Mol Biotechnol (2007) 37:120–126
DOI 10.1007/s12033-007-0007-3

RESEARCH

Time-course determination of plasmid content in eukaryotic and prokaryotic cells using Real-Time PCR

Elisabete Carapuça · Adriano R. Azzoni · Duarte M. F. Prazeres · Gabriel A. Monteiro · Filipe J. M. Mergulhão

Received: 7 June 2006 / Accepted: 3 October 2006 / Published online: 13 July 2007

Abstract A Real-Time PCR method was developed to monitor the plasmid copy number (PCN) in *Escherichia coli* and Chinese hamster ovary (CHO) cells. *E. coli* was transformed with plasmids containing a ColE1 or p15A origin of replication and CHO cells were transfected with a ColE1 derived plasmid used in DNA vaccination and carrying the green fluorescent protein (GFP) reporter gene. The procedure requires neither specific cell lysis nor DNA purification and can be performed in <30 min with dynamic ranges covering 0.9 pg–55 ng, and 5.0 pg–2.5 ng of plasmid DNA (pDNA) for *E. coli* and CHO cells, respectively. Analysis of PCN in *E. coli* batch cultures revealed that the maximum copy number per cell is attained in mid-exponential phase and that this number decreases on average 80% towards the end of cultivation for both types of plasmids. The plasmid content of CHO cells determined 24 h post-transfection was around 3×10^4 copies per cell although only 37% of the cells expressed GFP one day after transfection. The half-life of pDNA was 20 h and around 100 copies/cell were still detected 6 days after transfection.

Keywords Plasmid copy number · Real-Time PCR · SYBR Green · Plasmid DNA vectors · Eukaryotic and prokaryotic cells · Plasmid DNA decay

E. Carapuça · A. R. Azzoni · D. M. F. Prazeres · G. A. Monteiro · F. J. M. Mergulhão
Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisboa, Portugal

F. J. M. Mergulhão (✉)
Chemical Engineering Department, LEPAE, Faculty of Engineering of the University of Porto, Porto, Portugal
e-mail: filipem@fe.up.pt

Introduction

Plasmids from *Escherichia coli* have been traditionally used as expression vectors for the overproduction of proteins [19] and more recently as vehicles for gene delivery and DNA vaccination [21].

Plasmid copy number (PCN) is largely controlled by the origin of replication although host physiology also plays a major role in plasmid replication [30]. High copy number plasmids are generally preferred for recombinant protein expression to take advantage of the gene dosage effect [19] although sometimes the use of low and moderate copy plasmids can be beneficial. This is due to a tighter control of gene expression, extreme segregational stability, the ability to replicate large pieces of DNA and low metabolic burden on the host strain [2].

High copy number plasmid DNA (pDNA) obtained from *E. coli* fermentations accounts for approximately 25% of the ongoing gene therapy trials [22] with a demand expected to increase several fold as soon as the first commercial pharmaceutics reach the market.

The major bottleneck in DNA vaccination is inefficient nuclear delivery of pDNA [26]. This occurs in part because delivery of genes to the nucleus of eukaryotic cells is a complex, multistage process, still poorly understood [14]. However, it is known that in its trafficking to the nucleus, pDNA must overcome a series of physical and biochemical barriers including endocytosis, entrapment inside endosomes and lysosomes, and degradation by a variety of endo/exo nucleases. These barriers, specially the one imposed by the presence of nucleases, may be partially circumvented by the use of adjuvants (e.g. cationic lipids or polymers), by using nuclease inhibitors, or by modifying pDNA secondary structures in order to eliminate nuclease labile sequences [6, 8, 26]. Fluorescence microscopy is

currently the most used technique in transfection studies for the detection of pDNA trafficking and decay inside mammalian cells [13, 27]. However, despite the extremely valuable information obtained from this technique, it lacks analytical precision and imposes the use of pDNA labelled with fluorophores or containing fluorescent DNA intercalators. These fluorescent dyes may be toxic to the cells or modify pDNA conformation and, consequently, may change the fate of the pDNA molecules under study.

Plasmid copy number determination has been traditionally performed in two steps starting with pDNA isolation from genomic DNA (gDNA) and quantitation by CsCl-Ethidium bromide centrifugation [9], gel electrophoresis [24, 25], high performance liquid chromatography [4], capillary electrophoresis [1] or DNA hybridization using radioactively labelled probes [7, 11]. These methods are laborious, require large amounts of biological sample, have low reproducibility and are only applicable on a narrow dynamic range [15]. Furthermore they are usually not indicated for high-throughput analysis and since they are time-consuming and labour-intensive they can not be used for process control. Real-Time PCR technology has numerous advantages over the aforementioned processes, including a wide range of quantification, high sensitivity and precision, no requirement for post-PCR steps, use of small amounts of biological sample and amenability to high-throughput formats and automation [10]. Recently, two methods for PCN determination using Real-Time PCR have been published [15, 16]. Both methods require an initial sample preparation step, which is performed by purifying pDNA with a commercial kit [16] or DNA isolation through a multi-step protocol involving mechanical and enzymatic cell disruption, DNA extraction, precipitation, washing and rehydration [15]. Since the amount of plasmid will be assayed after all these steps, the plasmid content of the samples is likely to under-represent the original amount of pDNA present in the cells. In fact, it has been reported that the steps of cell lysis, clarification and concentration, and column binding can seriously affect the DNA recovery yield [23] and thus have a negative impact on PCN determination. In addition, differences in plasmid and gDNA physico-chemical properties may lead to different recovery yields during isolation steps (e.g., alcohol precipitation or resin adsorption). This could induce errors in PCN determination, especially when relative quantification based on a reference gene present in the gDNA is used. Furthermore, this initial preparation may introduce additional errors in sample manipulation and increases the time and cost of the assay.

Determining the plasmid content in prokaryotic cells during pDNA production and in eukaryotic cells after transfection is crucial for DNA vaccine development and also for protein production. Existing methods are often labour intensive, time-consuming and lack analytical precision. Alternative methods are sought that can provide *quasi*-online results without sample pre-treatment enabling the optimisation of fermentation conditions, downstream processing and monitoring of plasmid trafficking.

Materials and methods

Plasmids

pFM20 (5301 bp) is a high copy number secretion vector for ZZ-GFP that contains a ColE1 origin of replication [18]. Plasmid pFM46 (6043 bp) is a low copy number secretion vector for ZZ-GFP, bearing a p15A origin of replication. It was constructed as described in [18, 20] using the pACYC177 derived vector bearing the *lacUV5-lacZ* fusion described in [29] as backbone.

pVAX1GFP (3697 bp) plasmid was constructed by modification of the commercial plasmid pVAX1*lacZ* (6050 bp, Invitrogen, Carlsbad, CA). It contains the human cytomegalovirus (CMV) immediate-early promoter and a ColE1 type origin of replication. In this work, the *β*-galactosidase reporter gene was replaced by the enhanced Green Fluorescent Protein (eGFP) obtained from pFM20, cloned between the *Xba*I and *Eco*RI restriction sites. Automated DNA sequencing was performed on this construction by Stab Vida Inc., Oeiras, Portugal.

Cell culture

E. coli

Escherichia coli AF1000 [29] was grown in M9 medium [28] supplemented with 4 g/l glucose, amino acids (50× stock solution from Sigma, St Louis, MO, catalogue #7131) and 30 μg/ml of kanamycin for cells harbouring plasmid pFM46 or 100 μg/ml ampicillin for cells transformed with pFM20. Cells were grown in 500 ml shake flasks containing 100 ml of medium incubated a 37°C on an orbital shaker (250 rpm). Culture volumes of 1.0 ml were periodically removed from the shake-flasks and immediately frozen for subsequent RT-PCR analysis.

CHO cells

Chinese hamster ovary (CHO) cells were grown in F-12 (Ham) nutrient mixture (Gibco, Paisley, UK) containing 10% (v/v) foetal bovine serum (Gibco, UK). All cell cultures were grown in 75 cm^2 culture flasks (5% CO_2 at 37°C) up to confluence, trypsinized and seeded in 24 well culture plates (4×10^4 cells per well). Cells were incubated for 48 h and then transfected with the plasmid

pVAX1GFP. Transfection was carried out using the Lipofectamine 2000™ reagent (Invitrogen, Carlsbad, CA), according to the reagent manufacturer instructions (1.0 μg pDNA plus 1.5 μl lipid in 100 μl of media per well). The media containing the transfection solution remained on the cells for 12 h and was then replaced by regular media. As a negative control, media containing pDNA but no transfection lipid was added to specific control wells. After appropriate periods, transfected and non-transfected cells were washed 4 times with 1.0 ml PBS to remove non-specifically bound pDNA as described by [31], trypsinized from wells, and centrifuged at 160*g* for 8 min. Cell pellets were then washed with PBS buffer, resuspended in 0.6 ml of the same saline solution, and counted on a Neubauer chamber (counting was performed in duplicate). Finally, cells were centrifuged again, the supernatant solution was removed, and pellets were stored at –80°C for subsequent PCR analysis.

Preparation of pDNA standards

Plasmid DNA standards were prepared by growing *E. coli* DH5α cells (harbouring the desired pDNA) overnight in 5 ml shake-flasks containing LB medium and antibiotics (30 μg/ml of kanamycin or 100 μg/ml ampicillin). Plasmid extraction and isolation was performed according to the High Pure Plasmid Isolation Kit protocol (Roche, Mannheim, Germany). The concentration of purified pDNA solutions was assayed by UV absorbance and confirmed by densitometry of agarose gels stained with ethidium bromide (not shown).

Real-Time PCR for determination of plasmid content

Quantitative Real-Time PCR for determination of pDNA was performed by amplification of a 108 bp sequence from the *GFP* gene using the 5′-TCG AGC TGG ACG GCG ACG TAA A-3′ forward primer, and 5′-TGC CGG TGG TGC AGA TGA AC-3′ reverse primer. PCR reactions were carried out in a Roche LightCycler™ detection system using the FastStart DNA Master SYBR Green I kit™ (Roche, Germany) as recommended by the manufacturer. Each 20 μl of final reaction volume contained 2.0 μl of the 10× SYBR Green mixture, 0.4 μl of each primer (0.4 μM final concentration), 1.6 μl of $MgCl_2$ solution (3.0 mM final concentration), 13.6 μl PCR grade water, and 2.0 μl of sample prepared as described below. Reactions were incubated at 95°C for 10 min to activate FastStart DNA polymerase and lyse cells, followed by 30 cycles of 10 s at 95°C, 5 s at 55°C and 7 s at 72°C. Following the final cycle, reactions were kept at 70°C for 30 s, and heat-denatured over a temperature gradient at 0.1°C/s from 70 to 95°C.

Sample preparation

E. coli cells

Since the cell constituents released during the initial 10 min incubation at 95°C may inhibit the amplification of the target DNA sequence, the same number of cells was always used per reaction in order to keep the amount of these potential inhibitors constant. In the case of the high copy plasmid pFM20, two cell concentrations (2.5×10^5 and 5×10^4 cells/reaction) were used to enable different working ranges, whereas a larger number of cells (3.5×10^5/reaction) were used in the case of the low copy number plasmid pFM46. The cell number in each starting sample was determined by using a correlation between the optical density of the culture and cell concentration [17]. Appropriate dilutions were then made with milliQ water in order to obtain the desired cell concentration. One micro liter of these cell suspensions was then added to 1 μl of PCR grade water. This sample was then mixed with the other PCR reagents as described above.

Plasmid DNA samples were prepared for construction of the calibration curves by spiking 1 μl of purified pDNA standards with non-transformed AF1000 cells. One micro liter of suspensions of these cells with the required amount per reaction (2.5×10^5 and 5×10^4 cells for pFM20 and 3.5×10^5 cells for pFM46) was used. These samples were then mixed with the other PCR reagents as described above. Each calibration curve was obtained as an average of three independent assays. Calculation of the PCN was performed on the basis of the plasmid base pair number (5301 bp for pFM20 and 6043 bp for pFM46) and of the average molecular weight of a DNA base pair (660 daltons).

CHO cells

Prior to analysis, CHO cell pellets were ice-thawed and resuspended in PCR grade deionized water up to a cell concentration of 6,250 cells/μl. Two micro liter of such suspension were then mixed with the other PCR reagents as described above. Negative controls containing the same amount of non-transfected cells, exposed to the pDNA but not to Lipofectamine™ (control cells), were always included. Determination of pDNA concentration in transfected cells was done in triplicate.

Calibration curves were constructed by adding 1 μl of pDNA standards (pVAX1GFP, 3697 bp) to 1 μl of a suspension of non-transformed CHO cells (12,500 cells per reaction). These samples were then mixed with the other PCR reagents as described above. Negative controls using PCR grade water instead of control cells were also always run to detect undesired contamination.

Detection of GFP expression by flow cytometry

Culturing and transfection of the CHO cells with the plasmid vector pVAX1GFP was performed as described above. After selected periods post-transfection, cells contained in 24 well plates were trypsinized and centrifuged at 160*g* for 8 min. Cell pellets were washed with PBS buffer and resuspended in 1.0 ml of the same solution. Transfected and non-transfected cells were analysed in a FACscan Scalibur (Becton-Dickinson, Franklin Lakes, NJ), which recorded forward scatter (FSC), side scatter (SSC) and green fluorescence (FL1). From these data, cells were first isolated from debris by their FSC versus SSC characteristics, and transfection efficiency was determined by subtracting those cells displaying background FL1 (non-transfected cells) from the total cell population. Average green fluorescent intensities corresponding to the expression level of GFP were calculated using the CellQuest Pro software (Becton-Dickinson, USA).

Results and discussion

Determination of plasmid copy number in *E. coli*

The copy number of two plasmids with different origins of replication in *E. coli* was determined. Plasmid pFM20 is a ColE1 plasmid used for the secretion of ZZ-GFP [18] with predicted copy numbers of up to 700 copies per cell [5]. Plasmid pFM46 is based on plasmid pACYC177, contains the p15A origin of replication and usually has copy numbers ranging from 10 to 50 copies per cell [3]. The exact same PCR method was used for the determination of PCN in both plasmids. Nevertheless, a higher number of cells were used when working with the low copy pFM46 (3.5×10^5 cells/reaction), and two different cell quantities were used in the case of pFM20 to obtain different working ranges (2.5×10^5 and 5×10^4 cells/reaction)

Calibration curves show a linear response on all tested conditions (Fig. 1). Although three different cell concentrations were used for the establishment of these curves, the results indicate that cellular components released during the PCR reaction do not significantly inhibit amplification (Fig. 1). A variation of <20% was obtained on the line equations (confidence level higher than 99%) when the quantity of cells was increased five-fold (open symbols in Fig. 1). Calibration curves for each plasmid were obtained in triplicate and a standard deviation of 15% was obtained. For the high copy number plasmid, the method enables the detection of at least four copies per cell and for the low copy number plasmid, the method is able to detect a single copy of the *GFP* gene even if only 50% of the cells contain the target sequence. Samples presented threshold cycle (Ct) numbers between 12.3 and 22.4 whereas negative controls containing AF1000 cells devoid of plasmid yielded Ct numbers higher than 26 and were automatically censored by the LightCycler™ software.

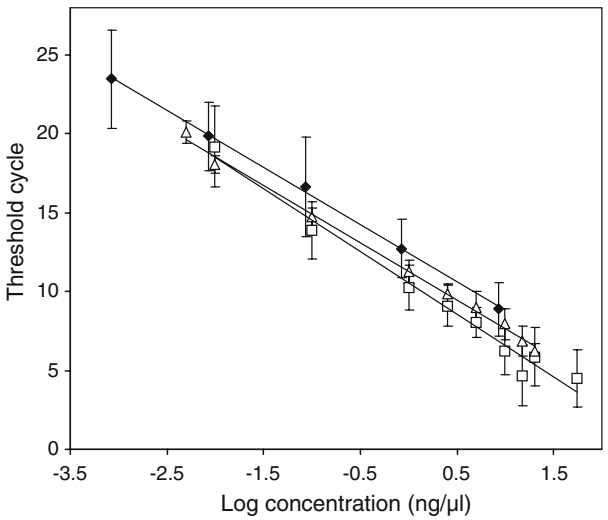

Fig. 1 Standard curves used to quantify pFM46 (closed symbols) and pFM20 (open symbols) in *E. coli* cells. Plasmid standards were spiked with non-transformed AF1000 cells at concentrations of 3.5×10^5 cells/reaction for pFM46 and 2.5×10^5 (triangles) and 5×10^4 (squares) cells/reaction for pFM20. Working linear range spans from 0.9 pg to 9 ng per reaction for pFM46 and from 5 pg to 55 ng per reaction for pFM20. Regression analysis presented r^2 values of 0.998 for pFM46 and of 0.980 and 0.996 for pFM20

The method was used to assess the variation of PCN during cell growth (Fig. 2). Plasmid copy number increases during the exponential phase for both, low and high copy number plasmids, with a sharp decrease of PCN observed upon entry into stationary phase (Fig. 2A, B). At the end of the cultivation, the measured PCN was around 20% of the maximum PCN found at the exponential phase. This is an indication that monitoring PCN when producing plasmids in *E. coli* can be extremely important for process development. The overall shape of the curves is very similar for both plasmids, with maximal values of PCN of approximately 400 copies/cell and 70 copies/cell for pFM20 and pFM46 respectively. The value for pFM20 falls within the range reported by other authors [5], whereas the value for pFM46 was slightly higher than expected. However, it has been reported that in certain conditions, plasmids harbouring the p15A origin of replication can reach copy numbers of around 200 per cell [3].

Determination of pDNA decay in CHO cells

The plasmid vector used for CHO cells transfection is a variant of the commercial pVAX1*LacZ* commonly used in DNA vaccination studies, in which the *GFP* gene replaced the *lacZ* reporter gene. A standard curve was generated by

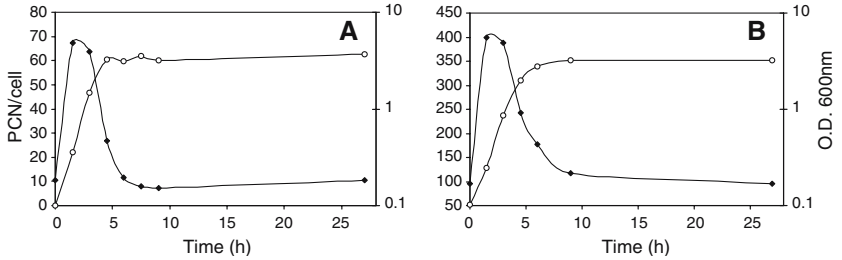

Fig. 2 Time-course evolution of plasmid copy number (PCN) in *E. coli*. **(A)** Plasmid pFM46 (p15A origin); (**B**) Plasmid pFM20 (ColE1 origin). Plasmid copy number per cell is represented by closed circles (left axis), and growth curve (cell culture O.D.) is represented by open circles (right axis)

spiking non-transfected CHO cells with increasing amounts of pDNA. The manipulation of the non-transfected cells used in the standard curve was the same as for the transfected cells, including contact with pDNA, with the difference that this plasmid solution did not contain the transfection agent (Lipofectamine). Using this approach, the ''control cells'' obtained still contained residual amounts of pDNA that could not be removed during the cell washing steps, as expected to occur in transfected cells. The standard curve obtained when using pDNA ranging from 5 pg to 100 ng per reaction (each reaction containing 12,500 cells) is presented in Fig. 3A. The data indicates that it is possible to determine pDNA in the complete concentration range studied. However, a narrow and linear working range (from 5 pg to 2.5 ng) was used in this work (Fig. 3B) enabling the detection of a minimum of 140 copies/cell. Standards presented Ct numbers between 13.0 and 18.2. Negative controls, containing the control CHO cells exposed to pDNA without the transfection agent, provided Ct's of 19.9 while runs containing no cells had Ct's higher than 26. This difference in Ct between the controls should be attributed to small amounts of pDNA non-specifically bound to the cells that could not be removed during the washing step.

The Real-Time PCR method was then used to quantitate the amount of pDNA present inside CHO cells at different times post-transfection (Fig. 4A) demonstrating the kinetics of plasmid degradation by cell endo/exonucleases. The efficiency of the cationic lipid-mediated transfection was verified by the expression of the GFP reporter (Fig. 4B). In agreement with the lipid-mediated transfection experiments reported by other authors [13, 31], our results indicate that on average a high number of pDNA copies (tens of thousands) is internalised by each cell via endocytosis during transfection. Nevertheless, only about 37% of the cells presented GFP expression during the first day after transfection. This result agrees with the observation that most of the pDNA that enters the cells remains aggregated in large vesicular compartments, never reaching the cell nucleus [16].

The number of plasmid copies per cell after transfection decreased sharply from approximately 30,000 at 24 h to <5000 copies at 72 h. On average, approximately 140 copies per cell were still detected 6 days after transfection. The calculated half-life of pDNA between 24 and 48 h post-transfection was 19.5 h, a value coherent with those found in literature [12, 27]. The half-life of pDNA inside CHO cells may vary significantly depending on several factors including the transfection method, cellular lineage, pDNA size and the type of transfection adjuvants used. Figure 4A and 4B also provide a comparison between the kinetics of pDNA decay and GFP expression, where the differences in the half-life of the expression vector and of the expressed reporter protein can be seen.

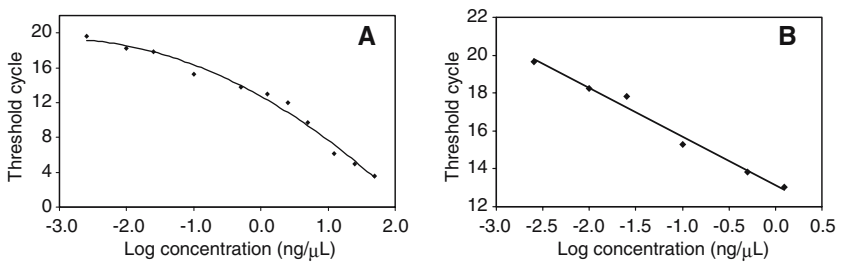

Fig. 3 Standard curves to quantify pVAX1GFP in transfected CHO cells. Plasmid standards were spiked with 12,500 non-transfected CHO cells per reaction. (**A**) The standard curve obtained when using pDNA masses ranging from 5 pg to 100 ng per reaction; and (**B**) linear working range, from 5 pg to 2.5 ng per reaction. Linear fitting curve presented an r^2 of 0.983

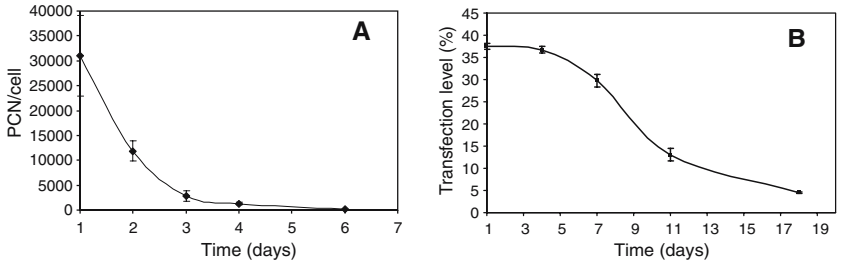

Fig. 4 Kinetics of plasmid decay quantified by Real-Time PCR measurement of plasmid copy number (**A**), and green fluorescent protein (GFP) expression detected by flow cytometry (**B**) after transfection of CHO cells with the pVAX1GFP using Lipofectamine™. Error bars indicate standard deviation between triplicates and deviation between duplicates for pDNA and GFP curves, respectively

Conclusions

The Real-Time PCR method presented here is suitable for determination of plasmid content in both CHO and *E. coli* cells. Adaptation to other eukaryotic and prokaryotic systems should be straightforward. Although the number of cells analysed in each PCR reaction had to be optimised in order to ensure that the output of the assay was within the operational range of the method, no additional optimisation was necessary for either type of cell. The procedure requires neither specific cell lysis nor DNA purification, since the first PCR step at 95°C disrupts cells, and the cell debris and impurities do not inhibit the amplification reaction. The elimination of sample pre-treatment steps from the protocol reduces errors associated with sample manipulation and is not dependent on the efficiency of DNA purification steps and recovery yields. Furthermore, the method is not labour intensive and provides results within 30 min.

Given the high sensitivity attained with bacterial cells it is likely that copy numbers of most multicopy plasmids (including those based on pBR3222) can be determined, as they are typically present in numbers between those determined for the plasmids used on this study. Since the method is rapid, it can be used for the monitoring of PCN during *E. coli* fermentations aimed at pDNA production. Given the time-course evolution of the PCN seen on this study, determining the endpoint of the fermentation may have important implications on downstream processing and productivity.

The method presented here was also shown to be a valuable analytical tool for monitoring pDNA decay in transfected CHO cells and may contribute, along with fluorescence microscopy techniques, to improve the current knowledge on the mechanisms of pDNA trafficking and gene delivery.

Acknowledgements The authors thank the Fundação para a Ciência e a Tecnologia, Portugal, for financial support (POCI/BIO/55799/2004, and post-doctoral grant BPD/14879/2003 to A.R. Azzoni).

References

1. Breuer, S., Marzban, G., Cserjan-Puschman, M., Durrschmid, E., & Bayer, K. (1998). Off-line quantitative monitoring of plasmid copy number in bacterial fermentation by capillary electrophoresis. *Electrophoresis, 19*, 2474–2478.
2. Carrier, T., Jones, K. L., & Keasling, J. D. (1998). mRNA stability and plasmid copy number effects on gene expression from an inducible promoter system. *Biotechnology and Bioengineering, 59*, 666–672.
3. Chang, A. C., & Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *Journal of Bacteriology, 134*, 1141–1156.
4. Coppella, S. J., Acheson, C. M., & Dhurjati, P. (1987). Isolation of high-molecular-weight nucleic acids for copy number analysis using high-performance liquid chromatography. *Journal of Chromatography, 402*, 189–199.
5. Cranenburgh, R. M., Lewis, K. S., & Hanak, J. A. (2004). Effect of plasmid copy number and lac operator sequence on antibiotic-free plasmid selection by operator-repressor titration in *Escherichia coli*. *Journal of Molecular Microbiology and Biotechnology, 7*, 197–203.
6. Crook, K., McLachlan, G., Stevenson, B. J., & Porteous, D. J. (1996). Plasmid DNA molecules complexed with cationic liposomes are protected from degradation by nucleases and shearing by aerosolisation. *Gene Therapy, 3*, 834–839.
7. Fujimura, H.-a., Sakuma, Y., & Weimer, T. (1996). A quantitative method for the determination of plasmid copy number in recombinant yeast. *Biologicals 24*, 67–70.
8. Glasspool-Malone, J., Steenland, P. R., McDonald, R. J., Sanchez, R. A., Watts, T. L., Zabner, J., & Malone, R. W. (2002). DNA transfection of macaque and murine respiratory tissue is greatly enhanced by use of a nuclease inhibitor. *Journal of Gene Medicine 4*, 323–332.
9. Hashimoto-Gotoh, T., & Sekiguchi, M. (1977). Mutations of temperature sensitivity in R plasmid pSC101. *Journal of Bacteriology, 131*, 405–412.
10. Klein, D. (2002). Quantification using real-time PCR technology: applications and limitations. *Trends in Molecular Medicine, 8*, 257–260.
11. Korpela, K., Buchert, P., & Soderlund, H. (1987). Determination of plasmid copy number with nucleic-acid sandwich hybridization. *Journal of Biotechnology, 5*, 267–277.
12. Lechardeur, D., & Lukacs, G. L. (2002). Intracellular barriers to non-viral gene transfer. Current Gene Therapy, 2, 183–194.
13. Lechardeur, D., Ming, X., & Lukacs, G. L. (2004). Contrasting nuclear dynamics of the caspase-activated DNase (CAD) in

dividing and apoptotic cells. *Journal of Cell Biology,* 167, 851–862.
14. Lechardeur, D., Verkman, A.S., & Lukacs, G. L. (2005). Intracellular routing of plasmid DNA during non-viral gene transfer. *Advanced Drug Delivery Reviews, 57*, 755–767.
15. Lee, C. L., Ow, D. S., & Oh, S. K. (2006). Quantitative real-time polymerase chain reaction for determination of plasmid copy number in bacteria. *Journal of Microbiological Methods 65*, 258–267.
16. Lee, C., Kim, J., Shin, S. G., & Hwang, S. (2006). Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. *Journal of Biotechnology, 123*, 273–280.
17. Mergulhão, F., & Monteiro, G. (2004). Secretion capacity limitations of the Sec pathway in *Escherichia coli*. *Journal of Microbiology and Biotechnoogyl, 14*, 128–133.
18. Mergulhão, F. J. M., & Monteiro, G. A. (2007). Analysis of factors affecting the periplasmic production of recombinant proteins in *Escherichia coli*. *Journal of Microbiology and Biotechnology, 17* (in press).
19. Mergulhão, F. J. M., Monteiro, G. A., Cabral, J. M. S., & Taipa, M.A. (2004). Design of bacterial vector systems for the production of recombinant proteins in *Escherichia coli*. *Journal of Microbiology and Biotechnology, 14*, 1–14.
20. Mergulhão, F. J. M., Monteiro, G. A., Larsson, G., Bostrom, M., Farewell, A., Nystrom, T., Cabral, J. M. S., & Taipa, M. A. (2003). Evaluation of inducible promoters on the secretion of a ZZ-Proinsulin fusion protein. *Biotechnology and Applied Biochemistry, 38*, 87–93.
21. Prather, K. J., Sagar, S., Murphy, J., & Chartrain, M. (2003). Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production and purification. *Enzyme and Microbial Technology, 33*, 865–883.
22. Prazeres, D. M. F., & Ferreira, G. N. M. (2004). Design of flowsheets for the recovery and purification of plasmids for gene therapy and DNA vaccination. *Chemical and Engineering Process, 43*, 615–630.
23. Prazeres, D. M., Ferreira, G. N., Monteiro, G. A., Cooney, C. L., & Cabral, J. M. (1999). Large-scale production of pharmaceutical-grade plasmid DNA for gene therapy: Problems and bottlenecks. *Trends in Biotechnology, 17*, 169–174.
24. Projan, S. J., Carleton, S., & Novick, R. P. (1983). Determination of plasmid copy number by fluorescence densitometry. *Plasmid, 9*, 182–190.
25. Pushnova, E. A., Geier, M., & Zhu, Y. S. (2000). An easy and accurate agarose gel assay for quantitation of bacterial plasmid copy numbers. *Analytical Biochemistry, 284*, 70–76.
26. Ribeiro, S. C., Monteiro, G. A., & Prazeres, D. M. (2004). The role of polyadenylation signal secondary structures on the resistance of plasmid vectors to nucleases. *Journal of Gene Medicine, 6*, 565–573.
27. Roth, C. M., & Sundaram, S. (2004). Engineering synthetic vectors for improved DNA delivery: insights from intracellular pathways. *Annual Review of Biomedical Engineering, 6*, 397–426.
28. Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. CSH Press, New York.
29. Sandén, A. M., Prytz, I., Tubulekas, I., Forberg, C., Le, H., Hektor, A., Neubauer, P., Pragai, Z., Harwood, C., Picon, A., Teixeira de Mattos, J., Postma, P., Farewell, A., Nystrom, T., Reeh, S., Pedersen, S., & Larsson, G. (2003). Limiting factors in *Escherichia coli* fed-batch production of recombinant protein. *Biotechnology and Bioengineering, 81*, 158–166.
30. Summers, D. (1998). Timing, self-control and a sense of direction are the secrets of multicopy plasmid stability. *Molecular Microbiology, 29*, 1137–1145.
31. Zabner, J., Fasbender, A. J., Moninger, T., Poellinger, K. A., & Welsh, M. J. (1995). Cellular and molecular barriers to gene transfer by a cationic lipid. *Journal of Biological Chemistry, 270*, 18997–19007.