protein synthesis)
			FreeStyle medium + sinngle feeding 10%(v/v) of 5% (w/v)casein peptone tryptone	4,6	ND	120	1		

Cell culture Strategy	Cell culture plattform	Cell line	Media	Maximal Cell Density ($x10^6$cell/mL)	μ_{max} ($x10^{-2}h^{-1}$)	Time to X_{max} (h)	Xv_0 (x106cell/mL)	Application	Reference
Fed-batch	5-L bioreactor (B. Braun, Melsungen, Germany) (wv=4L; feed rate=1-3L/h)	293H from GibcoBRL, Life Technologies, MD	293 SFM II. Feed medium: 10X calcium-free, glucose-free, and glutamine-free DMEM/F12 with 1X salt (Hyclone, Logan, UT) supplemented with 10 g/L of Hysoy (Quest International, Hoffman Estate, IL) soybean protein hydrolysate and 20 mM of glutamine (Sigma G5763).	4.3	2.8	120	0.3	Cell media study and adenovirus production	2003_BiotechProgress_Lee-Wong (Low-Glutamine Fed-Batch Cultures of 293-HEK Serum-Free suspension cells for adenovirus production)
			DMEM/F12. Feed medium: Feed medium: 10X calcium-free, glucose-free, and glutamine-free DMEM/F12 with 1X salt (Hyclone, Logan, UT) supplemented with soybean protein hydrolysate and 20 mM of glutamine (Sigma G5763).	4.1	2.9	80	0.3		
Fed-Batch	ND	HEK293-IFN (293-D9)	Freestyle F17 expression medium (Invitrogen) +4mM glutamine+0.1% anti-clumping agent+ 1% Pluronic F-68+1% penicillin–streptomycin (Invitrogen)	4.3	3.0	140	0.2	Metabolism study	2014_Jbiotech_Vallé-Henry (Exploiting the metabolism of PYC expressing HEK293 cells in fed-batch cultures)

continue next page

Cell culture Strategy	Cell culture plattform	Cell line	Media	Maximal Cell Density ($x10^6$cell/mL)	μ_{max} ($x10^{-2}h^{-1}$)	Time to X_{max} (h)	Xv_0 (x106cell/mL)	Application	Reference
Punctual Feeding	2L Bioreactor	293 (ATCC-CRL_1573)	CD293 (Invitrogen)+4mM Glutamate+serine	4.5	1.4	ND	ND	Metabolism study and adenovirus production	2004_Jbiotech_Ferreira-Alves
Punctual Feeding	3L bioreactor	293 SF-3F6	NSFM13+single feeding 1X MEM amino acids, 4 mM glutamine, and 25 mM of glucose	5	1.3	ND	0.25	Metabolism study	2001_Biotech&Bioeng_Nadeau-Kamen (Low-Protein Medium Affects the 293SF Central Metabolism During Growth and Infection with Adenovirus)
Complete Batch Replacement	3L Bioreactor Daily replacement from 120h cell culture	293E	FreeStyle (Invitrogen)+ 0.1% Pluronic F- 68 (Invitrogen) + 50 µg/mL Geneticin G418	7	ND	192	0.25	Transient transfection for lentivirus production	2007_Biotech&Bioeng_Segura-Kamen (Production of Lentiviral Vectors by Large-Scale Transient Transfection of Suspension Cultures and Affinity Chromatography Purification)
Complete Batch Replacement	Tubespins	HEK293E	Ex-cell 293 CDM (SAFC Biosciences)+4 mM glutamine+3mM NaBut	7	ND	240	1	Transient transfection for Antibody production	2008_Biotechnol&Bioeng_Backliwal-Wurm (Valproic Acid: A Viable Alternative to Sodium Butyrate for Enhancing Protein Expression in Mammalian Cell Cultures)
Perfusion	Perfusion rate 1volume/day	293S	DMEM (Gibco)+5% iron supplemented BCS+4mM glutamine+1mM pyruvate	14.3	ND	240	0.3	Adenovirus production	2004_BiotechProg_Cortin-Garnier (High Titer Adenovirus Vector prodcution in cell perfusion culture)
Perfusion	3L Bioeactor CellFerm-pro DasGIp (Jülich, Germany) (wv=1.2L) perfusion rate=0.5vol/day)	HEK293SF	SFM4Transfx-293TM (HyQ)	15	2.4	216	0.25	Cell metabolism study and Influenza virus production	2011_BMC Biotechnology_Petiot-Kamen (Metabolic and Kinetic analyses of influenza production in perfusion HEK293 cell culture)
Semi-Perfusion	miniPERMTM (Greiner bio-one)	HEK293-EBNA-LG4/5	Pro293s-CDM suppl. with 0.5% FCS and additives(non-described)	16	ND	600	0.25	Protein production by transient transfection evaluation	2006_ProtExpress&Purif_Belin-Rouselle (Production of a recombinantly expressed laminin fragment by HEK293-EBNA cells cultured in suspension in a dialysis-based bioreactor)

10.2. GENETIC SEQUENCES OF INTEREST

10.2.1. Porcine circovirus serotype 2 genome (universal sequence)

```
  1  accagcgcac ttcggcagcg gcagcacctc ggcagcacct cagcagcaac atgcccagca
     tggtcgcgtg aagccgtcgc cgtcgtggag ccgtcgtgga gtcgtcgttg tacgggtcgt
                                                Rep (ORF1) >>.......>

 61  agaagaatgg aagaagcgga ccccaaccac acaaaaggtg ggtgttcacg ctgaataatc
     tcttcttacc ttcttcgcct ggggttggtg tgttttccac ccacaagtgc gacttattag
     >.........................Rep (ORF1).........................>

121  cttccgaaga cgagcgcaag aaaatacggg agcttccaat ctccctttt gattatttta
     gaaggcttct gctcgcgttc ttttatgccc tcgaaggtta gagggaaaaa ctaataaaat
     >.........................Rep (ORF1).........................>

181  ttgttggcga ggagggtaat gaggaaggac gaacacccca cctccagggg ttcgctaatt
     aacaaccgct cctcccatta ctccttcctg cttgtggggt ggaggtcccc aagcgattaa
     >.........................Rep (ORF1).........................>

241  ttgtgaagaa gcaaacattt aataaagtga aatggtattt cggtgcccgc tgccacatcg
     aacacttctt cgtttgtaaa ttatttcact ttaccataaa gccacgggcg acggtgtagc
     >.........................Rep (ORF1).........................>

301  agaaagcgaa aggaactgat cagcagaata aagaatactg cagtaaagaa ggcaacttac
     tctttcgctt tccttgacta gtcgtcttat ttcttatgac gtcatttctt ccgttgaatg
     >.........................Rep (ORF1).........................>
                                                          ORF3 <<.<

361  tgatggaatg tggagctcct agatctcaag gacaacggag tgacctgtct actgctgtga
     actaccttac acctcgagga tctagagttc ctgttgcctc actggacaga tgacgacact
     >.........................Rep (ORF1).........................>
     <............................ORF3............................<

421  gtaccttgtt ggagagcggg agtctggtga ccgttgcaga gcagcaccct gtaacgtttg
     catggaacaa cctctcgccc tcagaccact ggcaacgtct cgtcgtggga cattgcaaac
     >.........................Rep (ORF1).........................>
     <............................ORF3............................<

481  tcagaaattt ccgcgggctg gctgaacttt tgaaagtgag cgggaaaatg cagaagcgtg
     agtctttaaa ggcgcccgac cgacttgaaa actttcactc gccctttac gtcttcgcac
     >.........................Rep (ORF1).........................>
     <............................ORF3............................<

541  attggaagac caatgtacac gtcattgtgg ggccacctgg gtgtggtaaa agcaaatggg
     taaccttctg gttacatgtg cagtaacacc ccggtggacc cacaccattt tcgtttaccc
     >.........................Rep (ORF1).........................>
     <............................ORF3............................<

601  ctgctaattt tgcagacccg gaaaccacat actggaaacc acctagaaac aagtggtggg
     gacgattaaa acgtctgggc ctttggtgta tgacctttgg tggatctttg ttcaccaccc
     >.........................Rep (ORF1).........................>
     <............................ORF3............................<

661  acggttacca tggtgaagaa gtggttgtta ttgatgactt ttatggctgg ctgccgtggg
     tgccaatggt accacttctt caccaacaat aactactgaa aataccgacc gacggcaccc
     >.........................Rep (ORF1).........................>
     <...ORF3..<<

721  atgatctact gagattgtgt gatcgatatc cattgactgt agagactaaa ggtggaactg
     tactagatga ctctaacaca ctagctatag gtaactgaca tctctgattt ccaccttgac
     >.........................Rep (ORF1).........................>

781  tacctttttt ggcccgcagt attctgatta ccagcaatca gaccccgttg gaatggtact
     atggaaaaaa ccgggcgtca taagactaat ggtcgttagt ctggggcaac cttaccatga
     >.........................Rep (ORF1).........................>
```

(continue next page)

Figure 10.2.1. PCV2 genome sequence. Primers used for Cap gene obtention are depicted.

10.2.2. Putative Capsid PCV2 gene

```
   1  gataaaatga gcaatagagg agagagacac cgcccccgca gccatcttgg ccagatcctc
      ctattttact cgttatctcc tctctctgtg gcgggggcgt cggtagaacc ggtctaggag

  61  cgccgccgcc cctggctcgt ccacccccgc caccgttacc gctggagaag gaaaaatggc
      gcggcggcgg ggaccgagca ggtgggggcg gtggcaatgg cgacctcttc ctttttaccg

 121  atcttcaaca cccgcctctc ccgcaccttc ggatatacta tcaagcgaac cacagtcaaa
      tagaagttgt gggcggagag ggcgtggaag cctatatgat agttcgcttg gtgtcagttt

 181  acgccctcct gggcggtgga catgatgaga ttcaatatta atgactttct tcccccagga
      tgcgggagga cccgccacct gtactactct aagttataat tactgaaaga agggggtcct

 241  gggggctcaa acccccgctc tgtgcccttt gaatactaca gaataagaaa ggttaaggtt
      cccccgagtt tgggggcgag acacgggaaa cttatgatgt cttattcttt ccaattccaa

 301  gaattctggc cctgctcccc gatcacccag ggtgacaggg gagtgggctc cagtgctgtt
      cttaagaccg ggacgagggg ctagtgggtc ccactgtccc ctcacccgag gtcacgacaa

 361  attctagatg ataactttgt aacaaaggcc acagccctca cctatgaccc ctatgtaaac
      taagatctac tattgaaaca ttgtttccgg tgtcgggagt ggatactggg gatacatttg

                                                                    Cap2Rv
                                                                    C
 421  tactcctccc gccataccat aacccagccc ttctcctacc actcccgcta ttttaccccc
      atgaggaggg cggtatggta ttgggtcggg aagaggatgg tgagggcgat aaaatggggg

           Cap2Rv
      AAACCTGTCC TAGATTC
 481  aaacctgtcc tagattccac tattgattac ttccaaccaa acaacaaaag aaatcagctg
      tttggacagg atctaaggtg ataactaatg aaggttggtt tgttgttttc tttagtcgac

 541  tggctgagac tacaaactgc tggaaatgta gaccacgtag gcctcggcac tgcgttcgaa
      accgactctg atgtttgacg acctttacat ctggtgcatc cggagccgtg acgcaagctt

 601  aacagtatat acgaccagga atacaatatc cgtgtaacca tgtatgtaca attcagagaa
      ttgtcatata tgctggtcct tatgttatag gcacattggt acatacatgt taagtctctt

 661  tttaatctta aagacccccc acttaaccct taatgaataa taaaaaccat tactgttgtt
      aaattagaat ttctgggggg tgaattggga attacttatt atttttggta atgacaacaa

 721  ttaggaagga ggaaagggtc ttactcccga caactaaaaa gacaga
      aatccttcct cctttcccag aatgagggct gttgattttt ctgtct
```

Figure 10.2.2. Putative sequence of Capside gene from PCV2 used in this particular work. Primer for extensive characterization of 3'end is depicted.

```
   1  gtgttagtaa gagtgcagat ggggggtctt taagattaaa ttctctgaat tgtacataca
      cacaatcatt ctcacgtcta ccccccagaa attctaattt aagagactta acatgtatgt

  61  tggttacacg gatattgtat tcctggtcgt atatactgtt ttcgaacgca gtgccgaggc
      accaatgtgc ctataacata aggaccagca tatatgacaa aagcttgcgt cacggctccg

 121  ctacgtggtc tacatttcca gcagtttgta gtctcagcca cagctgattt cttttgttgt
      gatgcaccag atgtaaaggt cgtcaaacat cagagtcggt gtcgactaaa gaaaacaaca

 181  ttggttggaa gtaatcaata gtggaatcta ggacaggttt gggggtaaaa tagcgggagt
      aaccaacctt cattagttat caccttagat cctgtccaaa cccccatttt atcgccctca

 241  ggtaggagaa gggctgggtt atggtatggc gggaggagta gtttacatag gggtcatagg
      ccatcctctt cccgacccaa taccataccg ccctcctcat caaatgtatc cccagtatcc

 301  tgagggctgt ggcctttgtt acaaagttat catctagaat aacagcactg gagcccactc
      actcccgaca ccggaaacaa tgtttcaata gtagatctta ttgtcgtgac ctcgggtgag

 361  ccctgtcacc ctgggtgatc ggggagcagg gccagaattc aaccttaacc tttcttattc
      gggacagtgg gacccactag cccctcgtcc cggtcttaag ttggaattgg aaagaataag

 421  tgtagtattc aaagggcaca gagcgggggt ttgagccccc tcctggggga agaaagtcat
      acatcataag tttcccgtgt ctcgccccca aactcggggg aggacccccc tctttcagta

                                                  Cap2_Fw
                                          CGT TTTGACTGTG GTTC
 481  taatattgaa tctcatcatg tccaccgccc aggagggcgt tttgactgtg gttcgcttga
      attataactt agagtagtac aggtggcggg tcctcccgca aaactgacac caagcgaact

 541  tagtatatcc gaaggtgcgg gagaggcggg tgttgaagat gccatttttc cttctccagc
      atcatatagg cttccacgcc ctctccgccc acaacttcta cggtaaaaag gaagaggtcg

 601  ggtaacgggg gcgggggggg ggacgagcca ggggcggcgg cggaggatct ggccaagatg
      ccattgcccc cgcccccccc cctgctcggt ccccgccgcc gcctcctaga ccggttctac

 661  gctgcggggg cggtgtcttc ttctccggta acgcctcctt ggatacgtca tatctgaaga
      cgacgccccc gccacagaag aagaggccat tgcggaggaa cctatgcagt atagacttct

 721  aaaaaaggga ggtgggagat ttgtttacaa gggggggaga ataggataga gatgcacgga
      ttttttccct ccaccctcta aacaaatgtt ccccccctct tatcctatct ctacgtgcct

 781  agaagactcg gaaaatagaa ttttgagaga aaaaaaaaag gggaggggga ttcgtttata
      tcttctgagc cttttatctt aaaactctct ttttttttte ccctcccccc aagcaaatat

 841  aaagaaaaag aggcccctcc cccctcttat ccctttatg aggatggtca agagaagaaa
      tttcttttte tccggggagg ggggagaata gggaaaatac tcctaccagt tctcttcttt

 901  gagaagatct acagcgcccc ccggaaaaga aaaaattttt ggaagggaag tagtgtatat
      ctcttctaga tgtcgcgggg ggccttttct tttttaaaaa ccttcccttc atcacatata

 961  aaaaaatgat aattctcccc ccccgccgtg gttttgtctg ttttttgttg gactacgatg
      ttttttacta ttaagagggg ggggcggcac caaaacagac aaaaaacaac ctgatgctac

1021  atctttttat gtattggtga acct
      tagaaaaata cataaccact tgga
```

Figure 10.2.3. Putative sequence of Capsid gene from PCV2 used in this particular work. Primer for extensive characterization of 5'end is depicted.

10.2.3. CapPCV2 consensus sequence (current project particular strain)

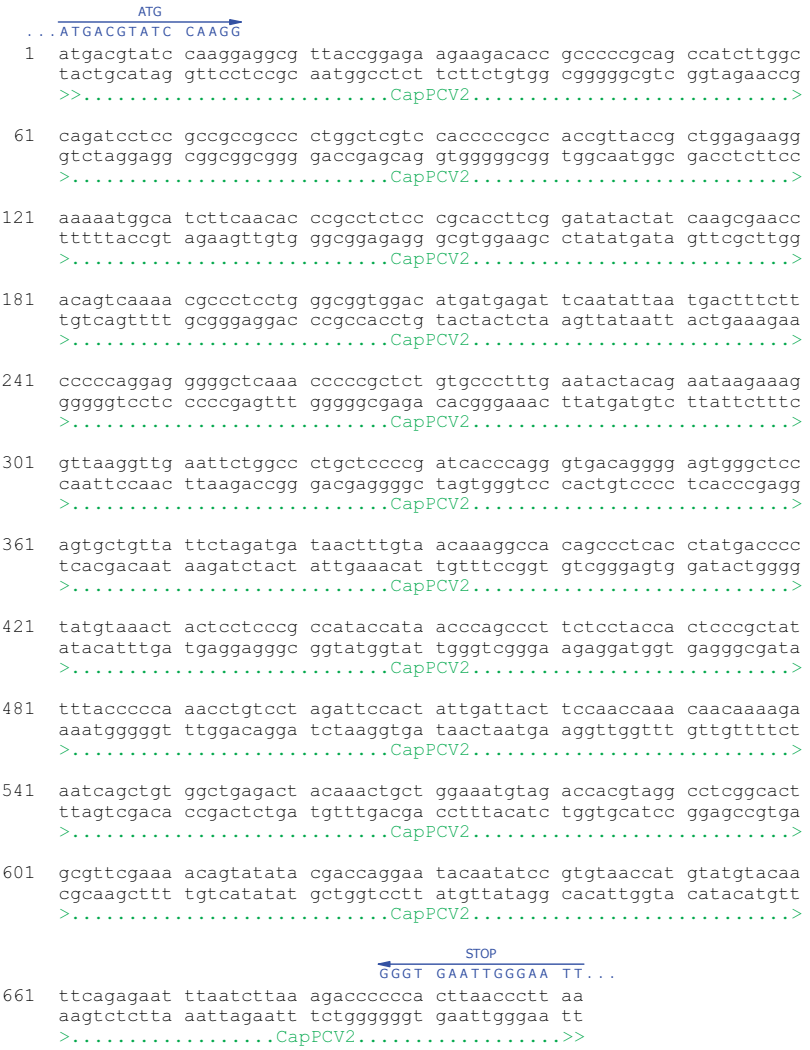


Figure 10.2.4. CapPCV2 gene consensus sequence.

10.3. IMPLEMENTATION OF QUANTIFICATION PROTOCOLS FOR GFP AND VIRAL VECTORS

In Chapter2 the bioprocess based on adenoviral infection for obtaining the vaccine candidate has been evaluated. In order to correctly design the experiments and for the correct evaluation of the results, it was mandatory to set up the techniques for GFP quantification and for rAdV-GFP titration. Regarding CapPCV2 quantification and rAdV-CapPCV2 titration techniques, they have been previously implemented and they are specified in Materials and Methods Section.

10.3.1. GFP quantification.

Among the different available methods to detect and quantify fluorescence, fluorimetry was the one selected for GFP quantification. Different parameters were addressed in order to define a protocol for protein assessment and they are specified and evaluated below.

10.3.1.1.Determination of media interference with GFP fluorescence detection

It was found out that at the GFP emission wavelength (λ=509nm), cell media used for HEK293 culture (SFMTransFx-293 5%FBS 10% CB5 (80g/L) supplemented) significantly cover up GFP signal, resulting in the impossibility of GFP detection when infecting at low MOI (i.e. MOI≈1) (Figure 10.3.1A). Also, the emission peak of GFP fluorescence was distorted and moved to higher wavelengths when it was measured on the supernatant, as it was noticed when the evolution of fluorescence signal on the supernatant along the infection progression was measured. After the replacement of cell media by PBS, GFP fluorescence of the infected cultures became detectable and differences between uninfected cell cultures and infected cultures at high and low MOI could be distinguished (Figure 10.3.1 B). Therefore, intracellular GFP quantification was performed on PBS and the evaluation of GFP release to the extracellular space was only qualitatively performed.

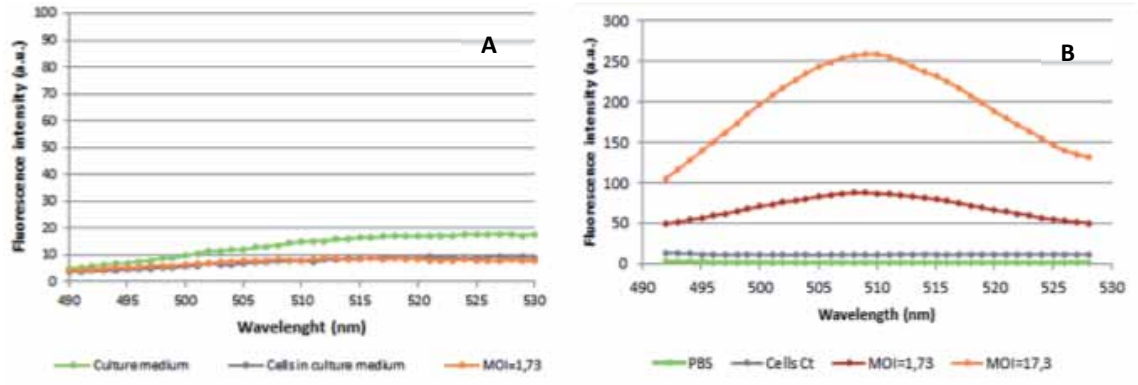


Figure 10.3.1. Fluorescence signal within the emission range scanned (490-530nm) of Right pane infected and unifected cell cultures in SFMTransFx-293 5%FBS 10%CB5 and of (B) infected and uninfected cell cultures with media replacement by PBS.

10.3.1.2. Time frame for Harvesting for fluorescence measurement (TOF_M)

It is known that viral infection progresses differently depending on infection parameters. In general terms, viral DNA replication will start approximately 10hpi and protein production will occur until viral assembly, which would occur 20-48hpi. In order to reduce this time frame to ease the study of infection parameters presented in Chapter 2, we followed intracellular fluorescence after infection at different MOIs from 16hpi until 46hpi (Figure 10.3.2)

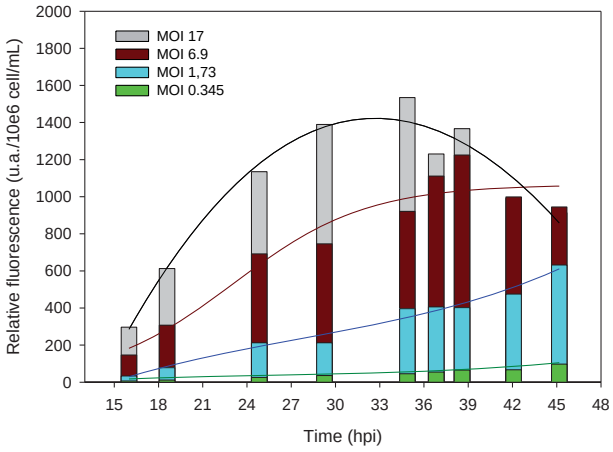


Figure 10.3.3. Evolution of the relative fluorescence signal of cell cultures infected at different MOIs from 16hpi until 46hpi

For the highest MOI (i.e. 17.25), a decrease on relative fluorescence was detected at the end of the studied time frame, indicating GFP leaking to extracellular space. For MOI=5, a "plateau stage" is reached approximately at 39hpi. At MOI<1 (specifically MOI=0.05) fluorescence signal was really low within the time range analysed. This fact was in good correlation with previous results obtained in

which it was shown that there are no significant differences in fluorescence signal between cell cultures with <20%$GFP_{positive}$ and uninfected cell cultures. Fluorescence of samples infected at MOI 1.73 exponentially increased within the selected time frame. It was then decided that for MOIs evaluated in the infection studies presented on Chapter 2, the time frame for fluorescence detection will be set at 32-46hpi.

10.3.1.3. Correlation of GFP concentration with fluorescence signal

For the correlation between GFP concentration ([GFP]) and fluorescence signal detected, serial dilutions of commercial GFP (Roche) in PBS were carried out. From the estimated polynomial regression (Figure 10.3.3), the equation 8.3.1. was established and used for protein quantification in the samples.

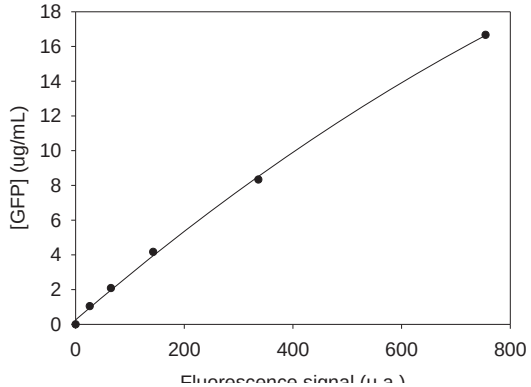


Figure 10.3.3. Relation between [GFP] and measured fluorescence**.**

$$y = -9e - 6x^2 + 0{,}0285x \qquad \text{Eq. 8.3.1}$$

10.3.2. rAdV-GFP titration protocol implementation

10.3.2.1.Gates determination

An important principle of flow cytometry data analysis is to selectively visualize the cells of interest while eliminating results from unwanted particles e.g. dead cells and debris. This procedure is called gating. Cells have traditionally been gated according to physical characteristics. For instance, subcellular debris and clumps can be distinguished from single cells by size, estimated by forward scatter. Also, dead cells have lower forward scatter and higher side scatter than living cells.

Four populations were clearly distinguished by flow cytometry analysis: (1) cell debris; (2) dead cells; (3) single cells and (4) aggregates. A gate comprising the three latter populations was defined and named Total cell (Figure 10.3.4). The percentage of positive cells within total population was used for viral titration.

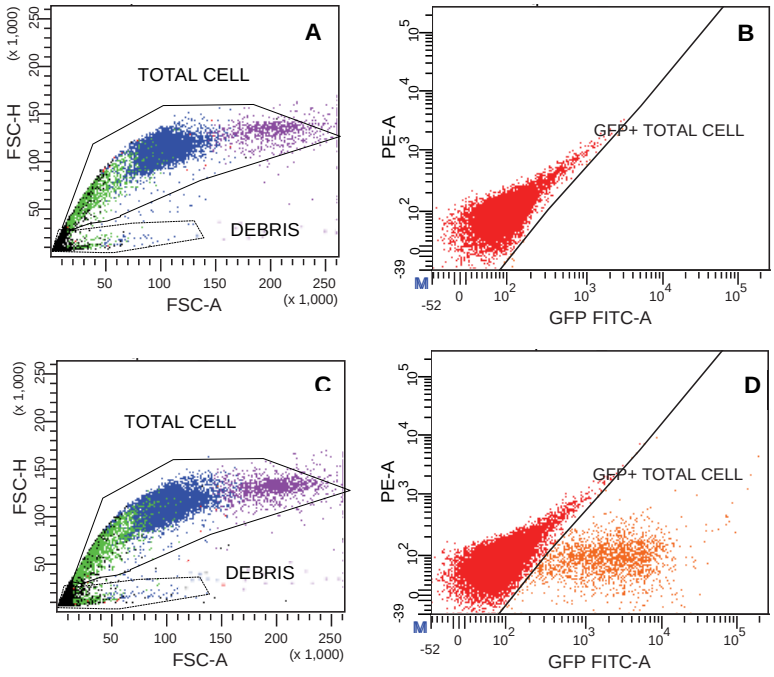


Figure 10.3.4. Example of flow citometry profile of uninfected cell culture (A and B) and an infected cell cutlure (C and D). The continuos line surrounds total cell population and cell debris is surrounded by the dashed line. Within the total cell population, three subpopulations can be distinguished: dead cells (green) , single cells (blue) and aggregates (purple).

The validation of this gates was performed by comparison of titration of the same sample by flow cytometry and fluorescence microscope. Viral titer with the former technique was 2.58e11±6.4e9 and 2.9e11 and 3.45e11 with the later technique (two independent titrations). Hence, the defined gates were considered as correct.

10.3.2.2.Calculation method for viral titer determination.

The equation 10.3.2. was used for correlation of the percentage of fluorescent cells with the adenovirus concentration[1]:

$$[\mathrm{IPU/mL}] = \frac{(\%\ \mathrm{Positive\ GFP} - \%\mathrm{Positive\ GFP\ Neg\ Gtrl})/100}{\mathrm{Viral\ Volume}} \cdot \mathrm{TCN} \cdot \mathrm{DF} \qquad \text{Eq.10.3.2.}$$

Where

Neg Ctrl is an uninfected cell culture under the same conditions (cell media, cell concentration and viability) as infected cell culture.

TCN is the total cell number per well

DF is the dilution factor of the sample

However, a slight modification on the inclusion criteria was introduced: while Côte et al considered all samples with ≤50% of positive cells, in the work here presented only samples with 2≤%positive cells≤20 were considered valid for titration This criteria was settled after the observation that good correlation between the theoretical percentage of GFP positive cells and real percentage of GFP positive cells could only be established when the real percentage was lower than 20 and higher than 2.

10.3.2.3. Determination of cell media interference with GFP fluorescent cells detection.

In order to determine a possible interference of cell media with GFP fluorescent cells detection, cell culture was replaced by PBS at TOH before flow cytometry analysis. The results were compared to GFP detection of the same samples in cell culture media. The experiment was performed as follows: Two independent dilution banks, each of them with three dilutions of a unique viral stock vial were performed. Duplicates of infection with each viral dilution were carried out (this would result in four samples for each viral dilution) and two time of harvesting were tested: 16hpi and 36hpi. At TOH half of the volume of each well was centrifuged at 200G for 8 minutes and pellet was gently resuspended in PBS. Results of titrations are presented in Table 10.3.1:

Table 10.3.1 Titration results with and without media replacement at time of titration.

		Dilution bank 1 ($x10^{11}$ AdV/mL)	Dilution bank 2 ($x10^{11}$ AdV/mL)
TOH=16hpi	Cell media	2.83±0.04	2.36±0.05
	PBS	2.41±0.30	2.29±0.09
TOH=36hpi	Cell media	2.74±0.02	2.50±0.04
	PBS	3.03±0.10	2.53±0.04

Any significant difference was detected between samples analysed in cell culture media and samples in which cell media was removed and replaced by PBS, indicating that for flow cytometry technology, cell media did not interfere with fluorescence detection.

10.3.2.4.Cell growth platform effect on infectivity

One of the main issues affecting the infection efficiency in suspension cell cultures is cell aggregation. If cells used for titration are highly aggregated, they will be infected less efficiently resulting in an underestimation of viral titer of the sample. Therefore, the study of cell growth and infection of HEK293 cells in different cell cultures platform was performed. Initially, three culture platforms were selected: 6 well plate, 12 well plate and 24 well plate. Shake flask platform was discarded for titration platform for unfeasibility matters (i.e. thirty-two infections were routinely performed for the titration of one sample). After the initial 12hpi, cells in 24 well plate presented high aggregation level. Therefore, this platform was also discarded for viral titration. 3 independent samples were titrated in parallel on 6well plate and 12 well plate and the results are depicted in Figure 10.3.5.

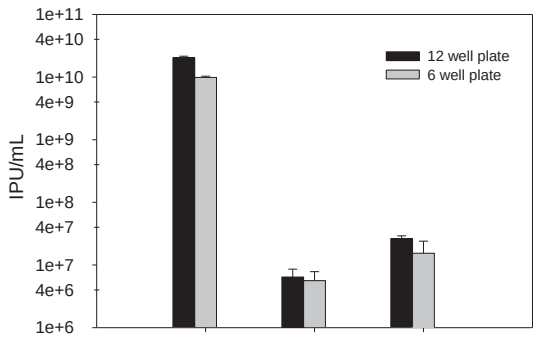


Figure 10.3.5. Effect of the titration plattform on final viral titer.

As any significant difference on final viral titer was detected, the selected titration platform was 12well plate. This selection would result in reduction of scale up for cell inocula for titration, reduction of culture media expense, reduction of space and consequently, an increment of the number of samples that can be titrated in the same experiment.

10.3.2.5.Time of titration

AdV viral cicle is about 36h[2]. Beyond this time, cell lysis will start increasing cell debris population in flow cytometer pattern, which will be discarded for AdV titration. Therefore, for avoiding underestimation in AdV titration, time of harvesting for titration (TOT) must be settled before 36hpi. In order to determine time frame in which TOT could be done, cell cultures were infected at different MOIs and harvested at 12, 16 and 24hpi. Results showed that the percentage of infected cells did not significantly vary within the selected time range, whereas the fluorescence intensity increased from 12hpi to 24hpi indicating the accumulation of GFP within the cells before cell lysis and virus release (Figure 8.3.6). However, at 24hpi cells presented aberrant morphology and the population of dead cells slightly but significantly increased. Therefore, the time frame 12-16hpi for TOT was selected.

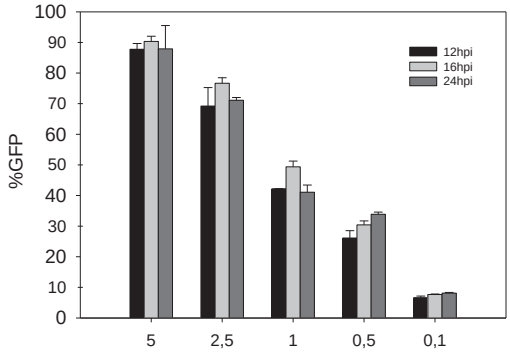
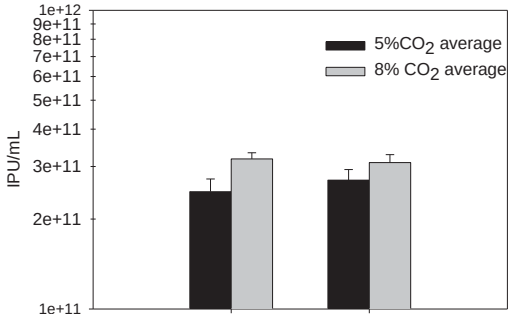


Figure 10.3.6. Percentage of GFP positive cells within total cell population detected by flow cytometry at different TOHs and different MOIs

10.3.2.6.%CO_2 effect on infectivity.

In previous studies of adenovirus production in HEK293 cells, some differences on infection



efficiency had been related to CO_2 percentage in the atmosphere of the incubator where cells are cultured[3]. We evaluated the impact of this parameter with the particular HEK293 strain used in this work by infecting cells at two different CO_2 percentages (5% and 8%). As it van be noticed in Figure 10.3.7, no significant differences were detected between infection at the two conditions tested. Therefore, the first one was selected as the preferred one because it was the $\%CO_2$ used for cell culture.

10.3.2.7.Fixation protocol evaluation.

Fixation of cells is a methodology that enables GFP signal preservation during a determinate period after cell harvesting. The fixation protocol explained in Material and Methods section was evaluated in terms of GFP signal lost and how long can GFP signal be maintained. Besides, it has been reported the necessity of fixation buffer replacement by PBS 24 hours post fixation in order to avoid GFP lost due to cell damage. The requirement of this step was also assessed. From the obtained results (Fig.10.3.8A) we concluded that the fixation protocol is suitable for the cell line used in this work as any diminution of % GFP positive cells or fluorescence intensity signal was detected after the fixation of samples infected at different MOIs within the range tested (0.1-5). Also, preservation of GFP signal was ensured at least for 72 hours post fixation (Figure 8.3.8B) and the replacement of fixation buffer by PBS could be avoided as no difference on GFP signal was encountered within the evaluated time frame.

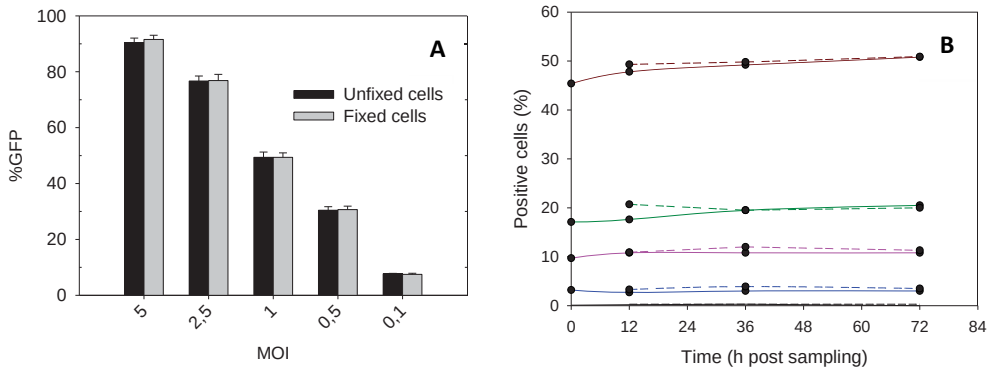


Figure 10.3.8. (A) Evaluation of cell fixation protocol in terms of lost of fluorescence. (B) Evaluation of GFP signal maintenance after fixation without fixation buffer replacement (solid lines) and after fixation buffer replacement by PBS (dashed lines).

10.3.3. Titration methods comparison and validation

Two different adenoviruses were used in this research project: AdV-GFP and AdV- CapPCV2. The titration of the first viral vector was done by flow cytometry, as this technique is less time consuming and is higher cost-effective in comparison to other cell-based titration assays. Conversely, titration of the second viral vector could not be done by the same method, due to the lack of a gene reporter within adenoviral genome. Among all the titration alternatives, two methods were initially chosen and compared: (1) Viral Titer Kit (Agilent), which is an immunohystochemical assay and (2) $TCID_{50}$ dilution assay, which is widely used in virology field. Finally, the comparison of viral titration by Viral Titer Kit (Agilent) and by flow citometry technology was made, in order to ensure that the results obtained with both metods are comparable.

10.3.3.1. $TCID_{50}$ vs. pAdEasy Viral titer kit

$TCID_{50}$ is an endpoint dilution assay that quantifies the amount of virus required to kill 50% of infected hosts or to produce a cytopathic effect in 50% of inoculated tissue culture cells. In the context of tissue culture, host cells are plated and serial dilutions of the virus are added. After incubation (up to two weeks, depending on cell infectivity time), the percentage of cell death (i.e. infected cells) is observed and recorded for each virus dilution, and results are used to mathematically calculate a $TCID_{50}$ result. The most commonly used methods to calculate $TCID_{50}$ are Spearman-Karber and Reed-Muench method, which were used in the comparison of titration methods presented below.

The AdEasy Viral Titer Kit is an enzyme-linked immunoassay for the determination of adenoviral titers. The immunoassay detects an adenoviral capsid protein called hexon in the intracellular space. The AdEasy viral titer kit can determine the titer of the stock in 24 to 48 hours, compared to two weeks for traditional endpoint dilution assays. Titer determination is achieved in three steps. First, AD-293 cells are fixed 24 to 48 hours post-adenoviral infection and then the expression of the hexon protein is detected with an anti-hexon antibody. Next, a horseradish peroxidase (HRP) conjugated secondary antibody is added to amplify the signal. The HRP enzyme catalyses a colorimetric reaction with the third reagent, a metal-enhanced substrate diaminobenzidine (DAB), which produces a dark precipitate in the cells expressing the hexon protein. Since only infected cells express the hexon protein, these are the only cells in culture that contain the dark brown precipitate indicative of the HRP-catalyzed reaction. The dark-colored, infected cells can therefore be counted under a standard laboratory microscope and used to calculate viral titer. The whole titration protocol is specified in Material and Methods chapter.

In order to compare both techniques, a commercial viral sample previously quantified by the manufacturer was used. Serial dilutions of the sample were performed and triplicates of each dilution were tested. The results obtained with the $TCID_{50}$ assay (regardless the calculation method) were one log-unit lower than the indicated by the manufacturer (Table 10.3.2) whereas the titer obtained with pAdEasy viral titer kit was comparable to reference titer. Hence, the latter method was selected for titration of rAdV-CapPCV2, due to its higher accuracy and the shorter incubation time needed for sample quantification.

Table 10.3.2. Titers obtained from the same viral sample using different methodologies

Reference titration	$TCID_{50}$ Spearman Karber	$TCID_{50}$ Reed München	pAdEasy viral titer kit
$3.45x10^{11}$	$2.39x10^{10}$	$2.69x10^{10}$	$3.51x10^{11}$

10.3.3.2. pAdEasy Viral titer kit vs. flow cytometry

The same quantified rAdV-GFP aliquot of prior section was used for the comparison of the two assays. Due to the characteristics of each methodology, different dilutions of the initial sample should be performed in order to ensure a final measure within the valid range for titration. Hence, the two experiments could be made independently.

a) pAdEasy Viral titer kit

In the case of Viral Titration Kit (Agillent), only the wells with ≤10% of positive cells can be considered for titration. In order to provide a positive internal control of the titration kit in future sample titrations, different theoretical MOIs were tested in this experiment for titration method validation. Hence, four independent titrations of the same sample were performed, and duplicates for each MOI were tested in each titration. The results of the titrations confirm the accuracy of this method and also show its high precision (Table 10.3.3.)

Table 10.3.3. Evaluation of viral titration with pAdEasy Viral titer kit

	TITRATION1			TITRATION2			TITRATION3		TITRATION4	
	MOI*			MOI*			MOI*		MOI*	
	0.5	0.1	0.05	0.1	0.02	0.05	0.02	0.05	0.02	0.05
%PC† in range? (Y/N)	N	N	Y	N	Y	Y	Y	Y	Y	Y
Final titer (IFU/mL)	$3.08x10^{11}$			$2.02x10^{11}$			$3.5x10^{11}$		$2.7x10^{11}$	
Final titer averaged	$(2.83\pm0.62)x10^{11}0$									

* MOI was calculated on basis the titer indicated by the manufacter and the cells seeded 12h pre titration

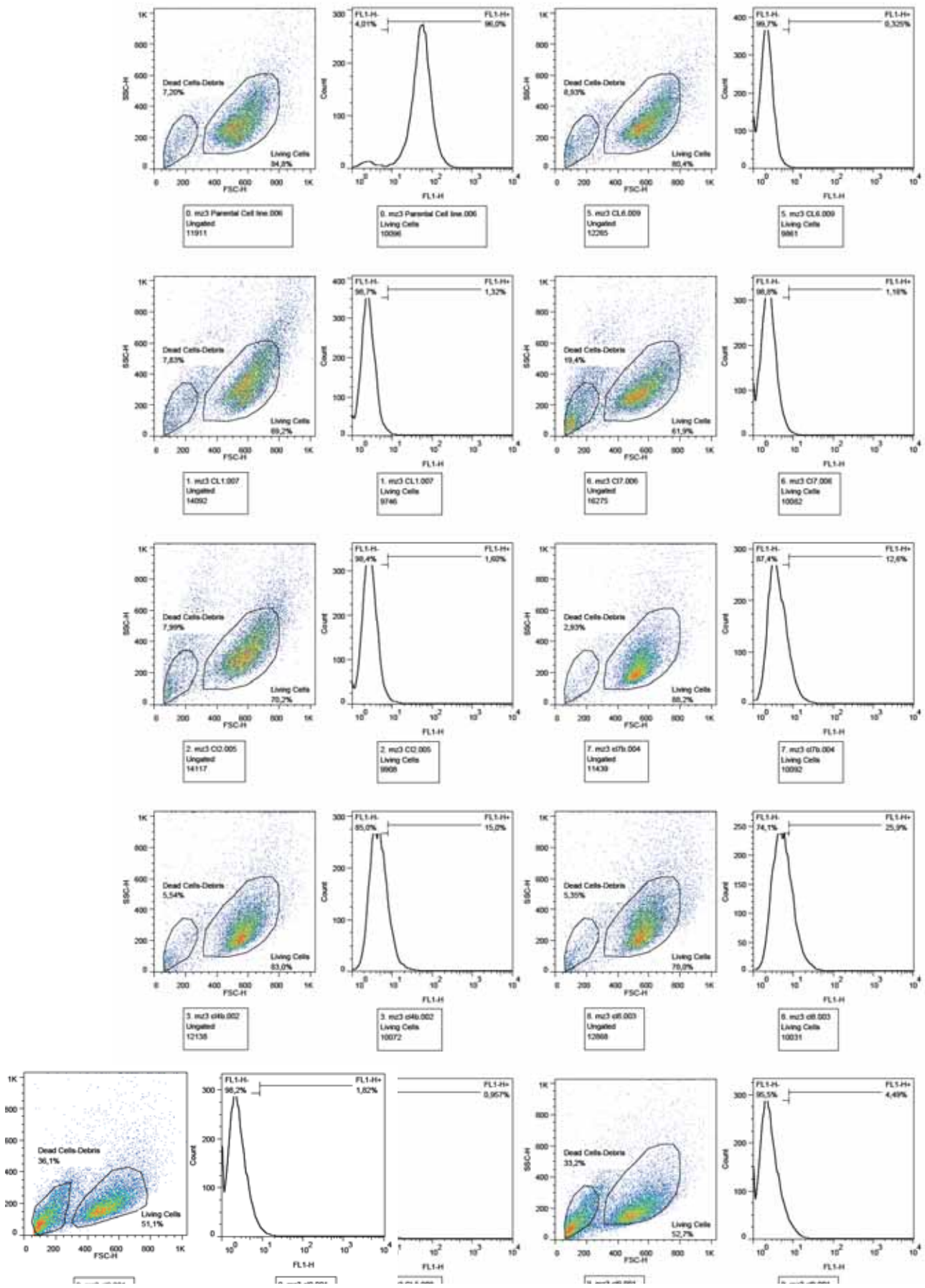
† PC refers to positive cells after the colorimetric reaction

b) Flow Cytometry

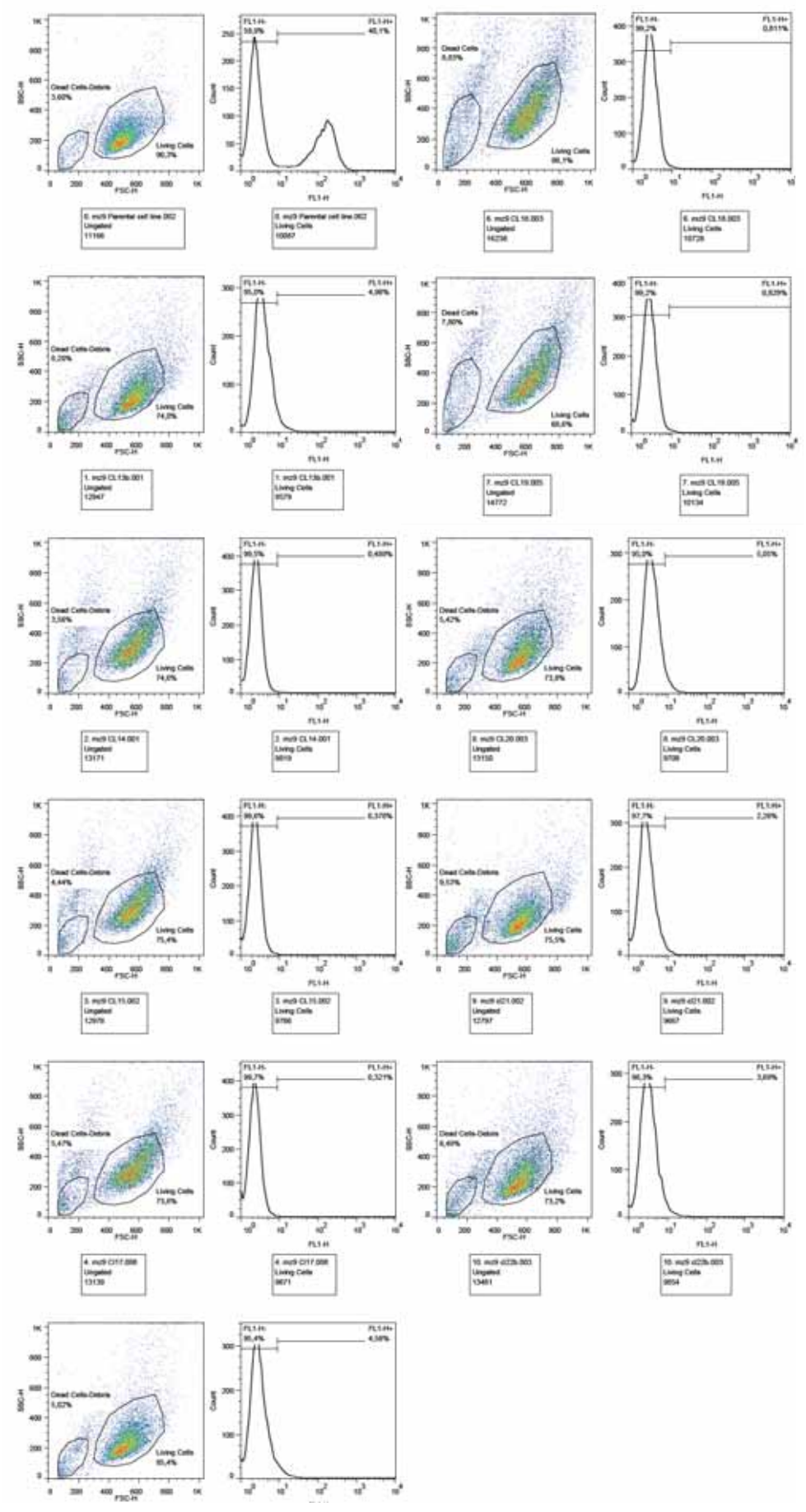
A single titration of the same viral sample used for pAdEasy Viral Titration Kit validation was performed by flow citometry following the protocol specified in Materials and Methods chapter. In this particular case, two independent viral dilution banks were prepared. Three diluitons from each bank were tested in quatriplicate. The final titer ($(2.58\pm0.06)x10^{11}$) was in good correlation with the previous method and with the viral titer indicated by the manufacter. It was then concluded that the selected titration methods could be used for the quantification of the viral vectors and that the results will be comparable.

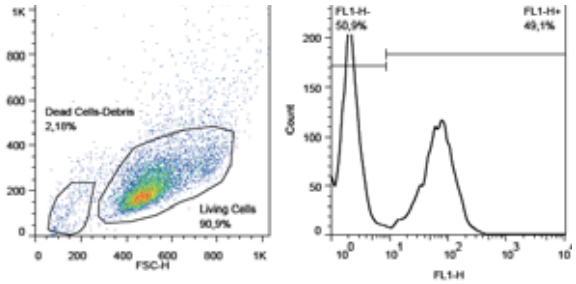
10.4. CHARACTERISATION OF 293 MZs DERIVED CLONES BY GFP EXPRESSION

10.4.1. 293 MZ3 DERIVED CLONES

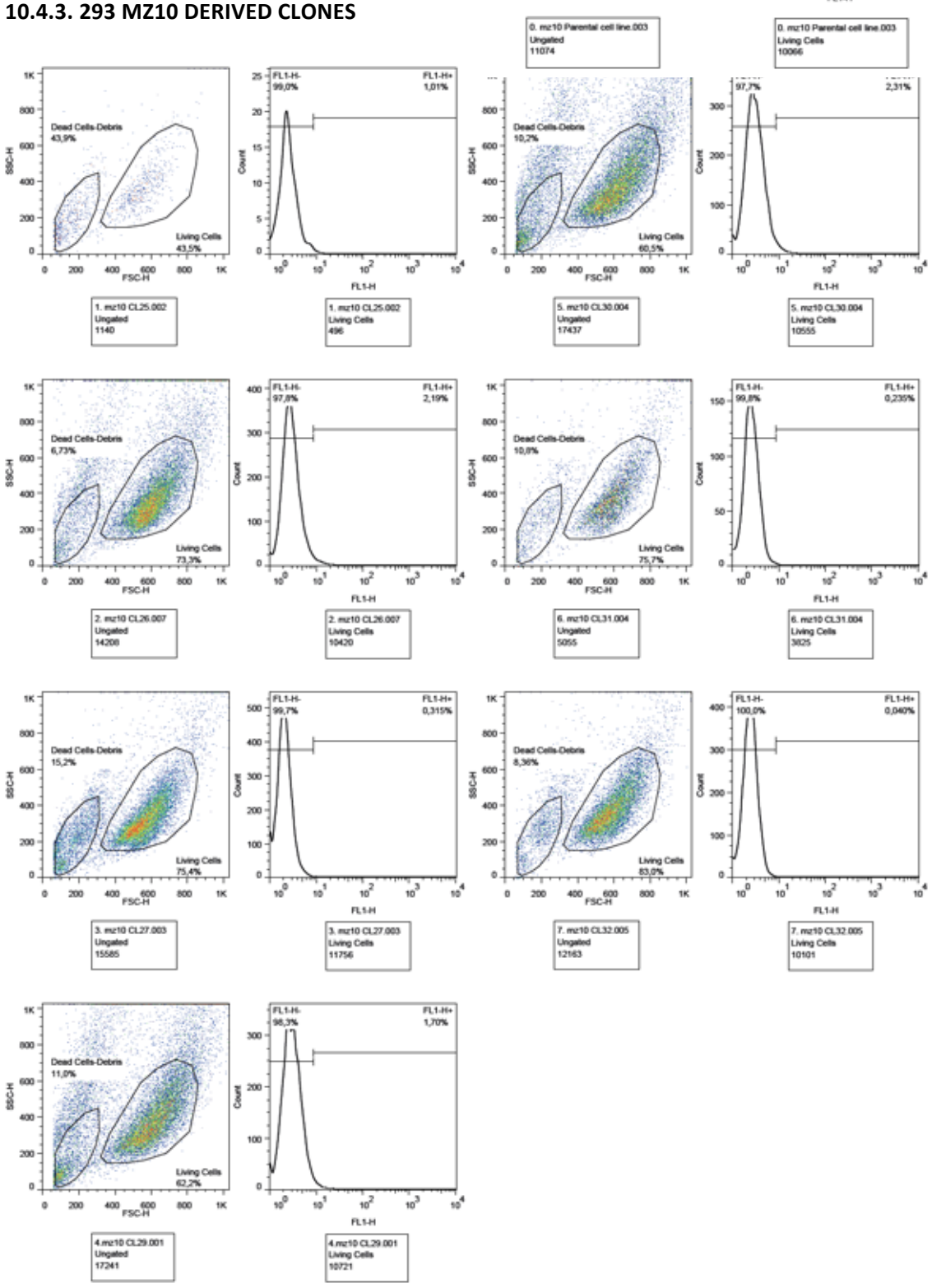


10.4.2. 293 MZ9 DERIVED CLONES





10.4.3. 293 MZ10 DERIVED CLONES



10.5. DATA USED FOR pFBA

10.5.1. METABOLIC REACTIONS CONSIDERED FOR METABOLIC NETWORK

ID	Reaction
R_EX_leu_L_LPAREN_e_RPAREN_	1 M_leu_L_e = 1 M_leu_L_b
R_EX_h_LPAREN_e_RPAREN_	1 M_h_e = 1 M_h_b
R_EX_lac_L_LPAREN_e_RPAREN_	1 M_lac_L_e = 1 M_lac_L_b
R_EX_pro_L_LPAREN_e_RPAREN_	1 M_pro_L_e = 1 M_pro_L_b
R_EX_nh4_LPAREN_e_RPAREN_	1 M_nh4_e = 1 M_nh4_b
R_EX_ile_L_LPAREN_e_RPAREN_	1 M_ile_L_e = 1 M_ile_L_b
R_EX_lys_L_LPAREN_e_RPAREN_	1 M_lys_L_e = 1 M_lys_L_b
R_EX_thr_L_LPAREN_e_RPAREN_	1 M_thr_L_e = 1 M_thr_L_b
R_EX_trp_L_LPAREN_e_RPAREN_	1 M_trp_L_e = 1 M_trp_L_b
R_EX_cys_L_LPAREN_e_RPAREN_	1 M_cys_L_e = 1 M_cys_L_b
R_EX_gln_L_LPAREN_e_RPAREN_	1 M_gln_L_e = 1 M_gln_L_b
R_EX_pe_hs_LPAREN_e_RPAREN_	1 M_pe_hs_e = 1 M_pe_hs_b
R_EX_phe_L_LPAREN_e_RPAREN_	1 M_phe_L_e = 1 M_phe_L_b
R_EX_tyr_L_LPAREN_e_RPAREN_	1 M_tyr_L_e = 1 M_tyr_L_b
R_EX_pi_LPAREN_e_RPAREN_	1 M_pi_e = 1 M_pi_b
R_EX_ser_L_LPAREN_e_RPAREN_	1 M_ser_L_e = 1 M_ser_L_b
R_EX_urea_LPAREN_e_RPAREN_	1 M_urea_e = 1 M_urea_b
R_EX_orn_LPAREN_e_RPAREN_	1 M_orn_e = 1 M_orn_b
R_EX_o2_LPAREN_e_RPAREN_	1 M_o2_e = 1 M_o2_b
R_EX_co2_LPAREN_e_RPAREN_	1 M_co2_e = 1 M_co2_b
R_EX_his_L_LPAREN_e_RPAREN_	1 M_his_L_e = 1 M_his_L_b
R_EX_asp_L_LPAREN_e_RPAREN_	1 M_asp_L_e = 1 M_asp_L_b
R_EX_asn_L_LPAREN_e_RPAREN_	1 M_asn_L_e = 1 M_asn_L_b
R_EX_glu_L_LPAREN_e_RPAREN_	1 M_glu_L_e = 1 M_glu_L_b
R_EX_hco3_LPAREN_e_RPAREN_	1 M_hco3_e = 1 M_hco3_b
R_EX_gly_LPAREN_e_RPAREN_	1 M_gly_e = 1 M_gly_b
R_EX_glc_LPAREN_e_RPAREN_	1 M_glc_D_e = 1 M_glc_D_b
R_EX_h2o_LPAREN_e_RPAREN_	1 M_h2o_e = 1 M_h2o_b
R_EX_val_L_LPAREN_e_RPAREN_	1 M_val_L_e = 1 M_val_L_b
R_EX_met_L_LPAREN_e_RPAREN_	1 M_met_L_e = 1 M_met_L_b
R_EX_HC00250_LPAREN_e_RPAREN_	1 M_HC00250_e = 1 M_HC00250_b
R_EX_GLTX	1 M_GLTX_e = 1 M_GLTX_b
R_EX_arg_L_LPAREN_e_RPAREN_	1 M_arg_L_e = 1 M_arg_L_b
R_EX_ala_L_LPAREN_e_RPAREN_	1 M_ala_L_e = 1 M_ala_L_b
R_IMPC	1 M_h2o_c + 1 M_imp_c = 1 M_fprica_c
R_ORNTArm	1 M_akg_m + 1 M_orn_m = 1 M_glu_L_m + 1 M_glu5sa_m
R_PHEtec	1 M_phe_L_e = 1 M_phe_L_c
R_DTMPK	1 M_atp_c + 1 M_dtmp_c = 1 M_adp_c + 1 M_dtdp_c
R_TYRt	1 M_tyr_L_e = 1 M_tyr_L_c
R_UREAt	1 M_urea_e = 1 M_urea_c
R_GLUt2m	1 M_h_c + 1 M_glu_L_c = 1 M_h_m + 1 M_glu_L_m
R_VALTAm	1 M_akg_m + 1 M_val_L_m = 1 M_glu_L_m + 1 M_3mob_m
R_r1146	2 M_h2o_r + 1 M_HC02110_r = 1 M_co2_r + 1 M_zymst_r
R_GHMT2rm	1 M_thf_m + 1 M_ser_L_m = 1 M_h2o_m + 1 M_gly_m + 1 M_mlthf_m
R_H2CO3D	1 M_h2o_c + 1 M_co2_c = 1 M_h_c + 1 M_hco3_c
R_DGTPtn	1 M_dgtp_c = 1 M_dgtp_n
R_ASPTA	1 M_akg_c + 1 M_asp_L_c = 1 M_glu_L_c + 1 M_oaa_c

R_O2tm	1 M_o2_c = 1 M_o2_m
R_L_LACt2r	1 M_h_e + 1 M_lac_L_e = 1 M_h_c + 1 M_lac_L_c
R_r0666	1 M_atp_c + 1 M_fpram_c = 1 M_adp_c + 1 M_air_c + 2 M_h_c + 1 M_pi_c
R_GLUDxm	1 M_h2o_m + 1 M_nad_m + 1 M_glu_L_m = 1 M_h_m + 1 M_akg_m + 1 M_nadh_m + 1 M_nh4_m
R_HACD9m	1 M_nad_m + 1 M_3hmbcoa_m = 1 M_h_m + 1 M_nadh_m + 1 M_2maacoa_m
R_r1664	1 M_orn_c + 1 M_lys_L_e = 1 M_lys_L_c + 1 M_orn_e
R_PGI	1 M_g6p_c = 1 M_f6p_c
R_PGK	1 M_3pg_c + 1 M_atp_c = 1 M_adp_c + 1 M_13dpg_c
R_ARTPLM2	1 M_Rtotal2coa_c = 1 M_pmtcoa_c
R_H2CO3Dm	1 M_h2o_m + 1 M_co2_m = 1 M_h_m + 1 M_hco3_m
R_PGM	1 M_2pg_c = 1 M_3pg_c
R_MTHFC	1 M_h2o_c + 1 M_methf_c = 1 M_10fthf_c + 1 M_h_c
R_MTHFD	1 M_nadp_c + 1 M_mlthf_c = 1 M_nadph_c + 1 M_methf_c
R_r1135	3 M_h_r + 1 M_nadp_r + 1 M_4mzym_int2_r = 1 M_nadph_r + 1 M_HC02110_r
R_r0525	1 M_h2o_m + 1 M_nad_m + 1 M_saccrp_L_m = 1 M_h_m + 1 M_nadh_m + 1 M_glu_L_m + 1 M_L2aadp6sa_m
R_VALt5m	1 M_val_L_c = 1 M_val_L_m
R_COAtm	1 M_coa_c = 1 M_coa_m
R_HMGCOARc	2 M_h_c + 2 M_nadph_c + 1 M_hmgcoa_c = 2 M_nadp_c + 1 M_coa_c + 1 M_mev_R_c
R_PROtm	1 M_pro_L_c = 1 M_pro_L_m
R_DCTPtn	1 M_dctp_c = 1 M_dctp_n
R_3HCO3_Nat	FALSO
R_PGMT	1 M_g1p_c = 1 M_g6p_c
R_ASPCTr	1 M_asp_L_c + 1 M_cbp_c = 1 M_h_c + 1 M_pi_c + 1 M_cbasp_c
R_CYTK1	1 M_atp_c + 1 M_cmp_c = 1 M_adp_c + 1 M_cdp_c
R_CHSTEROLtrc	1 M_chsterol_r = 1 M_chsterol_c
R_GLUDym	1 M_h2o_m + 1 M_nadp_m + 1 M_glu_L_m = 1 M_h_m + 1 M_nadph_m + 1 M_akg_m + 1 M_nh4_m
R_SUCOASm	1 M_coa_m + 1 M_atp_m + 1 M_succ_m = 1 M_adp_m + 1 M_pi_m + 1 M_succoa_m
R_PEt	1 M_pe_hs_e = 1 M_pe_hs_c
R_CO2tm	1 M_co2_c = 1 M_co2_m
R_HMGCOAtm	1 M_hmgcoa_c = 1 M_hmgcoa_m
R_2OXOADPTm	1 M_akg_m + 1 M_2oxoadp_c = 1 M_akg_c + 1 M_2oxoadp_m
R_ENO	1 M_2pg_c = 1 M_h2o_c + 1 M_pep_c
R_r0781	3 M_o2_r + 2 M_h_r + 3 M_nadph_r + 1 M_lanost_r = 4 M_h2o_r + 1 M_44mctr_r + 3 M_nadp_r + 1 M_for_r
R_r0780	1 M_44mzym_r + 1 M_nadp_r = 1 M_h_r + 1 M_44mctr_r + 1 M_nadph_r
R_TYRTA	1 M_akg_c + 1 M_tyr_L_c = 1 M_34hpp_c + 1 M_glu_L_c
R_O2ter	1 M_o2_c = 1 M_o2_r
R_MGCHrm	1 M_h2o_m + 1 M_3mgcoa_m = 1 M_hmgcoa_m
R_GARFT	1 M_10fthf_c + 1 M_gar_c = 1 M_h_c + 1 M_thf_c + 1 M_fgam_c
R_RPI	1 M_r5p_c = 1 M_ru5p_D_c
R_ORPT	1 M_ppi_c + 1 M_orot5p_c = 1 M_prpp_c + 1 M_orot_c
R_PIter	1 M_pi_r = 1 M_pi_c
R_TALA	1 M_g3p_c + 1 M_s7p_c = 1 M_f6p_c + 1 M_e4p_c
R_ACACT10m	1 M_coa_m + 1 M_2maacoa_m = 1 M_accoa_m + 1 M_ppcoa_m
R_RPE	1 M_ru5p_D_c = 1 M_xu5p_D_c

R_ADK1	1 M_amp_c + 1 M_atp_c = 2 M_adp_c
R_LEUTAm	1 M_akg_m + 1 M_leu_L_m = 1 M_glu_L_m + 1 M_4mop_m
R_ILEtec	1 M_ile_L_e = 1 M_ile_L_c
R_r1418	1 M_h_e + 1 M_hco3_e = 1 M_h2o_e + 1 M_co2_e
R_GALU	1 M_h_c + 1 M_g1p_c + 1 M_utp_c = 1 M_ppi_c + 1 M_udpg_c
R_H2Oter	1 M_h2o_c = 1 M_h2o_r
R_H2Ot	1 M_h2o_e = 1 M_h2o_c
R_ECOAH9m	1 M_h2o_m + 1 M_2mb2coa_m = 1 M_3hmbcoa_m
R_r0034	1 M_h2o_m + 1 M_co2_m + 2 M_atp_m + 1 M_nh4_m = 2 M_adp_m + 1 M_cbp_m + 3 M_h_m + 1 M_pi_m
R_H2Otm	1 M_h2o_c = 1 M_h2o_m
R_MCCCrm	1 M_atp_m + 1 M_hco3_m + 1 M_3mb2coa_m = 1 M_3mgcoa_m + 1 M_adp_m + 1 M_h_m + 1 M_pi_m
R_G5SADrm	1 M_glu5sa_m = 1 M_h_m + 1 M_h2o_m + 1 M_1pyr5c_m
R_AMETtd	1 M_amet_c = 1 M_amet_m
R_GAPD	1 M_pi_c + 1 M_nad_c + 1 M_g3p_c = 1 M_h_c + 1 M_nadh_c + 1 M_13dpg_c
R_G6PDH2r	1 M_nadp_c + 1 M_g6p_c = 1 M_h_c + 1 M_nadph_c + 1 M_6pgl_c
R_O2t	1 M_o2_e = 1 M_o2_c
R_FBA	1 M_fdp_c = 1 M_dhap_c + 1 M_g3p_c
R_NDPK8	1 M_atp_c + 1 M_dadp_c = 1 M_adp_c + 1 M_datp_c
R_NDPK7	1 M_atp_c + 1 M_dcdp_c = 1 M_adp_c + 1 M_dctp_c
R_NDPK5	1 M_atp_c + 1 M_dgdp_c = 1 M_adp_c + 1 M_dgtp_c
R_NDPK4	1 M_atp_c + 1 M_dtdp_c = 1 M_adp_c + 1 M_dttp_c
R_NDPK3	1 M_atp_c + 1 M_cdp_c = 1 M_adp_c + 1 M_ctp_c
R_NDPK2	1 M_atp_c + 1 M_udp_c = 1 M_adp_c + 1 M_utp_c
R_NDPK1	1 M_atp_c + 1 M_gdp_c = 1 M_adp_c + 1 M_gtp_c
R_LEUtec	1 M_leu_L_e = 1 M_leu_L_c
R_L_LACt4r	1 M_na1_e + 1 M_lac_L_e = 1 M_na1_c + 1 M_lac_L_c
R_IPDDI	1 M_ipdp_c = 1 M_dmpp_c
R_DATPtn	1 M_datp_c = 1 M_datp_n
R_THFtm	1 M_thf_c = 1 M_thf_m
R_TKT1	1 M_r5p_c + 1 M_xu5p_D_c = 1 M_g3p_c + 1 M_s7p_c
R_TKT2	1 M_xu5p_D_c + 1 M_e4p_c = 1 M_g3p_c + 1 M_f6p_c
R_r0450	1 M_akg_m + 1 M_L2aadp_m = 1 M_2oxoadp_m + 1 M_glu_L_m
R_GLYtm	1 M_gly_c = 1 M_gly_m
R_HIBDm	1 M_nad_m + 1 M_3hmp_m = 1 M_h_m + 1 M_nadh_m + 1 M_2mop_m
R_r0645	1 M_h2o_c + 1 M_nad_c + 1 M_am6sa_c = 2 M_h_c + 1 M_nadh_c + 1 M_amuco_c
R_GLCt1r	1 M_glc_D_e = 1 M_glc_D_c
R_MMEm	1 M_mmcoa_R_m = 1 M_mmcoa_S_m
R_LDH_L	1 M_nad_c + 1 M_lac_L_c = 1 M_h_c + 1 M_pyr_c + 1 M_nadh_c
R_MALtm	1 M_pi_m + 1 M_mal_L_c = 1 M_pi_c + 1 M_mal_L_m
R_ASPte	1 M_asp_L_c = 1 M_asp_L_e
R_MDHm	1 M_nad_m + 1 M_mal_L_m = 1 M_h_m + 1 M_nadh_m + 1 M_oaa_m
R_L_LACtm	1 M_h_c + 1 M_lac_L_c = 1 M_h_m + 1 M_lac_L_m
R_ASPGLUm	1 M_h_c + 1 M_glu_L_c + 1 M_asp_L_m = 1 M_h_m + 1 M_glu_L_m + 1 M_asp_L_c
R_r0074	1 M_h2o_m + 1 M_nad_m + 1 M_glu5sa_m = 2 M_h_m + 1 M_nadh_m + 1 M_glu_L_m
R_NH4t3r	1 M_nh4_c + 1 M_h_e = 1 M_h_c + 1 M_nh4_e

R_AIRCr	1 M_co2_c + 1 M_air_c = 1 M_h_c + 1 M_5aizc_c
R_UMPK	1 M_atp_c + 1 M_ump_c = 1 M_adp_c + 1 M_udp_c
R_PRASCS	1 M_5aizc_c + 1 M_asp_L_c + 1 M_atp_c = 1 M_25aics_c + 1 M_adp_c + 1 M_h_c + 1 M_pi_c
R_PYRt2m	1 M_h_c + 1 M_pyr_c = 1 M_h_m + 1 M_pyr_m
R_TPI	1 M_dhap_c = 1 M_g3p_c
R_AICART	1 M_10fthf_c + 1 M_aicar_c = 1 M_fprica_c + 1 M_thf_c
R_C3STDH1Pr	1 M_nadp_r + 1 M_4mzym_int1_r = 1 M_h_r + 1 M_nadph_r + 1 M_4mzym_int2_r + 1 M_co2_r
R_C4CRNCPT2	1 M_coa_m + 1 M_c4crn_m = 1 M_btcoa_m + 1 M_crn_m
R_ILETAm	1 M_akg_m + 1 M_ile_L_m = 1 M_glu_L_m + 1 M_3mop_m
R_AKGMALtm	1 M_akg_m + 1 M_mal_L_c = 1 M_akg_c + 1 M_mal_L_m
R_METtec	1 M_met_L_e = 1 M_met_L_c
R_Htr	1 M_h_c = 1 M_h_r
R_AHCYStd	1 M_ahcys_m = 1 M_ahcys_c
R_FORtr	1 M_for_c = 1 M_for_r
R_ARGSL	1 M_argsuc_c = 1 M_fum_c + 1 M_arg_L_c
R_CITRtm	1 M_citr_L_m = 1 M_citr_L_c
R_ALAt2r	1 M_h_e + 1 M_ala_L_e = 1 M_h_c + 1 M_ala_L_c
R_PRPPS	1 M_atp_c + 1 M_r5p_c = 1 M_h_c + 1 M_amp_c + 1 M_prpp_c
R_MTHFCm	1 M_h2o_m + 1 M_methf_m = 1 M_10fthf_m + 1 M_h_m
R_LYStm	1 M_h_m + 1 M_lys_L_c = 1 M_h_c + 1 M_lys_L_m
R_r0193	1 M_h2o_c + 1 M_cys_L_c = 1 M_nh4_c + 1 M_h_c + 1 M_pyr_c + 1 M_HC00250_c
R_AHC	1 M_h2o_c + 1 M_ahcys_c = 1 M_adn_c + 1 M_hcys_L_c
R_LEUt5m	1 M_leu_L_c = 1 M_leu_L_m
R_r0838	1 M_nh4_c = 1 M_nh4_m
R_r0940	1 M_HC00250_c = 1 M_HC00250_e
R_r0941	1 M_hco3_c = 1 M_hco3_m
R_MDH	1 M_nad_c + 1 M_mal_L_c = 1 M_h_c + 1 M_nadh_c + 1 M_oaa_c
R_r0947	1 M_orn_m + 1 M_citr_L_c = 1 M_orn_c + 1 M_citr_L_m
R_PE_HStm	1 M_pe_hs_c = 1 M_pe_hs_m
R_CYStec	1 M_cys_L_e = 1 M_cys_L_c
R_TRPt	1 M_trp_L_e = 1 M_trp_L_c
R_DTTPtn	1 M_dttp_c = 1 M_dttp_n
R_ASPTAm	1 M_akg_m + 1 M_asp_L_m = 1 M_glu_L_m + 1 M_oaa_m
R_ACACT1r	2 M_accoa_c = 1 M_coa_c + 1 M_aacoa_c
R_SUCD1m	1 M_fad_m + 1 M_succ_m = 1 M_fadh2_m + 1 M_fum_m
R_ACONTm	1 M_cit_m = 1 M_icit_m
R_PSSA1_hs	1 M_ser_L_c + 1 M_pchol_hs_c = 1 M_chol_c + 1 M_ps_hs_c
R_r2534	1 M_thr_L_e = 1 M_thr_L_c
R_ILEt5m	1 M_ile_L_c = 1 M_ile_L_m
R_r2532	1 M_asn_L_e = 1 M_asn_L_c
R_DHFR	1 M_h_c + 1 M_nadph_c + 1 M_dhf_c = 1 M_nadp_c + 1 M_thf_c
R_CDIPTr	1 M_cdpdag_hs_c + 1 M_inost_c = 1 M_h_c + 1 M_cmp_c + 1 M_pail_hs_c
R_r1552	1 M_gly_e + 1 M_pro_L_c = 1 M_gly_c + 1 M_pro_L_e
R_MTHFD2m	1 M_nad_m + 1 M_mlthf_m = 1 M_nadh_m + 1 M_methf_m
R_r2525	1 M_gln_L_e = 1 M_gln_L_c
R_VALtec	1 M_val_L_e = 1 M_val_L_c
R_r2526	1 M_ser_L_e = 1 M_ser_L_c
R_CITtam	1 M_cit_c + 1 M_mal_L_m = 1 M_cit_m + 1 M_mal_L_c
R_ALATA_L	1 M_akg_c + 1 M_ala_L_c = 1 M_pyr_c + 1 M_glu_L_c

R_HACD1m	1 M_h_m + 1 M_nadh_m + 1 M_aacoa_m = 1 M_nad_m + 1 M_3hbcoa_m
R_ACACt2m	1 M_h_c + 1 M_acac_c = 1 M_h_m + 1 M_acac_m
R_10FTHFtm	1 M_10fthf_c = 1 M_10fthf_m
R_C4STMO1r	3 M_o2_r + 3 M_h_r + 3 M_nadph_r + 1 M_44mzym_r = 4 M_h2o_r + 3 M_nadp_r + 1 M_4mzym_int1_r
R_G3PD1	1 M_nad_c + 1 M_glyc3p_c = 1 M_h_c + 1 M_nadh_c + 1 M_dhap_c
R_ECOAH12m	1 M_h2o_m + 1 M_2mp2coa_m = 1 M_3hibutcoa_m
R_CO2t	1 M_co2_e = 1 M_co2_c
R_KHte	1 M_h_c + 1 M_k_e = 1 M_h_e + 1 M_k_c
R_PRAGSr	1 M_atp_c + 1 M_gly_c + 1 M_pram_c = 1 M_adp_c + 1 M_gar_c + 1 M_h_c + 1 M_pi_c
R_ECOAH1m	1 M_3hbcoa_m = 1 M_h2o_m + 1 M_b2coa_m
R_r2419	1 M_akg_c + 1 M_pi_m = 1 M_pi_c + 1 M_akg_m
R_ACACT1rm	2 M_accoa_m = 1 M_coa_m + 1 M_aacoa_m
R_LDH_Lm	1 M_nad_m + 1 M_lac_L_m = 1 M_h_m + 1 M_nadh_m + 1 M_pyr_m
R_r1670	1 M_orn_e + 1 M_his_L_c = 1 M_orn_c + 1 M_his_L_e
R_OCOAT1m	1 M_acac_m + 1 M_succoa_m = 1 M_aacoa_m + 1 M_succ_m
R_GLYt2r	1 M_h_e + 1 M_gly_e = 1 M_h_c + 1 M_gly_c
R_FRDPtcr	1 M_frdp_c = 1 M_frdp_r
R_C40CPT1	1 M_crn_c + 1 M_btcoa_c = 1 M_coa_c + 1 M_c4crn_c
R_PIt2m	1 M_h_c + 1 M_pi_c = 1 M_h_m + 1 M_pi_m
R_GK1	1 M_atp_c + 1 M_gmp_c = 1 M_adp_c + 1 M_gdp_c
R_GLCt2_2	2 M_h_e + 1 M_glc_D_e = 2 M_h_c + 1 M_glc_D_c
R_PROt2r	1 M_h_e + 1 M_pro_L_e = 1 M_h_c + 1 M_pro_L_c
R_CO2ter	1 M_co2_c = 1 M_co2_r
R_MMMm	1 M_mmcoa_R_m = 1 M_succoa_m
R_FUMtm	1 M_pi_m + 1 M_fum_c = 1 M_pi_c + 1 M_fum_m
R_DHORTS	1 M_h2o_c + 1 M_dhor_S_c = 1 M_h_c + 1 M_cbasp_c
R_HIStiDF	1 M_his_L_e = 1 M_his_L_c
R_FUMm	1 M_h2o_m + 1 M_fum_m = 1 M_mal_L_m

10.5.2. METABOLIC RATES VALUES

	Production rate (mg/(gDW·h))			
	pH controlled		pH free	
	Phase 1	Phase 2	Phase 1	Phase 2
Glucose	-760,2	0	-354	-97,7
Lactate	995,4	-52,9	600	-57,2
Ammonia	27	-7,6	30	10
O_2	-699,8	-141,8	-801	-526,4
Alanine	19,1	0,8	48,1	-2,5
Argininie	-17,2	-2,1	-25,6	-10
Asparagine	-9,3	-2,2	-27,4	-8,1
Aspartic acid	-19,8	-1,9	-41,3	-7,8
Cystein	-4,6	-0,13	-2,8	-1,3
Glutamine	-6,6	-0,8	58,6	-0,6
GlutaMAX	-2,4	-1,2	-30,6	-1,3
Glutamic acid	-7,1	-1,7	-20	-5,5
Glycine	17,6	3,2	27	1,7
Histidine	-12	-1,5	-7,7	-2,6
Isoleucine	-23,6	-3,6	-22,9	-10
Leucine	-34,4	-3,7	-23	-12,2
Lysine	-35,8	-5,5	-20	-9,1
Methionine	-9,2	-1,3	-11	-4,1
Ornitine	2,68	-0,7	10,3	-2,8
Phenilalanine	-9,4	-1,7	-11	-4,3
Proline	-8,1	-0,8	-33	0,8
Serine	-80,7	-8,3	-40	-15,6
Threonine	-15,9	-2	-18	-4,9
Tryptophan	-2,8	-0,037	-3,6	-0,9
Tyrosine	-6,3	-1	-10,2	-3
Urea	1,02	3,06	21,8	6,05
Valine	-23,4	-3,8	-27,7	-10,2