



UNIVERSITY OF BEIRA INTERIOR

Sciences

Validation of an analytical method using an anion-exchange monolithic column for the assessment of supercoiled plasmid DNA

Élia Marília da Fonte Mota

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Supervised: Professor Fani Sousa and Professor Cândida Teixeira Tomaz

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*“Nothing in life is to be feared, it is only to be understood.
Now is the time to understand more, so that we may fear less.”*

Marie Curie

To my greatest love,

My father...

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Resumo

Nos últimos anos tem aumentado a exigência para obtenção de DNA plasmídico superenrolado de elevada pureza de forma a ser aplicado como vector em novas estratégias terapêuticas, como a terapia génica ou as vacinas de DNA. Assim, é necessário implementar uma técnica analítica adequada para controlar a qualidade do plasmídeo superenrolado, como um produto farmacêutico, durante o processo de produção. O presente estudo descreve o desenvolvimento de uma nova metodologia para quantificar e controlar a pureza do plasmídeo superenrolado, usando uma coluna monolítica que se baseia em cromatografia de troca aniónica. Este método analítico com detecção UV permite a separação das isoformas do plasmídeo, usando um gradiente por passos de NaCl. Avaliou-se a selectividade, linearidade, exatidão, reprodutibilidade e repetibilidade do método, e também se estabeleceram os limites inferiores de quantificação e de detecção. A validação foi realizada de acordo com as directivas, sendo demonstrado que o método é preciso e exato até uma concentração de plasmídeo superenrolado de 200 µg/mL. O principal avanço alcançado ao usar este método é a possibilidade de quantificar plasmídeo superenrolado numa amostra contendo outras topologias do plasmídeo, num ensaio de 4 minutos. Esta coluna também possibilita a avaliação de plasmídeo superenrolado presente em amostras mais complexas, permitindo o controlo ao longo do bioprocesso. Assim, estes resultados confirmam a possibilidade de utilizar esta coluna monolítica associada a um método analítico poderoso no controlo do processo “downstream” do plasmídeo superenrolado para aplicações terapêuticas.

Palavras-chave

Coluna monolítica, cromatografia de troca aniónica, DNA plasmídico superenrolado, método analítico, validação.

Abstract

The demand of high-purity supercoiled (sc) plasmid DNA (pDNA) to be applied as a vector for new therapeutic strategies, such as gene therapy or DNA vaccination has increased in the last years. Thus, it is necessary the implementation of an analytical technique suitable to control the quality of the sc pDNA as a pharmaceutical product, during the manufacturing process. The present study describes a new methodology to quantify and monitor the purity of sc pDNA, using a monolithic column based on anion-exchange chromatography. This analytical method with UV detection allows the separation of the plasmid isoforms by using a NaCl stepwise gradient. The selectivity, linearity, accuracy, reproducibility and repeatability of the method have been evaluated, and the lower quantification and detection limits were also established. The validation was performed according to the guidelines, being demonstrated that the method is precise and accurate for a sc plasmid concentration up to 200 µg/mL. The main advance achieved by using this monolithic method is the possibility to quantify the sc plasmid in a sample containing other plasmid topologies, in a 4 minutes experiment. This column also permits the assessment of the sc pDNA present in more complex samples, allowing the control of pDNA throughout the bioprocess. Thus, these findings strengthen the possibility of using this monolithic column associated with a powerful analytical method to control the downstream process of sc pDNA for therapeutic applications.

Keywords

Analytical method, anion-exchange chromatography, monolithic column, supercoiled plasmid DNA, validation.

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List of acronyms

A	Adenine
AEX	Anion-exchange
AIDS	Acquired immunodeficiency syndrome
BCA	Bicinchoninic acid
C	Cytosine
CGE	Capillary gel electrophoresis
CIM	Convective Interaction Media
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
<i>E. coli</i>	<i>Escherichia coli</i>
E.U.	Endotoxin units
EDTA	Ethylene-diamine tetraacetic acid
EMA	European Agency for the Evaluation of Medical Products
FDA	Food and Drug Administration
G	Guanine
gDNA	Genomic DNA
h	Hour
HIC	Hydrophobic interaction chromatography
HPLC	High-performance liquid chromatography
kbp	Kilo base pairs
LAL	Lymulus amebocyte lysate
LB	Luria-Bertani
ln	Linear
LPS	Lipopolysaccharide
M	Molar
min	Minute
mL	Milliliter
mm	Millimeter
M_r	Relative molecular mass
mRNA	Messenger RNA
NaCl	Sodium chloride
nm	Nanometer
NaOH	Sodium hydroxide
oc	Open circular
OD	Optical density

PCR	Polymerase chain reaction
pDNA	Plasmid DNA
pDERMATT	Plasmid DNA encoding recombinant MART-1 and tetanus toxin fragment-c
Q	Quaternary amino groups
®	Registered trademark
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
rRNA	Ribosomal RNA
s	Second
sc	Supercoiled
SDS	Sodium dodecylsulphate
T	Thymine
TB	Terrific Broth
Tris	Tris(hydroxymethyl)methylamine
tRNA	Transfer RNA
UV	Ultraviolet
µm	Micrometer

Chapter I - Introduction

Section I - Clinical application of plasmid DNA: gene therapy or DNA vaccination

In the last decades, a technological advance in medical and pharmaceutical areas allowed the accomplishment of several human clinical trials, testing the ability to inject plasmids providing therapeutic benefits for untreatable pathologies [1-4]. The rapid evolution of gene therapy and DNA vaccination promises to revolutionize the treatment of inherited and acquired diseases, by the development of specific DNA vectors in large quantities under pharmaceutical grade [5].

Briefly, DNA vaccines use the plasmid DNA (pDNA) to express specific antigens on cell membranes, stimulating and enhancing the immune system's response and memory [6], providing immunity against major killers, such as malaria [7]. On the other hand, gene therapy is based on the introduction of functional genes into human cells and expression of correct copy [8] to repair genetic defects, such as cystic fibrosis, or acquired diseases like cancer and AIDS [9].

An effective application of pDNA for therapeutic benefits has been made difficult by issues associated with intracellular delivery, transfection efficiency and purity degree required for pDNA expression vectors, to avoid adverse immune responses [10]. Effective DNA vaccination and gene therapy require the control of both the location and function of therapeutic genes at specific target sites within the patient's body [11]. Thus, one of the main challenges in the development of molecular medicine is to introduce naked DNA into target cells overcoming the extra- and intracellular barriers.

Most gene administration methods are based on viral vectors [9,12] but this delivery system has raised safety and regulatory concerns because of their toxicity and immunogenicity [10]. Non-viral vectors, such as pDNA vectors have been considered to be safer, simpler to use and easier to produce on a large-scale [13,14]. Thus, the non-viral vectors overcome the problems associated to the viral vector-mediated therapy [11,12,15], becoming the most attractive gene-transfer systems to be used as biopharmaceutical products [9,11,16].

The clinical application of pDNA is progressing and the commercial availability for immunotherapeutic and gene therapy purposes is approaching reality. Plasmid DNA is considered a new generation biotechnology product, and several clinical trials are currently in progress using these vectors to treat or to prevent a wide range of diseases with complex

etiologies including: cancer, AIDS, allergic diseases, infectious diseases like malaria, neurological disorders such as Parkinson's disease and Alzheimer's disease, and cardiovascular disorders [17,18]. The consequent use of pDNA vectors in these therapeutic approaches has increased the need for large amounts of highly pure pDNA with a therapeutic or pharmaceutical-grade and the development of large-scale and efficient pDNA purification processes is also required [19].

Section II - Characteristics of pDNA biomolecule

Plasmids are extra-chromosomal covalently closed double stranded DNA molecules found most frequently but not exclusively in bacterial cells [20]. They replicate independently of the cell chromosome, and typically carry genes that encode for antibiotic resistance [20]. On a molecular level, pDNA molecules can be considered pro-drugs that upon cellular internalization employ the DNA transcription and translation apparatus in the cell to biosynthesize the specific protein [17].

Plasmid DNA molecules are very large ($M_r > 10^6$, sizes in the μm range) when compared with proteins [15]. Each strand of a pDNA molecule is a linear polymer of deoxyribonucleotides linked by phosphodiester bonds. The phosphate groups are negatively charged for $\text{pH} > 4$ [21]. The winding of the two anti-parallel DNA strands around each other and around a common axis originates the classic right handed double helix structure, which is stabilized by Watson-Crick hydrogen bonds between AT and GC base pairs and by stacking forces [21]. The inside of the double helix is highly hydrophobic due to the close packing of the aromatic bases [19]. When the helix axis of pDNA is coiled in space appears sc pDNA.

Despite pDNA being a very stable biomolecule, during the manufacturing and recovery processes, it can undergo several types of stress that may disrupt its structural stability [22]. In this way, plasmid molecules that are mainly recovered in the sc isoform can also exist in a non-sc or relaxed form, such as the oc conformation resulting from the damage of the sc form. Other variants, such as linear (ln), denatured or oligomeric conformations can also be found in cell lysates [19]. If the manipulation induce nicks in one DNA strand (cleavage of the phosphodiester bonds), the sc pDNA uncoils to become the oc plasmid isoform; on the other hand, if the manipulation induce double-stranded nicks it produces the ln plasmid form [23]. The denatured forms exhibit a conformation where the hydrogen bonding between complementary strands at certain locations has been disrupted and oligomers are a consequence of homologous recombination [24].

Supercoiled pDNA, due to its structure and conformation extremely compact and functional, is considered the most efficient isoform at inducing gene expression comparing with other conformational variants [25]. Thus, the sc pDNA isoform is the desired topological form since it induces the most efficient access to the nucleus of the cell and consequently improves gene expression in eukaryotic cells [25]. Since the diversity of pDNA forms results from the damage of the sc isoform, and also because this is the only naturally intact form adapted to play an active role in eukaryotic cell physiology [1,25,26], the attainment of the isolated and purified sc plasmid isoform becomes essential.

Section III - Biotechnological process for pDNA manufacturing

Process development for pDNA manufacturing usually starts on a bench scale, and includes three stages: upstream processing, fermentation and downstream processing [27] (Fig. 1). Generally almost all processes, for the manufacture of pDNA in sufficient quantities to clinical applications, need to improve the productivity [18], mainly in the following operations: fermentation, cell lysis, isolation and purification [28, 29].

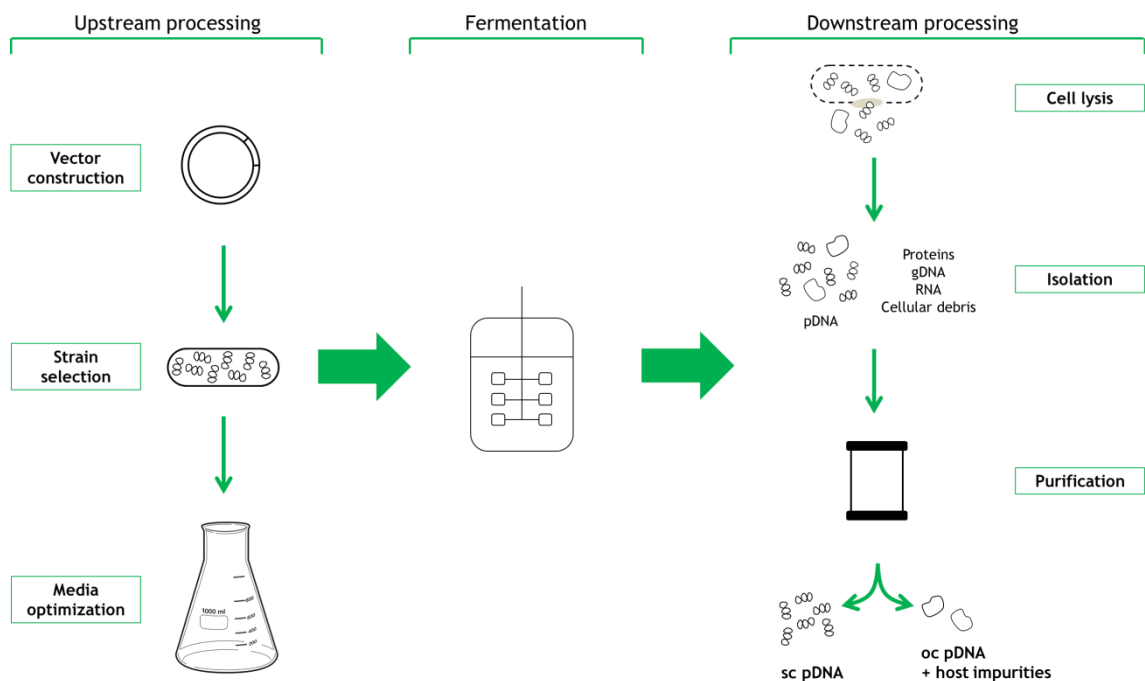


Figure 1 - Schematic representation of the three stages involved in process development for pDNA manufacturing. (Adapted from [20, 30])

In order to obtain pDNA is necessary the design of the vector to ensure safety, efficacy and cost-efficient production. After completing the vector construction, the transformation of a bacterial cell, typically *Escherichia coli* (*E. coli*), is performed with the plasmid encoding the gene of interest. Plasmid molecules are usually biosynthesized by autonomous replication in *E. coli* host fermentation, intending to maintain high plasmid copy number per bacterium [1]. After selection and optimization of growth conditions (upstream processing), the production is then developed by fermentation of the host cells in a bioreactor to achieve high yields of pDNA [31]. Finally, the isolation and purification steps (downstream processing) (Fig. 1) consist on the disruption of *E. coli* cells to recover the pDNA, which is then purified using a suitable methodology [19].

There are several techniques that can be used to disrupt *E. coli* cells, and hence, release pDNA molecules, but the most widely used method is alkaline lysis [32] or its variations [33]. This is the first critical step in the downstream processing of pDNA, because all the intracellular components, including all plasmid DNA conformations, RNA, genomic DNA (gDNA), endotoxins and proteins, are released [27]. Moreover, this step can affect the ratio of supercoiled (sc) plasmid to other forms, the plasmid amount recovered, as well as the physicochemical characteristics of cellular impurities that must be removed during the downstream process.

The alkaline cell lysis relies on the disruption of cells at high pH with NaOH in the presence of SDS, followed by the release and denaturation of gDNA, cell wall material and most of the cellular proteins. After the lysis step, the solution is neutralized with potassium acetate, which precipitates SDS together with denatured gDNA and cellular debris. Different operations can remove this insoluble material, whereas the majority of pDNA remains in the supernatant. During these manipulations care should be taken to avoid shear-induced cleavage of gDNA, which may result in small fragments that will not aggregate. The resulting clarified alkaline lysate normally contains proteins, RNA, lipopolysaccharides (LPS), gDNA fragments and pDNA. Thus, it is generally performed the reduction of impurities content, like proteins and host nucleic acids, and the reduction of volume to increase the plasmid mass fraction by clarification and concentration operations, respectively [19,34,35] Throughout the process, the mechanical stress should be avoided (for example, by stirring or pumping) in order to minimize the risk for open circular (oc) pDNA formation [18].

The final step to obtain pDNA as a highly pure product for therapeutic applications is the recovery and purification of pDNA molecules from the clarified cell lysate, through of liquid chromatography, which stands as a central purification technique, both at laboratory and process-scale [19,28]. Several studies have been made to develop adsorbents based on different chromatographic principles that allow a fast and efficient separation of pDNA [19]. Different approaches exploit one or more of the following purification processes: size exclusion, anion-exchange, hydrophobic interaction and affinity chromatography [19]. Liquid

chromatography is crucial in process-scale manufacturing of therapeutic pDNA, since the quality specifications set or recommended by regulatory agencies, such as the Food and Drug Administration (FDA) and the European Agency for the Evaluation of Medical Products (EMA) must be fulfilled [14]. The role of chromatography is then to remove those cellular host components (RNA, proteins, gDNA fragments, endotoxins) and non-sc pDNA variants (Fig. 1), in order to achieve the purification of sc pDNA [14]. Several chromatographic techniques have been integrated, individually or combined, into several processes for the manufacture of therapeutic pDNA [36].

The challenges in downstream processing are essentially related to eliminating host impurities that share common characteristics with pDNA, like negative charge (RNA, gDNA, and endotoxins), molecular mass (gDNA and endotoxins) and hydrophobicity (endotoxins) [18], as well as to isolate the sc pDNA from the other plasmid conformational variants. These facts render more evident the need to develop and implement efficient methods to quickly isolate and purify the sc plasmid isoform. Chromatographic processes can then explore some differences in properties, such as charge, molecular size and hydrophobicity [19].

Critical values for the acceptability of pharmaceutical-grade preparations are represented in table 1, showing all specifications that must be reliably reached in processes which are supposed to be used for production of therapeutic substances [18].

Table 1 - Specifications of bacterial cell lysates and accepted levels of impurities for the final product. (Adapted from [18])

Content of bacterial cell lysates	Final product		
	Issue in focus:	Range of acceptance:	Determined by:
55% Proteins	proteins	<3 µg/mg pDNA	BCA test
21% RNA	RNA	<0.2 µg/mg pDNA	Analytical HPLC
3% gDNA	gDNA	<2 µg/mg pDNA	TaqMan-PCR
3% LPS	endotoxins	<10 E.U./mg pDNA	LAL test
3% Plasmid	sc over oc form	>97%	CGE
15% Others			

Plasmid DNA represents less than 3% of the global content in the cleared lysate preparation (Table 1), even in the case of high copy number plasmid [18]. The removal of endotoxins is particularly important, since these LPS components of the *E. coli* cell wall can cause symptoms of toxic shock syndrome [37]. Furthermore, endotoxins may dramatically reduce transfection efficiencies in various cell lines and display cytotoxic effects on mammalian cells [37].

Finally, the plasmid product is formulated and delivered to the eukaryotic cells to express the protein of interest [23]. In general, all the unit operations involved in the global process are

adjusted in a way that pDNA can accomplish the properties to express the encoded information, preferably at the right place, time, and level [38].

The global process selected has to be safe and productive as recommended by the regulatory agencies [19]. Considering the clinical application of pDNA, the pharmaceutical development of a functional, stable and sterile product for clinical trials depends heavily on the availability of an assay for the quantification and purity determination of active plasmid products [39]. Due to the particular interest in the bio-active plasmid conformation, it is crucial that this analytical methodology quantify only the sc isoform. Liquid chromatography is also very useful as an analytical tool to monitor and control pDNA quality during processing and in the final formulations. This type of analysis is essential to guarantee that the final product meets the desired specifications [19,40].

According to regulatory agencies, such as FDA, the product-quality is defined as a percentage of the sc isoform compared to the total pDNA, being that the purity of the sc pDNA must be 100% with the homogeneity degree higher than 97% of sc isoform from *E. coli* host to apply in gene therapy and DNA vaccination [18]. Thus, it is essential that both research and industry improve the effectiveness of sc pDNA production and purification methodologies [28].

Section IV - Analytical methods for pDNA quantification

Monitoring the performance of a pDNA manufacturing process, as well as assessing the final product quality considering the product specifications, are key issues in process development, validation and product approval [40,41]. Biopharmaceuticals based on pDNA are chemically highly defined and so can be analysed by chemical, biochemical and physical assays [41]. Assessing the quality of the final plasmid preparation in terms of its safety, potency and purity, in order to ensure that the product specifications are met, is a key issue for process validation [40,41]. The development and set-up of reliable analytical methods for the quantification of pDNA and assessment of its purity and homogeneity are especially important.

Since methods for producing and purifying plasmids in large quantities are being developed [42], it is also important that quick, reliable, accurate, and inexpensive analytical methods can be established in order to ensure that purity levels are constantly met in each unit operation (Figure 2).

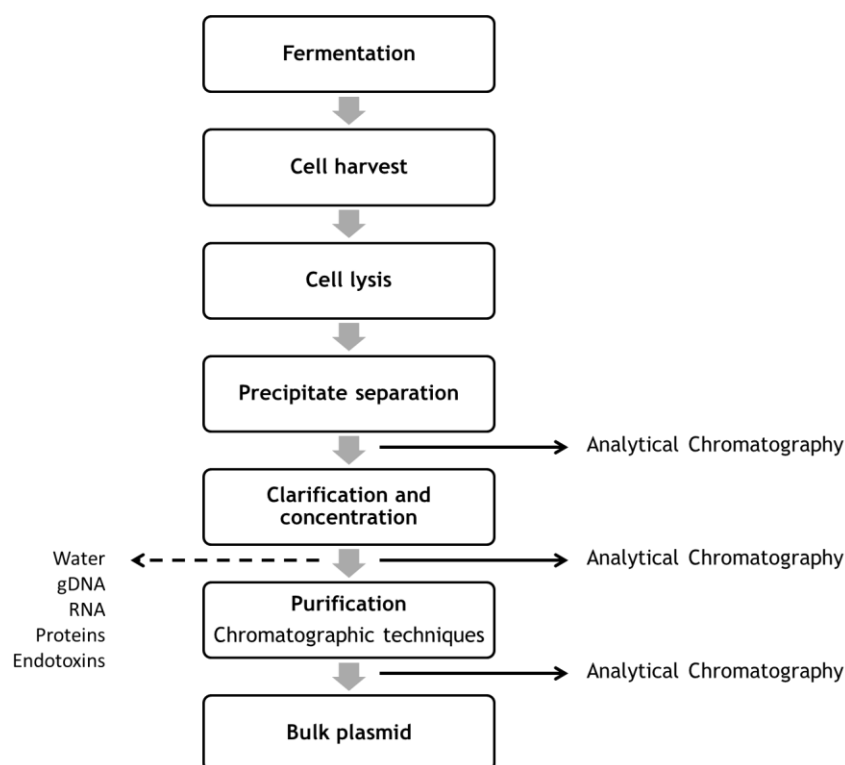


Figure 2 - The role of analytical chromatography in process-scale manufacturing of therapeutic pDNA. (Adapted from [19])

Although quantification of total pDNA in pure solutions is relatively easy to accomplish (for example, by spectrophotometry at 260 nm or fluorescence), mass estimation in impure process streams requires the separation of impurities such as RNA, gDNA fragments and proteins from plasmid molecules before quantitative detection. This separation of plasmid from impurities can be accomplished by electrophoresis carried out either in the conventional [43] or capillary operation modes [44-46].

Gel electrophoresis is used routinely to identify the presence of the various forms of a given plasmid [47], with the sc form almost always having the fastest migration rate. Gel electrophoresis of DNA plasmids of modest size (<50 kbp) is performed with agarose concentrations of 0.5-1%, while larger DNA plasmids have been separated by pulsed gel electrophoresis [48]. However conventional agarose gel electrophoresis is not sufficiently reproducible or accurate for quantitative purposes and is time consuming. Furthermore, for purity analysis the method is only generally accepted for a qualitative assessment of RNA contamination [49]. Although many researchers have quantified DNA plasmids by the imaging of stained gels [47], more sensitive, linear and rapid quantification techniques continue to be pursued [45].

Capillary electrophoresis on the other hand combines a high degree of resolution with sensitivity, reproducibility and a fast analysis time. This technique is becoming more and more used to quantify pDNA and its topoisomers (sc and oc) [44-46]. Unfortunately, the superior performance of capillary electrophoresis when compared with conventional gel electrophoresis has a high cost associated and its routine use in research laboratories is not likely to occur in the near future [50]. Furthermore capillary electrophoresis does not adequately handle process solutions that contain significant amounts of impurities [50]. Although the electrochemical methods can also provide an alternative, they are also restricted to laboratories equipped with voltammetric and chronopotentiometric analysers [51]. For this reason, various chromatographic methods for determining the concentration of the sc pDNA and assess the purity started to emerge. The method is based on measuring the peak area of sc pDNA, the bio-active plasmid conformation, which is proportional to the concentration of this isoform.

High-performance liquid chromatography (HPLC) is a routine technique in many research and industrial laboratories, which has been used for the separation and quantitation of nucleic acids [52]. HPLC is a fast, reproducible and robust methodology mainly based on anion-exchange [29,49,53-55], hydrophobic interaction [20] or reversed-phase binding modes [55-57]. Some HPLC columns can also selectively separate pDNA isoforms, thus providing a means to assess the heterogeneity of pDNA preparations [20,54,55].

Quantification of the plasmids eluting from the column is typically performed using UV detection at 260 nm. One recent study indicates that not only the concentration (i.e. amount), but also size (effective radius) and molecular mass of pDNA eluting from chromatography columns can be determined using light scattering [58].

With regard to the chromatographic methods, the hydrophobic interaction chromatography (HIC) allied to an HPLC system was already applied to quantify total pDNA in different samples [50] and to separate plasmid isoforms [20].

For the first time, Sousa and Queiroz (2011) have reported the assessment of the sc plasmid isoform using an analytical method based on arginine-affinity chromatography that allows the quantification and the quality control of sc plasmid biopharmaceuticals [23].

Anion-exchange (AEX) chromatography was also used by different authors to quantify or to determine the purity of plasmid samples [19,52]. In earlier experiments with anion exchangers, total pDNA analysis were described, using Poros HQ [59], Poros QE [29], Poros PI [53,60,61] and DMAE-LiChrospher [49]. An HPLC macroporous Nucleogen-DMA-4000 column (Macherey-Nagel) was reported to separate RNA and gDNA from pDNA, which eluted as a peak consisting of 95% of the sc isoform [62]. The separation and quantitation of oc and sc isoforms has also been achieved with DEAE-NPR and DEAE-5PW (Tosoh) columns [54]. The presence of nanograms of oc pDNA could be determined within 30 min [54]. One recent study documents

the validation of an AEX liquid chromatographic method with UV detection for the quantification and purity determination of the sc pDERMATT plasmid isoform (3.8 kbp). However, this method is time-consuming, since the total run time was 35 min [39]. Fast pDNA separations can be obtained by reducing the mass transfer resistance within the pores/channels of super-porous matrices [52]. High-performance monolithic columns [63] have been described as a good option to achieve this purpose. A much faster analysis of pDNA isoforms was obtained with a non-porous, micropellicular DEAE anion exchanger based on highly cross-linked PS-DVB particles [64].

Section V - Anion-exchange chromatography

AEX chromatography remains one of the most used techniques for capture, purification and quantitation of pDNA, both at preparative and analytical scale [18,19], as it offers the advantages of rapid separation, no solvent requirement, possibility to be sanitized with NaOH and a wide selection of process-grade stationary phases [65].

AEX chromatography takes advantage of the interaction between negatively charged phosphate groups in the DNA backbone and positively charged ligands on the stationary phase [29]. After binding, a salt gradient is used to displace the different nucleic acids that should elute in order of increasing charge density, a property, which in turn is a function of chain length and conformation [14]. The shape and size of the molecules clearly plays an important role in the separation of plasmid variants [52,66]. In this way, the more compact sc forms, which have higher charge density, elute later than the oc forms which have lower charge density [62,67,68]. The stronger binding of the more compact sc pDNA can be overall attributed to its higher charge density in comparison with the other isoforms [62,67,68] or to a better fit and interaction with ligands within the stationary phase pore curvatures [62]. Furthermore, the base sequence and composition also may affect the elution pattern of nucleic acids in anion exchangers [66].

When using AEX to separate pDNA, the clarified lysate feed should always be loaded at a sufficiently high salt concentration (typically >0.5 M NaCl) to avoid an unnecessary adsorption of low charge density impurities, such as low M_r RNA, oligonucleotides and proteins. Under these conditions, a significant amount of impurities elute in the flowthrough and capacity can be fully exploited for pDNA adsorption [62,65]. Molecules with a high charge density, such as pDNA isoforms, high M_r RNA and gDNA are retained and subsequently eluted by increasing the ionic strength of the mobile phase. Many AEX stationary phases display poor separation selectivity towards pDNA and impurities (high M_r RNA, pDNA concatamers, gDNA, non-sc pDNA

isoforms, endotoxins) due to their similar binding affinities. In the case of gDNA, separation will be even more difficult to accomplish if alkaline lysis generates fragments with sizes closer to pDNA size [15].

The use of pDNA chromatography has been reviewed by Diogo and co-workers (2005), being described that AEX, HIC and reversed phase ion-pair chromatography are the techniques commonly used for pDNA quantitation [19]. However AEX-HPLC is still most widely used [19,52]. Several groups have successfully separated the pDNA forms using AEX-HPLC [49,54,66,69,70].

Several authors have reported the use of weak anion-exchange ligands such as DEAE coupled to silica [62] or polymer-based matrices [68], and the use of strong ligands such as quaternary amines (Q) coupled to polymeric matrices [67] in analytical and/or preparative chromatography of plasmids. However none of these groups have validated monolithic columns for assessment of sc pDNA isoform.

Section VI - Monolithic columns

A typical monolith (BIA Separations) is a continuous bed constituted by a single piece of highly porous material, characterized by a highly interconnected network of channels [63], with large diameter (0.01 - 4 μm), whose pore size depends on the polymerization process [71,72]. In this way, all the mobile phase is forced to flow through the channels via convection [73], resulting in a very fast transfer between mobile and stationary phases [74]. This chromatographic support named as Convective Interaction Media (CIM) was developed with the aim of obtaining a short chromatographic layer, ensuring well-defined, narrow pore-size distributions, excellent separation power and exceptional chemical stability [71]. Thus, these innovative CIM disks present numerous advantages comparing to conventional supports applied on sc pDNA purification.

Monolithic supports exhibit very high binding capacity for pDNA [71,75] and excellent mass transfer properties, because present a huge quantity of accessible binding sites for large biomolecules as pDNA [76], due to their “superpores” which can easily accommodate large pDNA biomolecules. Moreover, these supports allow a fast and high-resolution separation of double stranded DNA molecules, making them attractive for pDNA chromatography [19].

In general, the plasmid purity, recovery and peak resolution are coherently influenced by chromatographic residence time, which consequently can be changed by manipulating buffer flow rate and column length [77]. In accordance, for the optimal purification of larger

biomolecules, the chromatographic column needs to be short, supporting higher flow rates [73,78] in order to reduce backpressure [63], unspecific binding, product degradation and minor changes in the structure of the biomolecule [79], without sacrificing resolution, due to short contact times with the chromatographic matrix [79].

The use of formats more adequate to handle large pDNA molecules, such as monoliths, will likely become more and more important in pDNA chromatography [19]. Monoliths are now being considered the material of choice for the purification and analysis of proteins, pDNA and viruses [76,80,81].

The purification of pDNA from an alkaline lysate without any addition of RNase using weak AEX chromatography on a CIM DEAE monolith (BIA Separations) has been described [63]. The pDNA quality was comparable to the quality of pDNA isolated with a commercial Qiagen kit. An additional advantage was the successful regeneration which allows a consecutive usage of the same CIM® DEAE monolithic column. The characteristic shortness of the monolith resulted in a fast separation (approximately 7 min) [63]. Furthermore, it was also shown that disk monolithic columns can be applied for fast analytical in-process control of the plasmid DNA purity [63].

Recently, it has been reported the application of monolithic innovative technology to purify sc pDNA suitable for pharmaceutical applications, at preparative scale, by exploiting affinity interactions [5,75]. Knowing that the monolithic columns have all these advantages, it will be interesting to validate an analytical methodology with this monolithic innovative technology.

Section VII - Aims

The present work aims to develop a new analytical method, efficient on sc pDNA quantification and purity evaluation, exploring the interaction with an anion exchange monolith. Since the separation and quantification of plasmid isoforms in final pDNA products is important, a method that can rapidly measure sc pDNA in impure solutions is very useful.

A CIM® pDNA analytical column from BIA Separations will be explored. The monolithic support will be tested first with a pre-purified pDNA sample (oc + sc) to verify the separation of both plasmid isoforms (selectivity).

The validation of the analytical method must be performed according to international guidelines [28]. Basic analytical parameters like linearity of the method, lower limits of

detection and quantification, accuracy and precision (intra- and inter-day) will be determined. In fact, the establishment of a new analytical method, able to monitor a biotechnological process in terms of controlling the sc pDNA quality, purity and yield, must guarantee the simplicity, linearity, accuracy and precision of the results.

If the monolithic column is validated for pre-purified sample (oc + sc), it becomes interesting to test this support for the quantification of sc pDNA isoform directly from a clarified *E. coli* lysate. Thus, the sc pDNA amount can be determined for different samples, which are collected from various process steps (in-process control) as well as in the final control.

Chapter II - Materials and Methods

Section I - Materials

Subsection I - Chemicals

The host strain used for obtaining the 6.05-kbp plasmid pVAX1-*LacZ* (Invitrogen, Carlsband, CA, USA) and the several nucleic acids was *E. coli* DH5 α obtained from Invitrogen (Carlsband, CA, USA). Kanamycin was purchased from Sigma (St. Louis, MO, USA). QIAGEN plasmid purification maxi kit was from QIAGEN (Hilden, Germany). Tris(hydroxymethyl)methylamine and isopropanol were purchased from Fisher (Leicestershire, UK). Sodium chloride and sodium hydroxide were obtained from Panreac (Barcelona, Spain). All solutions were freshly prepared using deionized water ultra-pure grade, purified with a Milli-Q system from Millipore (Billerica, MA, USA) and analytical grade reagents. The elution buffers were filtered through a 0.20 μ m pore size membrane (Schleicher Schuell, Dassel, Germany) and degassed ultrasonically. The samples to inject in monolith were filtered through 0.22 μ m pore size filter (Millipore, Bedford, MA, USA). All salts used were of analytical grade.

Subsection II - Instrumentation

All analytical chromatography experiments were performed using ÄKTATM purifier system (GE Healthcare Biosciences, Uppsala, Sweden) controlled by UNICORN software, Version 5.11. This equipment consists of two pumps with various sample loop volumes and a UV detector. All DNA concentration determinations were made spectrophotometrically with Ultrospec 3000 UV/Visible Spectrophotometer (Pharmacia Biotech, Cambridge, England). One unit of OD 260 nm in a 10 mm cuvette is assumed to correspond to 50 μ g/ml dsDNA.

Subsection III - Column

CIM[®] pDNA Analytical Column was kindly provided by BIA Separations (Ljubljana, Slovenia). CIM disk monolithic columns bearing weak anion exchanger (diethylamino) were used. CIM monolithic column consists of a disk-shaped poly(glycidyl methacrylate-co-ethylene dimethacrylate) highly porous polymer matrix with a defined morphology (pore size, and size distribution, porosity and shape). Their small volume (0.32 mL) and short column length (15.0 mm length and 5.2 mm diameter) allows operation at high flow rates (up to 10 column

volumes/min). The disk-shaped matrix is packed into a dedicated stainless steel housing (BIA Separations). Regeneration of CIM disk monolithic column was performed after approximately 50 chromatographic runs by washing it well with water followed by 10 column volumes of 0.5 M NaOH at a flow rate of 0.2 ml/min and finally washing with water.

Section II - Methods

Subsection I - Plasmid and bacterial growth conditions

The analytical method to quantify sc pDNA was developed using the 6.05-kbp plasmid pVAX1-*LacZ*, chosen as model vector. The plasmid amplification was obtained by fermentation of *E. coli* DH5 α after transformation, being obtained a high plasmid copy number enriched in sc isoform. In pre-cultivation, a stride from a selective plate of Luria-Bertani (LB) agar supplemented with 30 μ g/mL, was inoculated into a 250 mL shake flask with 62.5 mL of Terrific Broth (TB) medium (20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH₂PO₄, 0.072 M K₂HPO₄) supplemented with 30 μ g/mL kanamycin. *E. coli* DH5 α harboring pVAX1-*LacZ* was grown in a rotary shaker at 250 rpm under aerobic conditions at 37 °C. Cell growth was evaluated by measuring the optical density of the culture medium at a wavelength of 600 nm (OD₆₀₀). Pre-culture was harvested in the exponential growth phase, i.e., after approximately 3 h of cultivation (OD₆₀₀~2.6). Fresh TB medium was inoculated with the appropriate amount of pre-culture, in order to start all the cultures with an OD₆₀₀ of approximately 0.2. Growth was carried out in 1 L shake flasks containing 250 mL of TB medium, under the same conditions for 14 h (overnight), i.e., the cell growth was suspended at late log phase (OD₆₀₀~9). The culture medium was transferred to centrifuge bottles and the cells were recovered by centrifugation at 5445 x g for 30 min at 4 °C. The supernatants were discarded and the bacterial pellets were stored at -20 °C. *E. coli* DH5 α cells without plasmid were also grown under the same conditions as described before, but with no antibiotic present.

Subsection II - Alkaline cell lysis and pre-purification of pVAX1-*LacZ*

In order to obtain plasmid samples for initial experiments, pDNA was purified using the Qiagen (Hilden, Germany) plasmid maxi kit, according to the manufacturer's instructions. The protocol is based on a modified alkaline lysis procedure, being considered a critical unit operation, because this step can influence the final ratio of sc plasmid. Unfavorable environmental conditions, such as extreme pH and temperature, affect the helical repeat of

DNA promoting its unwinding, being generated other forms such as oc isoform. Bacterial pellets were thawed and resuspended in 20 mL of 50 mM Tris-HCl, 10 mM EDTA, pH 8.0 with 100 µg/mL RNase. Alkaline lysis was performed by adding very carefully 20 mL of a 200 mM NaOH, 1% (w/v) SDS solution. After 5 min of incubation at room temperature, cellular debris, gDNA and proteins were precipitated by gently adding 20 mL of prechilled 3 M potassium acetate solution, pH 5.5 followed by incubation on ice for 20 min. The precipitate was removed by centrifugation at $20\,000 \times g$ for 30 min at 4 °C, using a Sigma 3-18 K centrifuge. A second centrifugation step was carried out for 15 min under the same conditions to separate the remaining suspended material. Following lysis, binding of pDNA to the Qiagen anion exchange resin is promoted under appropriate low-salt and pH conditions (750 mM NaCl, 50 mM MOPS, 15% isopropanol (v/v), 0.15% Triton® X-100 (v/v), pH 7.0). Impurities are removed by a medium-salt wash (1.0 M NaCl, 50 mM MOPS, 15% isopropanol (v/v), pH 7.0), and pDNA is eluted in a high-salt buffer (1.25 M NaCl, 50 mM Tris-HCl, 15% isopropanol (v/v), pH 8.5). Plasmid DNA is then concentrated through an isopropanol precipitation, by adding 0.7 volumes of ice-cold isopropanol, followed by centrifugation at $15\,000 \times g$ for 30 min at 4 °C, using a Sigma 3-18 K centrifuge. Pellet was air-dried for 5-10 min at room temperature and redissolved in a suitable volume of buffer with adjusted ionic strength (approximately 3 mL of 10 mM Tris-HCl, 700 mM NaCl, pH 8.0). Finally, 260 and 280 nm absorbance of the samples was determined using spectrophotometer to assess pDNA quantity and purity. In general, the pDNA obtained at the end of these recovery and isolation steps contained around 90% of sc isoform, while the remaining 10% corresponded to the presence of oc isoform, as revealed by agarose electrophoresis. The pDNA samples were stored at -80 °C until analytical chromatographic studies.

Subsection III - Isolation of plasmid DNA isoforms

1. Supercoiled isoform

In order to improve the quantity of sc isoform obtained in the plasmid sample, the fermentation was suspended at exponential phase ($OD_{600} \sim 5$), reducing the culture time to 5 h. The sc isoform was purified after alkaline lysis using the Qiagen (Hilden, Germany) plasmid maxi kit, according to the manufacturer's instructions described above.

2. Open circular isoform

Open circular pDNA isoform was prepared by incubating a sc pDNA sample without NaCl at room temperature (20 - 25 °C). The sample was monitored over the time by electrophoresis analysis until the total conversion of sc plasmid to oc isoform was observed (about 3 days). Samples were stored at -80 °C until analysis. For the chromatographic analysis the ionic strength of the oc pDNA sample was adjusted, dissolving the required NaCl quantity to obtain a final concentration of 700 mM.

3. Linear isoform

The linear pVAX1-*LacZ* sample was prepared by enzymatic digestion with Hind III (Takara Bio, Shiga, Japan), a single-cutting restriction enzyme. The plasmid samples, without NaCl, were incubated at 37 °C for 1 h, according to the protocol of the manufacturer. Successful linearization of the plasmid was confirmed by agarose gel electrophoresis, being obtained only one band at 6050 bp. Linear plasmid was stored at -80 °C until analysis. For the chromatographic analysis the ionic strength of the ln pDNA sample was adjusted, dissolving the required NaCl quantity to obtain a final concentration of 700 mM.

Subsection IV - Isolation of host nucleic acids

Host nucleic acids (gDNA and RNA) were isolated from pDNA-free DH5α *E. coli* cells.

1. gDNA

Genomic DNA was isolated using the Wizard genomic DNA purification kit from Promega (Madison, WI, USA), according to the manufacturer's instructions. Briefly, bacterial pellets (65 mL) were resuspended in 25 mL of nuclei lysis solution and incubated at 80 °C for 5 min to lyse the cells. After cooling the solution to room temperature, 250 µL of 1 mg/mL RNase solution were added to the cell lysate, followed by incubation at 37 °C for 45 min. Then, 5 mL of protein precipitation solution were added to the RNase-treated cell lysate. The pellet was removed by centrifugation at 16 000 x g for 10 min. After centrifugation, 12.5 mL of room temperature isopropanol were added to the supernatant and the solution was mixed by inversion until the thread-like strands of DNA form a visible mass. The solution was centrifuged at 16 000 x g for 6 min and the supernatant discarded. The pellet was air-dried for 5-10 min and 12.5 mL of 70% ethanol were added to wash the DNA pellet. The solution was centrifuged at 16 000 x g for 6 min and, after discarded the supernatant, the pellet was air-dried for 5-10 min. Finally, 2.5 mL of DNA rehydration solution were added, followed by incubation at 65 °C for 1 h. The gDNA samples were stored at -20 °C until used in chromatographic studies.

2. RNA

For RNA isolation, the cells were lysed by a modification of the alkaline method [43]. Thus, after cell lysis, clarification was performed by adding 1 volume of 5 M solution of a chaotropic salt (ammonium acetate). After 30 min-incubation at room temperature the mixture was centrifuged at 21 000 x g for 40 min at 4 °C. Pellets were discarded and nucleic acids precipitated and concentrated by adding 1 volume of a solution of 15% poly(ethylene glycol) (PEG) 6000, 1.6 M NaCl. After 30 min on ice and centrifugation at 21 000 x g for 40 min, precipitates were resuspended in 3 mL of 700 mM NaCl solution in 10 mM Tris-HCl pH 8.0 [34].

Subsection V - Preparation of process samples

The evaluation of the applicability of the analytical method to quantify the sc pDNA isoform present in more complex samples required the preparation of *E. coli* lysates. Thus, *E. coli* cells were lysed using a modification of the alkaline method [43], as described by Diogo and co-workers (2000) [82]. Bacterial pellets (obtained from centrifugation of 250 mL cell broth at $5445 \times g$ for 30 min, 4 °C) were thawed and resuspended in 20 mL of 50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA, pH 8.0. Alkaline lysis was performed by gently adding 20 mL of a 200 mM NaOH, 1% (w/v) SDS solution. After 5 min of incubation at room temperature, cellular debris, gDNA and proteins were precipitated with 16 mL of prechilled 3 M potassium acetate, pH 5.0 and incubating on ice for 20 min. The precipitate was removed by centrifuging twice at $20\,000 \times g$ for 30 min at 4 °C with a Sigma 3-18 K centrifuge. Samples of this supernatant were then kept and stored at -80 °C for further analysis. The concentration of the lysate was performed according to a previously published method [82]. The pDNA present in the supernatant was precipitated by adding 0.7 volumes of ice-cold isopropanol and incubating on ice for 30 min. The pDNA was recovered by centrifugation at $16\,000 \times g$ for 30 min at 4 °C. After discarding the supernatant, the pellet was air-dried for 5-10 min and then redissolved in 6 mL of 10 mM Tris-HCl buffer, pH 8.0. Samples of this concentrated lysate were then kept and stored at -80 °C for further analysis. The same procedure was used to perform the lysis of a pDNA-free cell culture. Samples were adequately diluted prior to analysis.

Subsection VI - Analytical chromatography

All experiments were performed using an ÄKTA Purifier system (GE Healthcare Biosciences, Uppsala, Sweden) with the Unicorn control system Version 5.11. Briefly, the system was prepared with a 500 mM NaCl in 10 mM Tris-HCl buffer pH 8.0 in the pump A (mobile phase A), and 1 M NaCl in 10 mM Tris-HCl buffer pH 8.0 in the pump B (mobile phase B). The monolithic column was equilibrated with 52% of buffer B. Afterward, 20 µL of a plasmid sample suitably diluted in the equilibration buffer with the adjusted ionic strength were injected in the monolith and eluted at a flow rate of 1 mL/min. After elution of unbound species, the ionic strength of the buffer was instantaneously changed to 100% of buffer B to elute bound species. Fractions were pooled according to the chromatograms obtained and used for further electrophoresis analysis after concentration and desalting with Vivaspinn concentrators (Vivascience). Next, the column was re-equilibrated for 9 min with the equilibration buffer in order to prepare the column for the next run. In all separations and throughout the entire chromatographic run the absorbance of the eluate was monitored at 260 nm and the conductivity was continuously recorded, while 250 µL fractions were pooled according to the chromatograms obtained. All experiments were performed at room temperature (about 20 - 25 °C). The experiments developed with the *E. coli* lysate or with the

isolated impurities, namely, oc and ln pDNA isoforms and host nucleic acids (gDNA and RNA), were performed using the same elution gradient described above.

Subsection VII - Agarose gel electrophoresis

The identification of the nucleic acids samples was performed by agarose gel electrophoresis. Briefly, the fractions recovered from each chromatographic experiment, after being efficiently desalinized, were analyzed by horizontal electrophoresis using 15 cm 1% agarose gels (Hoefer, San Francisco, CA, USA) stained with green safe (1 µg/mL) and visualized under UV light in a Vilber Lourmat system (ILC Lda, Lisbon, Portugal). Electrophoresis was carried out in Tris-acetic acid (TAE) buffer (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA, pH 8.0) and run at 100 V for 40 min. Finally, the bands corresponding to nucleic acids were visualized under UV light. Hyper Ladder I (Bioline, London, UK) was used as a DNA molecular weight marker.

Chapter III - Results and Discussion

Section I - Establishment and validation of the analytical procedure for assessment of purity and quantification of supercoiled plasmid

According to the FDA guidelines, the quantification of pDNA and the analysis of its purity must be evaluated using suitable analytical methods [1], and the high performance chromatographic techniques are usually applied to ensure the plasmid quality considering the clinical application. The correct validation of an analytical procedure involves the control of several parameters. Thus, in this study, the specificity, the linearity, the accuracy, the reproducibility, the repeatability, and the lower quantification and detection limits were evaluated.

Subsection I - Specificity

The specificity of an analytical method is described as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present [83], which in this case can be considered as the ability of the method to distinguish between both plasmid isoforms. To achieve the specificity of the method to assess sc pDNA it was necessary to define an appropriate strategy for the optimization of chromatographic conditions allowing the separation of pDNA isoforms. In this way, the two main plasmid isoforms, oc and sc, were first isolated by QIAGEN plasmid maxi kit. With this plasmid sample, some initial screenings were performed in order to achieve optimal resolution between sc and oc pDNA isoforms. These experiments were carried out in order to achieve the most appropriate binding/elution conditions to separate the isoforms within a short time. Thus, linear and stepwise gradients were tested.

Initially, the linear gradient was evaluated by using a buffer A consisting in a Tris-HCl buffer at pH 8.0 and a buffer B containing 1 M NaCl in Tris-HCl buffer. Linear gradient, represented by the dashed line in Figure 3, was carried out in the range between 0 and 100% of buffer B, for 20 min (Figure 3a). After the equilibration of the column, the pDNA sample containing the oc and sc plasmid isoforms was applied to the analytic column. Figure 3a presents the chromatographic profile where it is observed only one peak with a retention time of 16.45 min. Since a unique peak was obtained, it was concluded that there was no separation of isoforms, and all pDNA was eluted at the same time, under these chromatographic conditions.

This result indicated that all biomolecules were retained at low NaCl concentrations and were eluted by increasing the ionic strength.

The next step was to evaluate the result of shortening the concentration range used in the linear gradient, to obtain a mild gradient. In order to choose the concentration range to be used in this second linear gradient strategy, it was considered that in the first assay the 16.45 min-peak corresponded to the elution of pDNA at about 84% of buffer B. Thus, the new gradient was initiated with 70% of buffer B (Figure 3b). In this way, the reduction of the gradient range (70-100%B) also allowed to decrease the length of the gradient to 15 min. Under these conditions, it was also obtained only a single peak and it was not possible to isolate sc isoform.

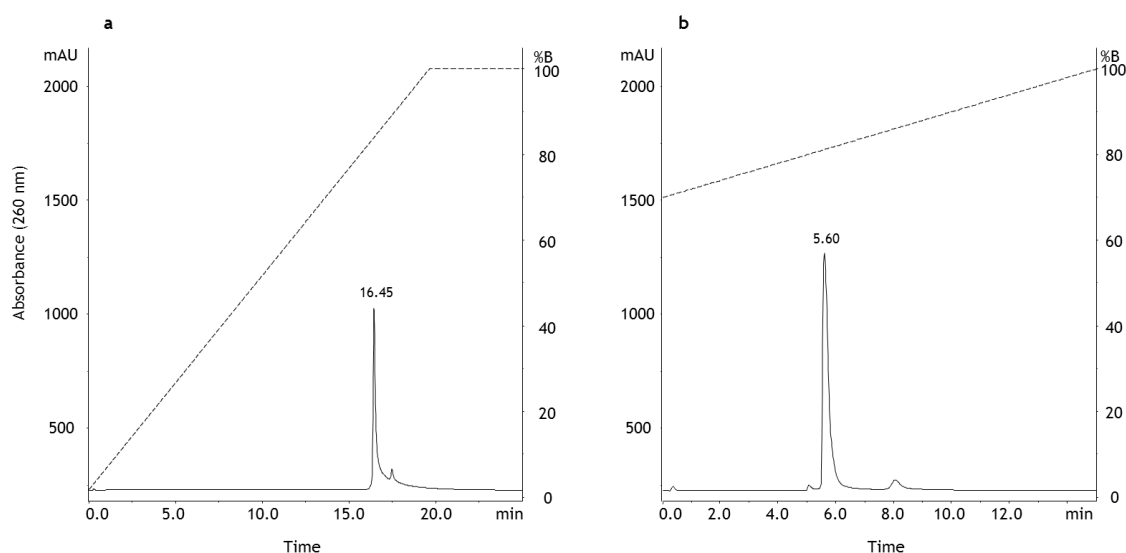


Figure 3 - Chromatographic analysis of native pDNA (oc+sc) using the monolithic column based on anion-exchange chromatography. (a) Chromatographic profile using a 20-min linear gradient in the range 0-100%B. (b) Chromatographic profile using a 15-min linear gradient in the range 70-100%B.

Thereby it was concluded that the NaCl linear gradients tested, led to the total pDNA elution with no separation of plasmid isoforms. So, further experiments were developed in order to find the exact NaCl concentration that allows the elution of each isoform, and a stepwise gradient was explored to facilitate the separation of sc and oc pDNA.

A stepwise gradient was tested and after some adjustments in the concentration of salt to be used for pDNA separation, it was possible to obtain two resolved peaks. The next step was to adjust the composition of the buffers and of the sample, to facilitate the complete separation of both isoforms. The NaCl concentration of buffer A was changed to 500 mM in the same Tris-HCl buffer and the concentration of buffer B was maintained at 1M of NaCl. As there was

an abrupt change in conductivity when injecting the sample, the ionic strength of the sample was adjusted to 700 mM NaCl. Although it could be possible to optimize the linear gradient strategy, the stepwise gradient was finally chosen because it enabled faster separations. In this case, after establishing the concentration of salt that allowed the separation of isoforms, it was possible to develop a rapid assay (Figure 4a). Figure 4b presents an agarose electrophoresis where it is observed in lane 1 the pDNA sample containing the oc and sc plasmid isoforms. This sample was applied to the analytic column and two peaks were obtained when using the stepwise gradient represented by the dashed line (Figure 4a). The elution of the first plasmid component was verified at 0.38 min using a gradient condition corresponding to 52% of buffer B. After 2 min of the chromatographic run, a stepwise gradient was created, by adjusting the elution conditions to 100% of buffer B, and the elution of the second component was observed with a retention time of 2.89 min (Figure 4a).

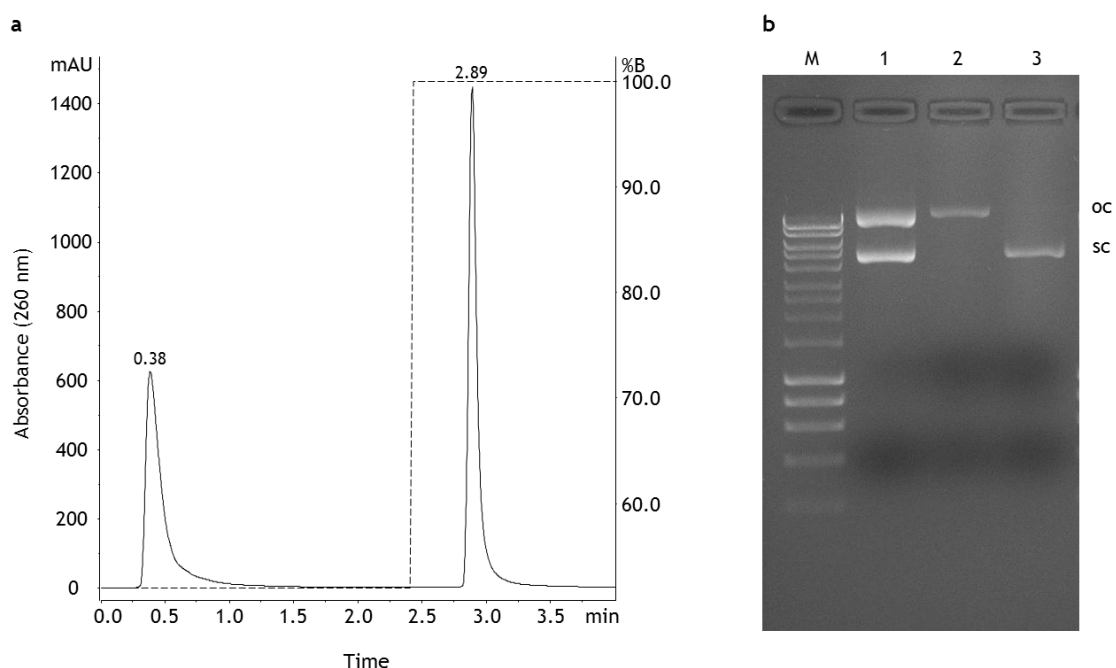


Figure 4 - Analysis of pDNA using the analytical method based on anion-exchange chromatography. (a) Chromatographic profile of a pDNA sample containing the open circular and supercoiled isoforms. (b) Agarose gel electrophoresis analysis of each peak obtained in respective chromatogram. Lane M - molecular weight marker; lane 1 - pDNA sample (oc + sc) injected onto the monolithic column; lane 2 - oc pDNA recovered from the first peak of the chromatogram; lane 3 - sc pDNA recovered from the second peak of the chromatogram.

Figure 4b presents the isolated isoforms, the oc conformation in lane 2 and the sc conformation in lane 3, which were recovered from analytic column in the first and second peaks, respectively. In this way, these results show that an analytical method can be

developed using the AEX monolithic column to specifically analyze sc pDNA. Briefly, a NaCl concentration of approximately 760 mM (52%B) can be used to recover the oc isoform and by increasing the ionic strength to 1 M the bound sc isoform is recovered.

The total run time of the analytical experiment was of 4 min, which represents an advantage because the separation of pDNA isoforms and the elution of sc pDNA occurs faster than the described for other methods, for example in an AEX-based method that requires 35 min to complete the run [39] and an analytical method based on arginine-affinity chromatography that requires 12 min to complete the run [23]. Moreover, comparing this column with another monolithic column described by Branovic and co-workers (2004), both have the same run time, however this method offers the possibility to isolate only the sc isoform [63].

To confirm the identification of the plasmid components eluting in both differentiated peaks, two experiments were also performed by injecting each isolated plasmid isoform into the analytic column, under the same gradient conditions (Figure 5). Thus, the isolated isoforms, oc and sc pDNA conformations were used to compare their retention patterns with the elution profiles and retention times obtained with the sample of total plasmid (oc + sc) represented in Figure 4a.

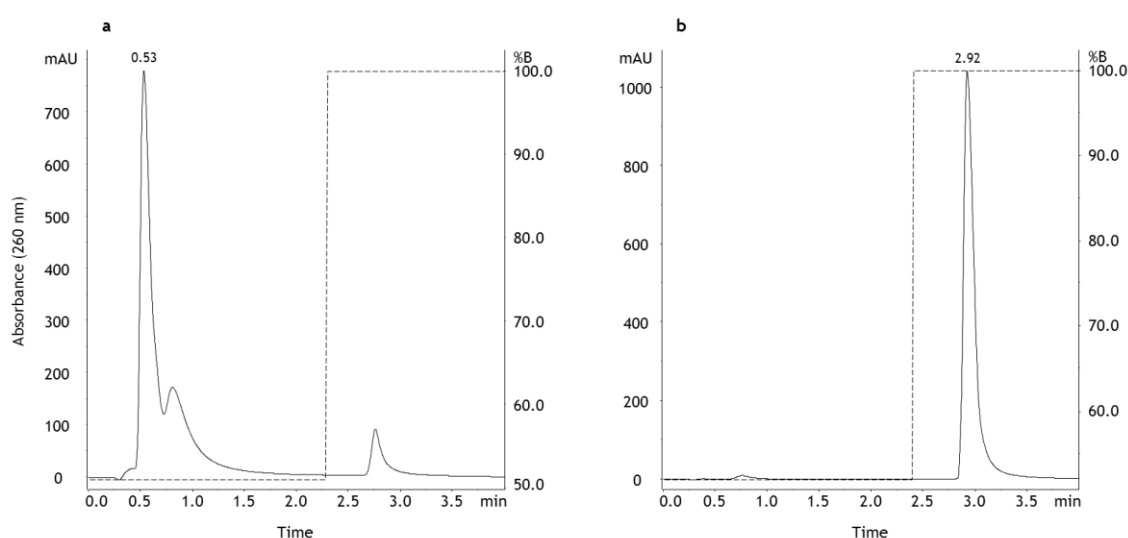


Figure 5 - Analysis of isolated pDNA isoforms using the analytical method based on anion-exchange chromatography. (a) Chromatographic profile of open circular pDNA isoform. (b) Chromatographic profile of supercoiled pDNA isoform.

In Figure 5a it is possible to observe the chromatographic profile obtained by the injection of the oc plasmid conformation. As it is visible, one peak was obtained with a retention time of 0.53 min, being coincident with the elution of the first peak in Figure 4a. Furthermore, the

application of the isolated sc plasmid isoform (Figure 5b) onto the monolithic column resulted in the elution of a single peak with a retention time of 2.92 min, corresponding to the second peak obtained in the chromatographic analysis of total pDNA. Thus, with these experiments it was possible to verify the specificity of the analytical method to efficiently distinguish between both major plasmid conformations.

Once the predominant attractive forces involved in AEX chromatography are electrostatic forces between positively charged ligands on the stationary phase and negatively charged phosphate groups in the pDNA backbone [52], the more compact sc isoform, which has a higher charge density, elute later than the oc isoform. This fact was verified with the elution strategy used, because the first step allowed the immediate elution of the non-retained oc isoform and the second step, with higher ionic strength, was suitable for the total recovery of the sc isoform. In fact, the stronger binding of the more compact sc pDNA to diethylamino ligands (weak anion exchangers) can be due to its higher overall charge density, in comparison to other isoforms, or to a better fit and interaction with ligands within the stationary phase pore curvatures.

In this context, the specificity found for sc pDNA is extremely relevant as it is required for the implementation of a new analytical technology able to ensure the quality and purity criteria of a bioproduct, like pDNA. However, other parameters have to be established to validate an analytical method.

Subsection II - Linearity

The linearity of this method was studied for two different concentration ranges. The first curve was performed with 5 sc pDNA standards in the range of 2 - 20 µg/mL (2, 5, 10, 15 and 20 µg/mL) using three replicates for each sc plasmid concentration. The second curve was performed with 6 sc pDNA standards in the range of 20 - 200 µg/mL (20, 50, 75, 100, 150 and 200 µg/mL) using four replicates for each sc plasmid concentration. The standards were prepared with the sc pDNA obtained from fermentation suspended at exponential phase ($OD_{600} \sim 5$) and purified with a Qiagen commercial kit. Considering that 1 unit of absorbance corresponds to a concentration of 50 µg/mL, the initial absorbance of the sc plasmid sample was measured in UV at 260nm. Each standard was obtained by dilution of the sc plasmid solution with the mobile phase used in the beginning of the chromatographic experiment. The pDNA standard samples were then injected into the analytic column using the procedure described above. The average plasmid peak area was determined and the standard deviation was calculated in order to construct each calibration curve.

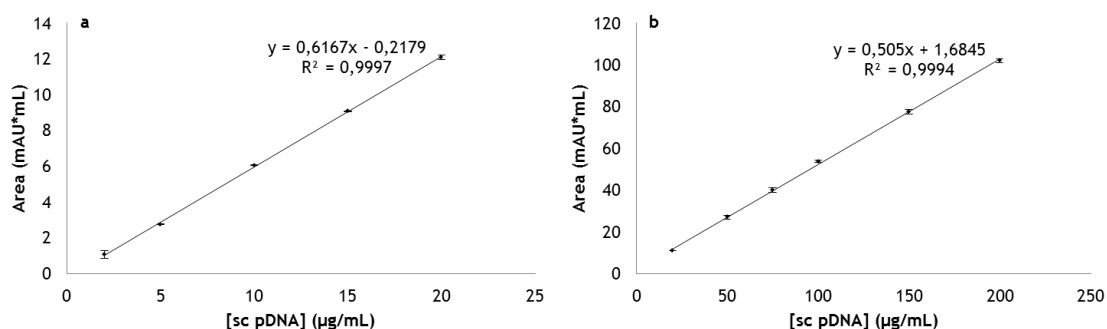


Figure 6 - Calibration curves obtained from the correlation between the supercoiled pDNA peak area and the concentration (a) in the range of 2 - 20 µg/mL (n=3) and (b) in the range of 20 - 200 µg/mL (n=4).

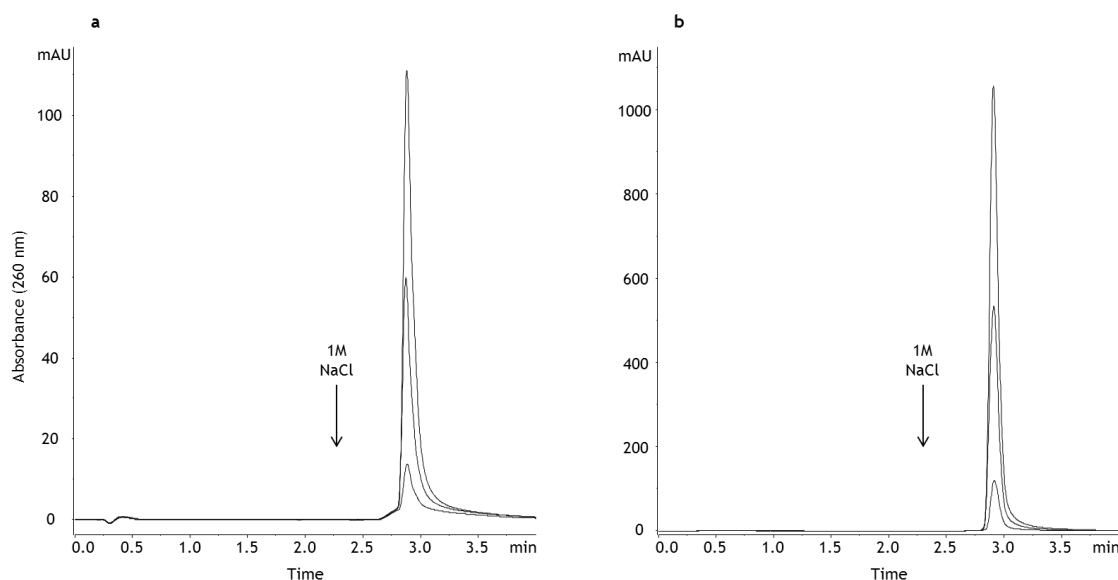


Figure 7 - Chromatographic profiles obtained for three supercoiled pDNA standards in each calibration curve, with the concentration of (a) 2, 10 and 20 µg/mL; and (b) 20, 100, 200 µg/mL.

With this study it was verified that the method is linear within the concentration ranges studied (2 - 20 and 20 - 200 µg/mL). A linear correlation was found for the sc pDNA concentration range considering either the height or area of the sc pDNA peak. In fact, the correlation coefficients for the first curve were of about 0.9984 and 0.9997, respectively and for the second curve were of about 0.9986 and 0.9994, respectively. The correlation found for the calibration curves relating the sc pDNA concentration range with the peak area is presented in Figure 6. In Figure 7 it is possible to observe an example of the chromatographic profiles obtained for three sc pDNA standards of each curve, where it is verified the elution of

the sc pDNA at 2.9 min in all experiments. The standard deviation was found to be lower than 0.22 µg/mL for all standards analyzed in the first curve and lower than 1.3 µg/mL in the second curve, providing a good indication of the method reproducibility.

In this study the linearity was found for the concentration range of 2 - 200 µg/mL. A similar range (2 - 150 µg/mL) was already established in AEX [39] and affinity methods [23]. Other authors have also described alternative methods based on hydrophobic interaction chromatography (HIC) [20,50] and AEX chromatography [82] that were not efficient on plasmid isoforms separation and assessment, and the linearity was only studied for a concentration range between 2.5 and 20 µg/mL [50]. Hence, this new method presents a great advantage over the hydrophobic-based methods because of the possibility to quantify specifically the sc plasmid isoform. The assessment of this conformation is a major concern for the industry focused on the production and purification of pDNA, since a higher than 97% of sc pDNA homogeneity is mandatory for this bioproduct to be applied in gene therapy and DNA vaccination [18]. For this reason, this methodology proved to be simple, selective and linear in a concentration range adequate to measure sc pDNA content and to assess its purity as a pharmacological product [39].

Subsection III - Accuracy, limit of detection and limit of quantification

In relation to the accuracy of the method, it was assessed by back-calculated concentration for all the standards and for an intermediate sc pDNA concentration of each curve (7.5 or 80 µg/mL depending on the curve). Thus, a comparison was made between the nominal concentration of the samples and the sc pDNA concentration obtained from the calibration curve. Furthermore, the coefficient of variation and the mean relative error [(measured concentration–nominal concentration)/nominal concentration×100] were determined to conclude about the accuracy of this method (Tables 2 and 3).

Table 2 - Accuracy for the assessment of sc pDNA in the range of 2 - 20 µg/mL (n=4).

Nominal concentration (µg/mL)	Mean concentration ± SD (µg/mL)	CV (%)	Mean relative error (%)
2	1.93 ± 0.12	6.23	-3.27
5	4.95 ± 0.13	2.69	-1.07
7.5	7.45 ± 0.20	2.74	-0.61
10	9.84 ± 0.14	1.41	-1.63
15	14.76 ± 0.22	1.46	-1.57
20	19.69 ± 0.30	1.52	-1.53

Table 3 - Accuracy for the assessment of sc pDNA in the range of 20 - 200 µg/mL (n=3).

Nominal concentration (µg/mL)	Mean concentration ± SD (µg/mL)	CV (%)	Mean relative error (%)
20	17.43 ± 0.26	1.50	-12.84
50	49.71 ± 0.84	1.69	-0.58
75	75.67 ± 0.43	0.57	0.89
80	80.24 ± 0.89	1.10	0.29
100	102.48 ± 1.37	1.33	2.48
150	153.27 ± 1.75	1.14	2.18
200	201.60 ± 1.58	0.79	0.80

In the first calibration curve (2 - 20 µg/mL) the lower value (2 µg/mL) shows the highest coefficient of variation (about 6 %) and the highest mean relative error (about -3%) (Table 2). In the second calibration curve (20 - 200 µg/mL) the highest coefficient of variation (about 2 %) also corresponds to the lower standards (20 and 50 µg/mL) and the highest mean relative error (about -13%) belongs to concentration of 20 µg/mL (Table 3). With regard to these results, it was possible to conclude that the accuracy is adequate for all the standards of the calibration curves and for a pDNA intermediate concentration of each curve (7.5 and 80 µg/mL), because both parameters (the coefficient of variation and the mean relative error) were within the range of ±15% of the nominal concentration, accepted by the guidelines [84].

The lower limit of quantification (LLOQ) was defined as the lowest concentration of sc pDNA that could be assessed with suitable precision (a coefficient of variation lower than 20%) and adequate accuracy, i.e. within ±20% of the nominal concentration [84]. The limit was established as 2 µg/mL, which shows a coefficient of variation of 6% that is below the value of 20% accepted by the guidelines [83]. Although some works describe LLOQ of 1 µg/mL for total pDNA using weak anion exchange [82] the limit found for this new analytical technique is in accordance with the limit described by other authors who have developed AEX [39] and analytical affinity methods [23]. On the other hand, higher values can be found, for example for HIC [50] which describes LLOQ of 2.5 µg/mL in quantification of total pDNA.

The limit of detection (LOD) of the method was determined to be 1 µg/ mL. In fact, the study of lower sc pDNA concentrations revealed an increased signal-to-noise that restricts the precise and accurate detection of the sc isoform peak. The determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of pDNA with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected [83]. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit [83], justifying the establishment of the value of 1 µg/ml for the LOD, because the height of the blank was 3.82 mAU and height of the standard of 1 µg/ml was 8.60 mAU (Figure 8). Some

works also describe limits of detection of 1 $\mu\text{g/mL}$ [50]. On the other hand, an AEX method [39] and an analytical affinity technique [23] describe LOD of 2 $\mu\text{g/ ml}$.

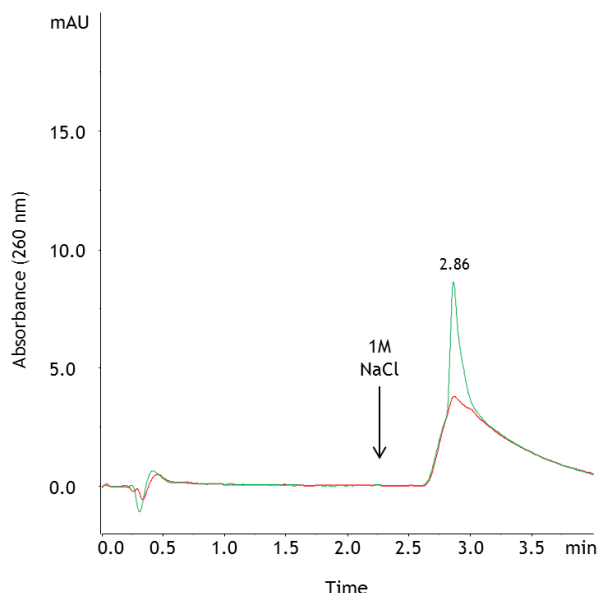


Figure 8 - Chromatographic profile obtained for blank and for sc plasmid standard with the concentration of 1 $\mu\text{g/mL}$.

The blank was performed by injecting a 700 mM NaCl solution in 10 mM Tris-HCl pH 8.0. The chromatographic profile of the blank shows a small peak at 2.86 min. This system peak results from the changes on the sodium chloride concentration in the eluent (Figure 8). This figure also shows the chromatographic profile of the standard of 1 $\mu\text{g/ ml}$. By comparing the height of both chromatograms (Figure 8, red and green lines) is possible to see that the standard of 1 $\mu\text{g/ ml}$ has more than twice the height of the blank.

Subsection IV - Precision

According to the guidelines, the precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the analytical conditions [83,84]. In the validation protocols, precision may be considered at two levels, the repeatability and the reproducibility, that are usually expressed as the variance, standard deviation, mean relative error or coefficient of variation of a series of measurements.

In this work, the precision of the method was evaluated on the basis of repeatability and reproducibility, also termed as intra-day and inter-day precision, respectively. The intra-day repeatability of the method was assessed by performing four consecutive injections of three sc pDNA samples at three different levels of concentration (low, medium and high) under selected conditions. The same samples were also analyzed over a period of four consecutive days to determine the inter-day precision. The data presented in Tables 4 and 5 show the coefficient of variation and mean relative error for the three sc pDNA concentrations of each curve (2, 7.5 and 20; and 20, 80 and 150 µg/mL, respectively).

Table 4 - Intra-day and inter-day precision for the assessment of sc pDNA in the range of 2 - 20 µg/mL (n=4).

Intra-day precision				Inter-day precision			
Nominal concentration (µg/mL)	Mean concentration ± SD (µg/mL)	CV (%)	Mean relative error (%)	Nominal concentration (µg/mL)	Mean concentration ± SD (µg/mL)	CV (%)	Mean relative error (%)
2	1.93 ± 0.12	6.23	-3.27	2	2.08 ± 0.17	8.12	3.75
7.5	7.45 ± 0.20	2.74	-0.61	7.5	7.18 ± 0.21	2.92	-4.29
20	19.69 ± 0.30	1.52	-1.53	20	19.28 ± 0.33	1.73	-3.61

Table 5 - Intra-day and inter-day precision for the assessment of sc pDNA in the range of 20 - 200 µg/mL (n=4).

Intra-day precision				Inter-day precision			
Nominal concentration (µg/mL)	Mean concentration ± SD (µg/mL)	CV (%)	Mean relative error (%)	Nominal concentration (µg/mL)	Mean concentration ± SD (µg/mL)	CV (%)	Mean relative error (%)
20	18.77 ± 0.35	1.87	-6.14	20	18.48 ± 1.43	7.72	-7.61
80	83.13 ± 2.48	2.99	3.91	80	83.77 ± 3.94	4.71	4.71
150	153.47 ± 4.22	2.75	2.31	150	155.74 ± 7.82	5.02	3.83

Table 4 shows the values of intra-day and inter-day precision for the first curve. The coefficients of variation for the intra-day precision were lower than 6.23% and for the inter-day precision a maximum value of 8.12% was obtained. The mean relative error for the intra-day experiments was between - 3.27 and - 0.61%, and the inter-day values did not deviate more than 4.29% from the nominal concentration (Table 4). In the second curve the coefficients of variation of intra-day precision were not higher than 3% and for the inter-day precision a value close to 8% was achieved. The mean relative error for the intra-day experiments was between - 6.14 and 3.91%, and the inter-day values deviated up to -7.61% from the nominal concentration (Table 5). With these results, it was possible to conclude that

the method is precise. In addition, these results also confirm the accuracy of this new analytical method.

Section II - Assessment of sc pDNA in process solutions

After the validation of the method to isolate and quantify sc pDNA present in samples containing sc and oc pDNA, it was also explored the possibility of applying this analytical method for assessment of complex samples recovered from different bioprocess steps. First, the elution profile of each impurity was investigated by injecting isolated oc pDNA, ln pDNA, RNA or gDNA, onto the column. After this, some complex samples recovered from different bioprocess steps were also analyzed and finally, the analytical method was validated for impure plasmid solutions.

Subsection I - Open circular pDNA isoform

In the previous section it was proved that it is possible to separate the oc isoform from the sc (Figure 4). It was also shown that by injecting only oc isoform that is not retained, the elution occurred immediately after injection (Figure 5a), in contrast to the sc isoform which is retained (Figure 5b). In the chromatogram of Figure 5a is possible to verify that the elution profile of the oc isoform results in two non-resolved peaks, with a slight difference from the chromatographic profile presented in Figure 4a. This is probably caused by the appearance of some ln pDNA in the oc pDNA sample. In fact, the room temperature incubation, previously described to induce the conversion of the sc pDNA into oc isoform [75] can also induce the formation of ln pDNA. Another similar technique has been described by Levy and co-workers (2000), in which the solutions were incubated at 60 °C for 48h, to accelerate chemical degradation of the sc isoform to oc and ln isoforms [51]. However there are more appropriate methods, but more expensive to prepare this isoform [39, 20]. Briefly, the oc isoform could be prepared by incubating sc pDNA with the nicking enzyme, which cuts only one strand of double stranded DNA. In this way, it would be controlled the formation of other isoforms that can influence the final chromatographic profile.

Subsection II - Linear pDNA isoform

To verify the elution pattern of the linear form of the plasmid in the chromatogram, this isoform was also injected in the column. The analytical chromatogram presented in Figure 9a shows the first peak of non-retained species at 0.32 min.

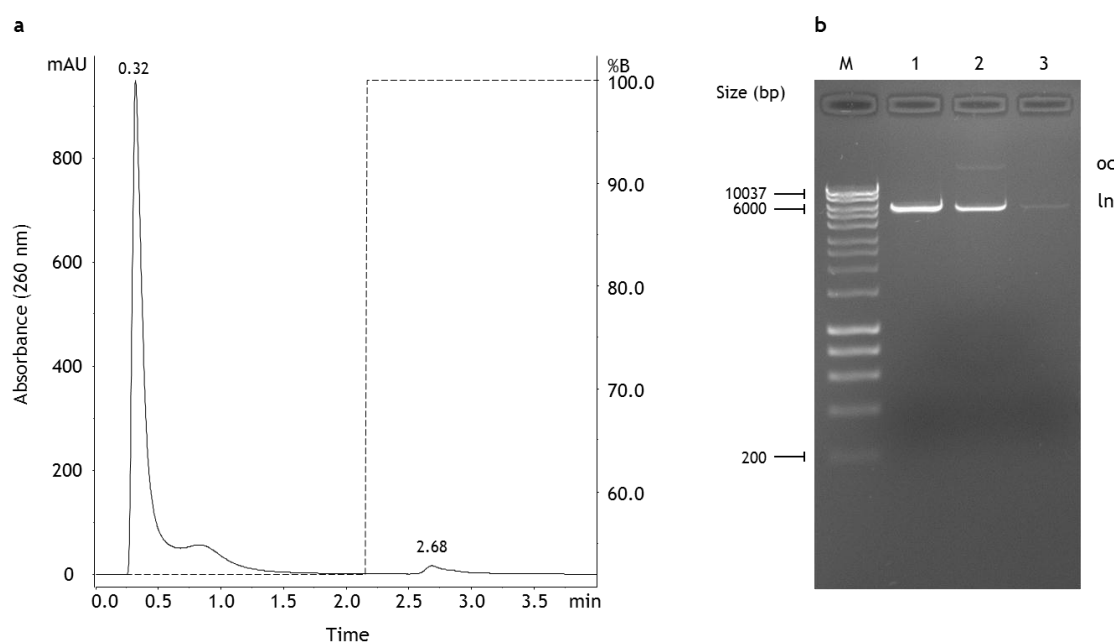


Figure 9 - Analysis of linear pDNA isoform using the analytical method based on anion-exchange chromatography. (a) Chromatographic profile of ln pDNA sample. (b) Agarose gel electrophoresis analysis of each peak obtained in the chromatogram. Lane M - molecular weight marker; lane 1 - ln pDNA sample injected onto the monolithic column; lane 2 - 0.32 min-peak; lane 3 - 2.68 min-peak.

The analytical chromatogram of Figure 9a shows the first peak of non-retained species at 0.32 min. This peak corresponds to ln isoform, as confirmed by agarose gel electrophoresis (Figure 9b), which shows in lane 2 the presence of one band with the size of approximately 6000 bp, corresponding to the size of the plasmid that has 6050 bp. In addition, the migration of this band coincides with the band of the ln pDNA sample injected into the column, represented in lane 1. Nevertheless, the lane 2 also shows a slight band above the previous one, which may correspond to some oc isoform present in the sample. In the 2.68 min-peak there is still present trace amounts of ln pDNA, probably because of the high ln pDNA concentration. In fact, the concentration of ln pDNA in samples to be analyzed is commonly low, so it is supposed that this will not influence the assessment of sc pDNA.

Subsection III - RNA

The chromatographic profile of RNA, under the same elution conditions, shows a single peak eluting at 0.34 min (Figure 10a). This peak was recovered and analyzed by agarose gel electrophoresis (Figure 10b, lane 2), being confirmed that RNA was not retained on the column.

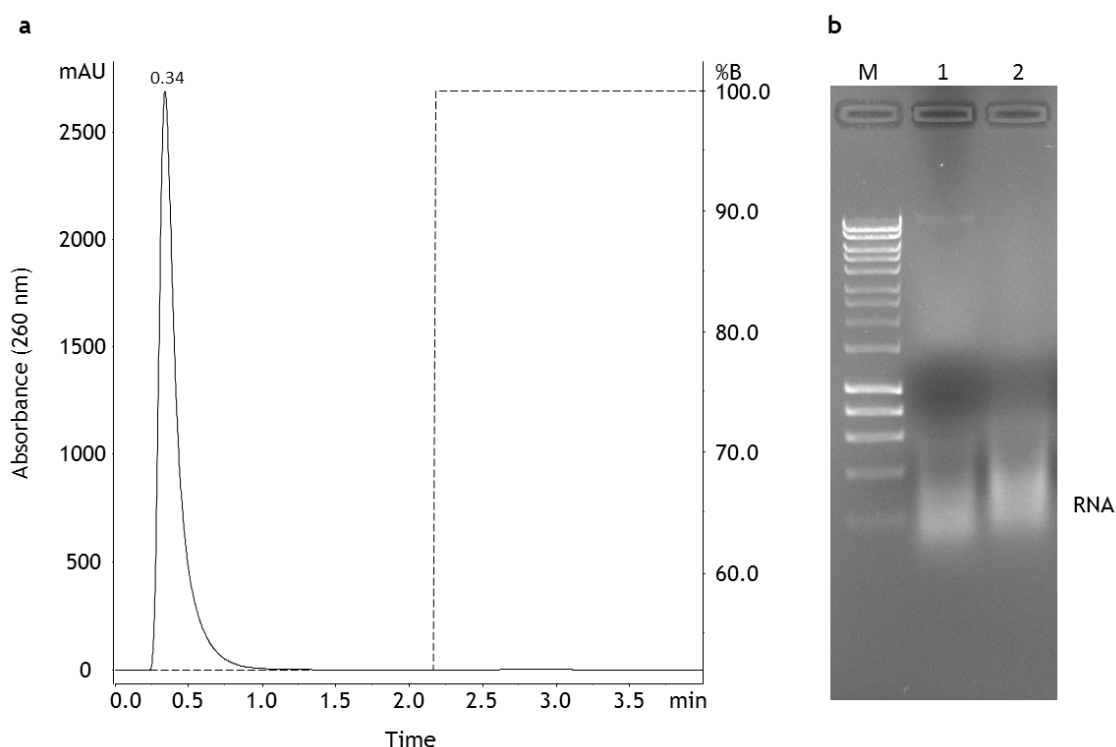


Figure 10 - Analysis of RNA using the analytical method based on anion-exchange chromatography. (a) Chromatographic profile of the RNA sample. (b) Agarose gel electrophoresis analysis of each peak obtained in the chromatogram. Lane M - molecular weight marker; lane 1 - RNA sample injected onto the monolithic column; lane 2 - 0.34 min-peak.

Furthermore, this result indicates the possibility of using this column to assess sc pDNA in samples prepared without any RNase addition. Actually, even using samples with high RNA concentration it was not verified any interference with the elution of sc pDNA.

Subsection IV - Genomic DNA

The chromatographic profile of the gDNA shows a main peak at 0.39 min (Figure 11a). This peak was recovered and injected on lane 2 of agarose gel electrophoresis (Figure 11b), but it was not possible to detect the nucleic acid in the electrophoresis.

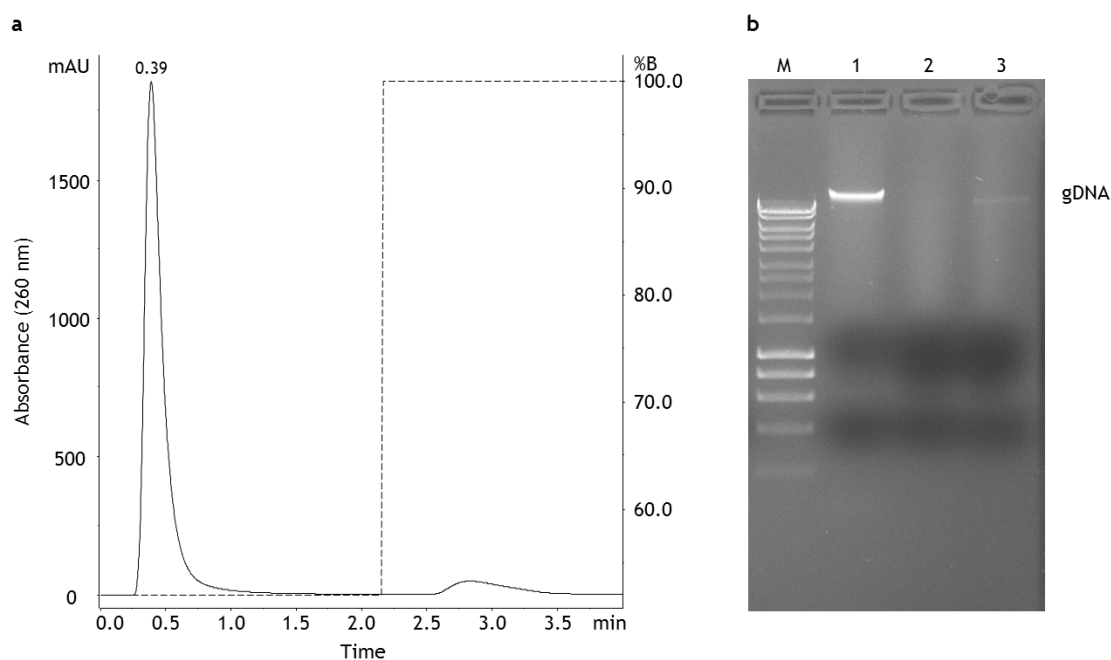


Figure 11 - Analysis of genomic DNA using the analytical method based on anion-exchange chromatography. (a) Chromatographic profile of a gDNA sample. (b) Agarose gel electrophoresis analysis of each peak obtained in the chromatogram. Lane M - molecular weight marker; lane 1 - gDNA sample injected onto the monolithic column; lane 2 - first chromatographic peak; lane 3 - second chromatographic peak.

Regarding the analysis of gDNA, it should be considered that during the manipulations of gDNA, care should be taken to avoid shear-induced cleavage of this molecule, which may generate small gDNA fragments with sizes closer to pDNA size. In this case, separation and detection could be even more difficult to accomplish. The preparation of the gDNA samples requires the use of alkaline lysis which can induce the product degradation. The fact that the species eluting in the first peak are not visible in the electrophoresis may be attributed to this degradation. However, to confirm this it could be used a PCR technique. In this way it would be quantified the gDNA present in the collected fraction corresponding to this large peak, confirming the elution of non-retained biomolecule.

In the second peak there is still present trace amounts of gDNA, as confirmed by agarose gel electrophoresis (Figure 11b), because the lane 3 shows one faint band that coincides with the band present in lane 1. However, if it is injected a sample with lower gDNA concentration this band is not visible, what can be advantageous when analyzing more complex mixtures of clarified lysates.

Subsection V - Clarified lysate

Since all isolated impurities eluted in the first gradient step defined for the separation of plasmid isoforms, and it was not verified a significant interference with the elution of sc pDNA, it was possible to test the application of the method to analyze a complex sample, such as an *E. coli* lysate, in the same elution conditions. Thus, it was first confirmed if the complex sample would present the same retention and elution behaviour that each isolated impurity.

Briefly, lysate samples of *E. coli* cells transformed and non-transformed with pDNA were recovered before and after isopropanol precipitation. So, samples collected throughout this clarification process were analyzed by the analytical method (Figures 12, 13, 14 and 15).

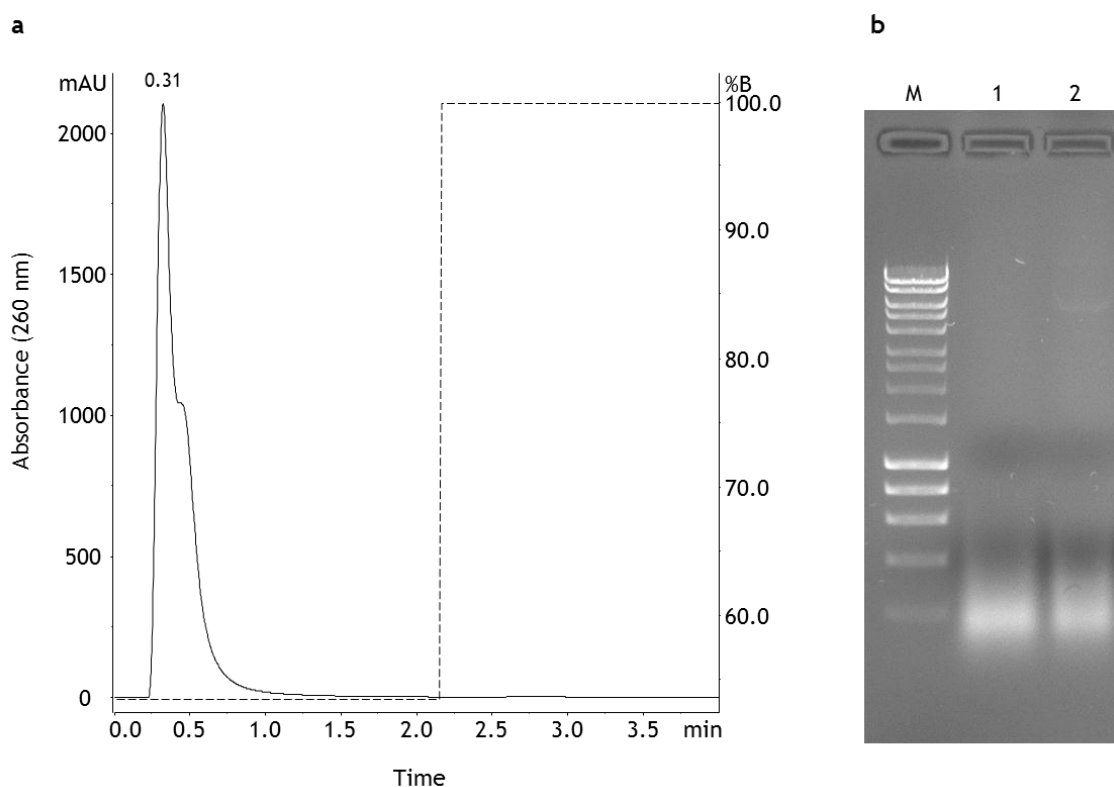


Figure 12 - Analysis of the lysate sample of non-transformed *E. coli* using the analytical method based on anion-exchange chromatography. (a) Chromatographic profile of the lysate sample before isopropanol precipitation (b) Agarose gel electrophoresis analysis of each peak obtained in the chromatogram. Lane M - molecular weight marker; lane 1 - sample injected onto the monolithic column; lane 2 - 0.31 min-peak.

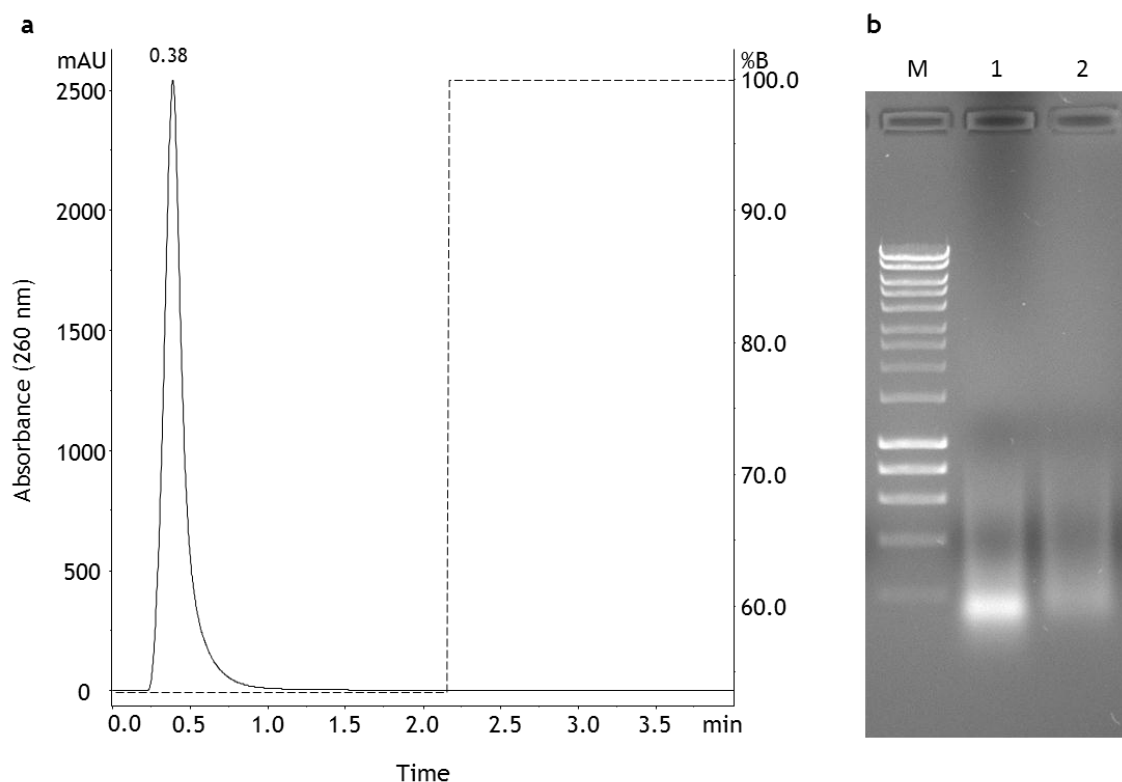


Figure 13 - Analysis of the concentrated lysate sample of non-transformed *E. coli* using the analytical method based on anion-exchange chromatography. (a) Chromatographic profile of the lysate sample after isopropanol precipitation (b) Agarose gel electrophoresis analysis of each peak obtained in the chromatogram. Lane M - molecular weight marker; lane 1 - sample injected onto the monolithic column; lane 2 - 0.38 min-peak.

The analytical chromatograms from Figures 12a and 13a show the peak of non-retained species at 0.31 and 0.38 min, respectively. These peaks correspond only to impurities, since the *E. coli* cells were not transformed with the plasmid. Moreover, the lane 2 of each agarose gel electrophoresis in Figures 12b and 13b revealed that the peak obtained and recovered in each chromatogram (Figures 12a and 13a) corresponds exactly to the sample that was injected onto the column (lane 1 in Figures 12b and 13b).

As the samples of non-transformed *E. coli* cells do not have pDNA, i.e., the isoform sc is not present in this extract, there is no species retained on the column. All this is confirmed since there is no elution in the second step of the gradient.

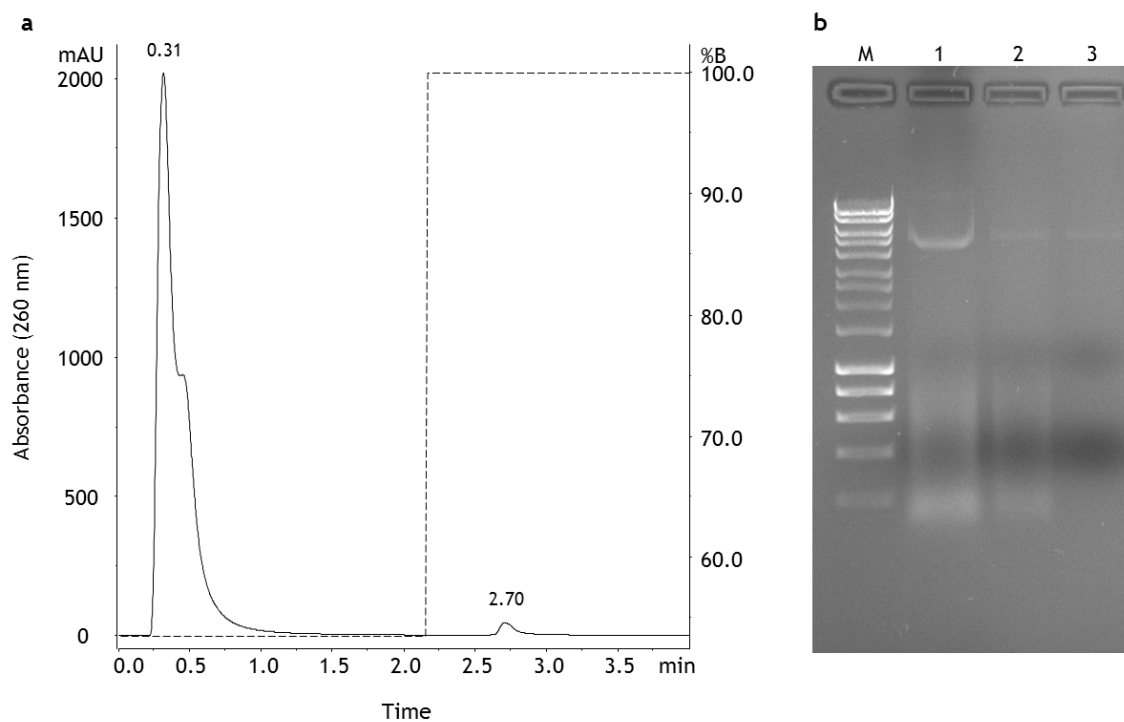


Figure 14 - Analysis of lysate sample of transformed *E. coli* using the analytical method based on anion-exchange chromatography. (a) Chromatographic profile of a lysate sample before isopropanol precipitation (b) Agarose gel electrophoresis analysis of each peak obtained in the chromatogram. Lane M - molecular weight marker; lane 1 - sample injected onto the monolithic column; lane 2 - first peak; lane 3 - second peak.

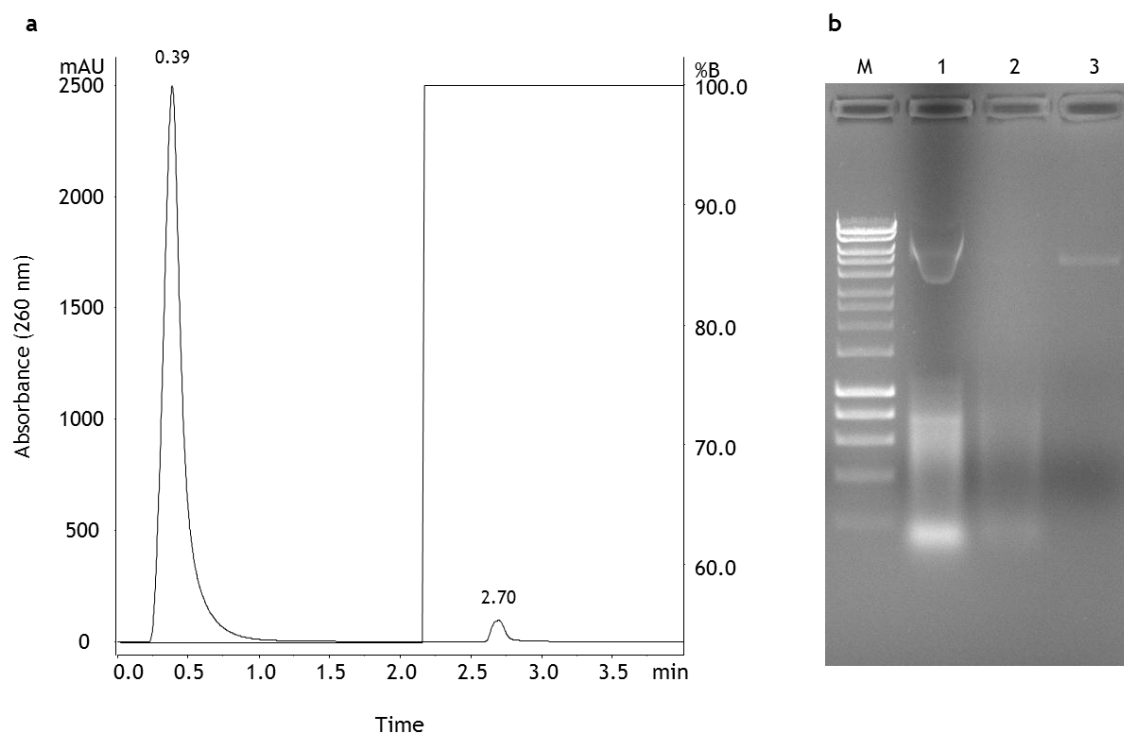


Figure 15 - Analysis of concentrated lysate sample of transformed *E. coli* using the analytical method based on anion-exchange chromatography. (a) Chromatographic profile of a lysate sample after isopropanol precipitation (b) Agarose gel electrophoresis analysis of each peak obtained in the chromatogram. Lane M - molecular weight marker; lane 1 - sample injected onto the monolithic column; lane 2 - first peak; lane 3 - second peak.

The analytical chromatograms from Figures 14a and 15a show the peak of non-retained species at 0.31 and 0.39 min, respectively. As revealed by injection of first recovered fraction of each experiment in agarose gel electrophoresis, these peaks correspond to impurities. Since, in this case, *E. coli* cells were transformed with the plasmid, it is justified the appearance of the second peak in the chromatograms, corresponding to the sc isoform elution, which was retained on the column as revealed by agarose gel electrophoresis (Figures 14b and 15b).

Thus, the monolithic column and the method described were tested for different process samples recovered at different stages of purification process and the elution pattern used was also appropriate to elute all non-bound impurities, without interfering with the sc pDNA elution.

Subsection VI - Clarified lysate enriched with pDNA

Supercoiled pDNA-containing samples collected in the clarification process after isopropanol precipitation (with the chromatographic profile presented in Figure 13a) were further enriched with pDNA (oc+sc) and analyzed by the analytical method (Figure 16). Again, the chromatogram shows the impurities eluting in the flowthrough, followed by the sc plasmid elution, when increasing the ionic strength of the mobile phase.

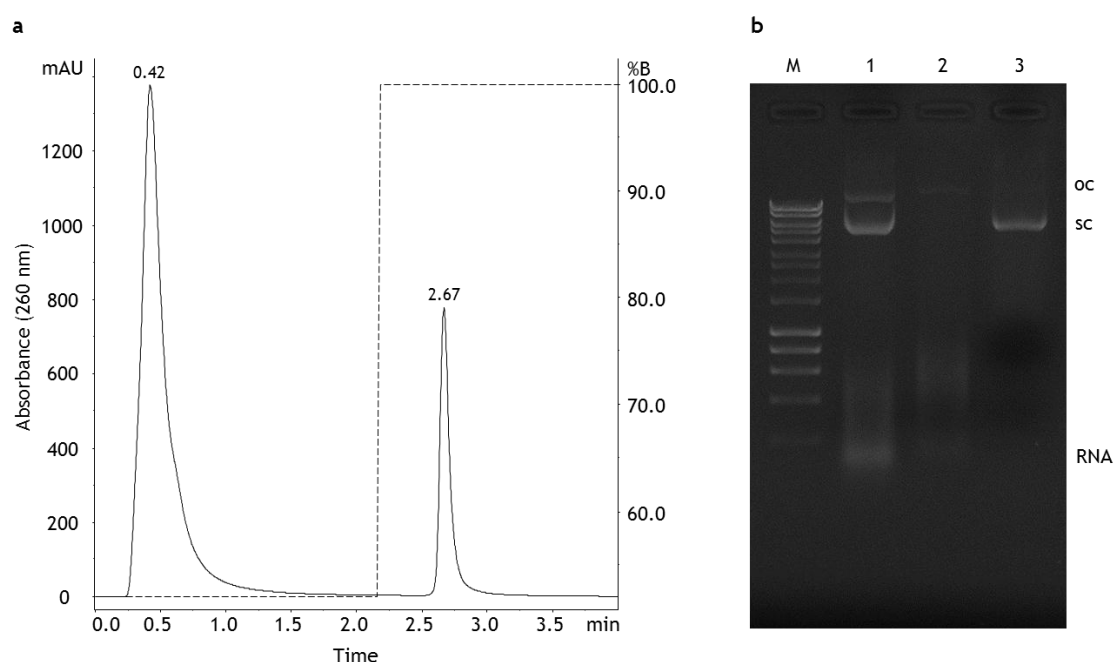


Figure 16 - Analysis of sc pDNA using the analytical method based on anion-exchange chromatography. (a) Chromatographic profile of a clarified lysate enriched with pDNA (oc + sc) (b) Agarose gel electrophoresis analysis of each peak obtained in the chromatogram. Lane M - molecular weight marker; lane 1 - sample injected onto the monolithic column; lane 2 -first peak; lane 3 - second peak.

Briefly, a clarified lysate diluted in 700 mM NaCl in 10 mM Tris-HCl pH 8.0 was injected into the monolithic column equilibrated with a mobile phase with 760 mM NaCl. In the presence of 760 mM of NaCl, the impurities were eluted from the column without being retained, whereas the sc plasmid showed a higher retention due to its higher charge density, since it presents a more compact form which allows a better fit and interaction with ligands within the stationary phase pore curvatures. After 2-min elution with the mobile phase used for equilibration, the eluent was changed to 1 M NaCl in 10 mM Tris-HCl pH 8.0. With the increase of the ionic strength only one resolved peak was eluted (Figure 16a). This peak corresponds to

sc pDNA isoform as proven in agarose electrophoresis (Figure 16b). This profile reflects the characteristic features of sc pDNA mentioned above.

The first peak in the chromatogram of Figure 16 was attributed to the impurities present in the lysate sample. In fact, control studies carried out at 760 mM NaCl using a clarified lysate solution without pDNA (Figures 12 and 13) proved that all impurities eluted with lower retention times (≤ 0.38 min) when compared with the sc pDNA isoform (about 2.7 min). The less charged molecules, such as impurities and other conformations of pDNA, are not retained in the column and elute immediately. On the other hand, the sc pDNA is retained in the column and elute at about 2.7 min due to the increase of the ionic strength of the mobile phase. This means that when using this NaCl gradient the sc plasmid peak is not overlapping with *E. coli* impurities.

Thus, it is possible to conclude that this monolithic column also showed specificity for the sc pDNA isoform, applying an *E. coli* lysate sample. In this way, it may be possible to quantify sc pDNA and to assess its purity in different bioprocess steps.

Subsection VII - Validation of the analytical method for impure plasmid solutions - Process monitoring

In order to validate the method developed for impure solutions, a clarified lysate solution obtained from a cell culture without plasmid supplemented with known amounts of a pure sc plasmid standard (5, 75 and 150 $\mu\text{g/mL}$ sc pDNA) was analyzed in the column (Figure 17). Two replicate analyses were performed for each sample.

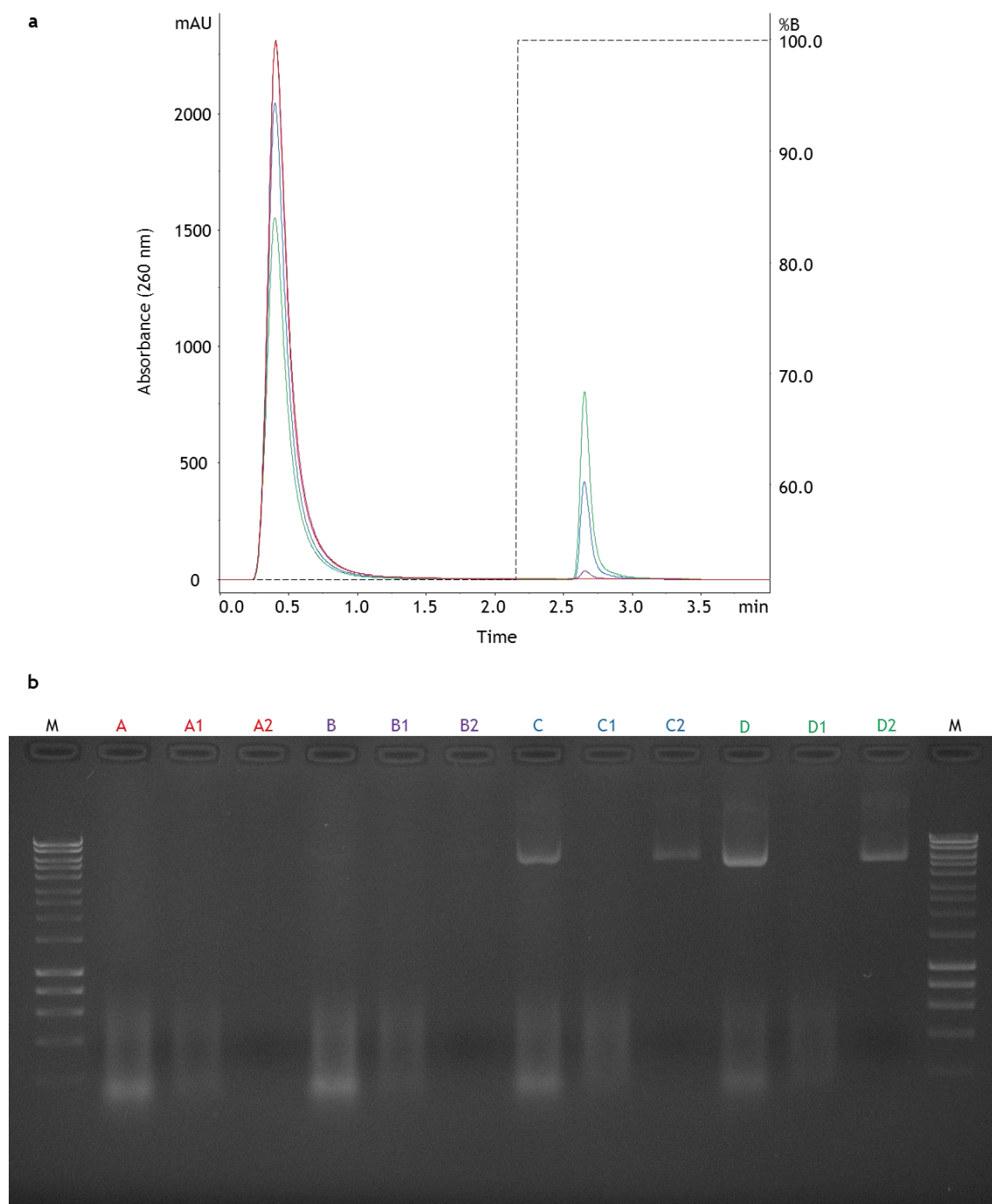


Figure 17 - Analysis of sc pDNA using the analytical method based on anion-exchange chromatography. (a) Chromatographic profiles of a clarified lysate enriched with sc pDNA in different concentrations (0, 5, 75, 150 µg/mL) (b) Agarose gel electrophoresis analysis of each peak obtained in the chromatograms. Lane M - molecular weight marker; lanes A, B, C and D - sample injected onto the monolithic column with a sc pDNA concentration of 0, 5, 75, 150 µg/mL, respectively; lane (A,B,C or D) 1 -first peak; lane (A,B,C or D) 2 - second peak.

Table 6 - Assessment of sc pDNA in lysate sample enriched with sc pDNA in different concentrations (0, 5, 75, 150 µg/mL) (n=2).

Nominal concentration (µg/mL)	Mean concentration \pm SD (µg/mL)	CV (%)	Mean relative error (%)
5	5.21 \pm 0.58	11.13	4.27
75	80.29 \pm 0.69	0.86	7.06
150	154.50 \pm 0.86	0.55	3.00

The sc plasmid concentration in all the samples analyzed was determined using the suitable standard calibration curve (Figure 6). The standard deviation was found to be lower than 0.86 µg/mL for all the samples, confirming the reproducibility of the method. The determination of the lower sc pDNA concentration (5 µg/mL) shows the highest coefficient of variation (about 11 %) and the mean relative errors were lower than 7%. Overall, these results confirm the validity of the monolithic method based on AEX chromatography for the quantification of sc pDNA in impure solutions. The analytical chromatograms also bear information about the purity degree of the samples, which can be estimated as the percentage of the plasmid peak area in the chromatogram.

One of the main advantages emerging from these results is the fact, that a successful separation of sc pDNA from cell RNA without any addition of exogenous RNase is feasible. In general, if the plasmids are used in the manufacture of pharmaceuticals it is desirable to avoid the addition of materials derived from animal, especially bovine sources due to concerns regarding bovine spongiform encephalopathies [85].

In addition, the applicability of the analytical method was tested for the quantification of plasmids prepared with different techniques, i.e., the plasmid samples were purified by AEX chromatography, using a commercial kit and by a modification of alkaline method. The analytical chromatographic profiles obtained in these assays were similar, because the same elution time for the oc and sc pDNA isoforms was achieved. Thus, it was concluded that the technique used to purify the plasmid samples, at preparative level, does not influence the analytical result.

Finally, a regeneration of monoliths was also tested and the results showed the possibility of a consecutive usage of the same disk with a periodic regeneration with 0.5 M NaOH.

Chapter IV - Conclusions

The development of new and optimized methods to quantify pDNA and to assess its purity is extremely important regarding the evolving technology to produce plasmids and the increased number of clinical trials using plasmids as a biopharmaceutical product. The first analytical methods purposed, were not efficient on pDNA isoforms differentiation; however, because of the improved relevance attributed to the sc pDNA isoform, new requirements were described, and the new methods must be able to isolate and specifically determine plasmid isoforms.

In this work, an analytical method based on AEX chromatography to quantify and monitor the sc pDNA quality was implemented and validated. The experiments performed in a monolithic column showed that the technique is specific in sc pDNA assessment. Indeed, the validation of the method was performed according to the guidelines, and it was proved that the method is linear in a suitable concentration range (2-20 and 20-200 µg/mL). Appropriate lower limits of detection and quantification were also obtained. In addition, the precision and accuracy were also achieved for this concentration range. Regarding the reproducibility and repeatability, it has been concluded that the method is precise and accurate.

Moreover, a relevant feature of the method is the ability to handle heavily contaminated samples without the need for any sample pre-treatment such as digestion of high-molecular-mass RNA with RNase. Therefore, it was demonstrated that this monolithic column can be successfully used for the control of purity and for the quantification of sc pDNA isoform in impure process solutions. Replicate analysis of impure samples containing plasmid DNA showed that the method is suitable for this purpose. Overall the analytical experiments showed that the method is robust. Additionally, a consecutive usage of the same CIM[®] DEAE monolithic column was possible due to a successful regeneration procedure. Moreover, the method developed in this work enabled an easy and fast analytical in-process control of the sc pDNA purity, completing the run in 4 min, which is an advantage for application in the quality control of a biotechnological process.

Chapter V - Future Trends

From the conclusions drawn from this thesis also arise some future perspectives to achieve an efficient quality control of a biotechnological process.

The applicability of the analytical method can also be tested for quantification of plasmids clarified and purified with other techniques. Thus, as the precipitation with ammonium sulphate is generally used as a clarification procedure, some samples prepared with this methodology could be assessed. Moreover, plasmid samples purified by hydrophobic interaction chromatography and affinity chromatography, at preparative level, could be analyzed to conclude if the technique used to purify the plasmid samples influences the analytical result. In addition, it could be also interesting to include some analysis of plasmids with different sizes and nucleotides composition, in order to understand the influence of these parameters on the interaction with the analytical AEX matrix. Large and small plasmids should be considered for this study. Furthermore, monitorization of sc pDNA quality could be performed for plasmids produced from other hosts.

On the other hand, some studies could also be developed in order to compare this analytical methodology with other techniques to conclude about their applicability in the monitorization of sc pDNA as a pharmaceutical product.

Chapter VI - Bibliography

- [1] M. Schleef, T. Schmidt, J. Gene Med. 6 (2004) S45.
- [2] A. Brave, K. Ljungberg, B. Wahren, M.A. Liu, Mol. Pharm. 4 (2007) 18.
- [3] R.J. Anderson, J. Schneider, Vaccine 25 (2007) B24.
- [4] M.G. Sebestyen, J.O. Hegge, M.A. Noble, D.L. Lewis, H. Herweijer, J.A. Wolff, Hum. Gene Ther. 18 (2007) 269.
- [5] A. Sousa, C.T. Tomaz, F. Sousa, J.A. Queiroz, J. Chromatogr. A 1218 (2011) 8333.
- [6] H. Tighe, M. Corr, M. Roman, E. Raz, Immunol. Today 19 (1998) 89.
- [7] H.L. Davis, M.L. Michel, R.G. Whalen, Ann. NY Acad. Sci. 772 (1995) 21.
- [8] R.G. Crystal, Nat. Med. 1 (1995) 15.
- [9] A. Mountain, Trends Biotechnol. 18 (2000) 119.
- [10] D. Luo, W.M. Saltzman, Nat. Biotechnol. 18 (2000) 33.
- [11] D.R. Gill, I.A. Pringle, S.C. Hyde, Gene Ther. 16 (2009) 165.
- [12] Y. Seow, M.J. Wood, Mol. Ther. 17 (2009) 767.
- [13] F.D. Ledley, Hum. Gene Ther. 6 (1995) 1129.
- [14] D.M.F. Prazeres, G.A. Monteiro, G.N.M. Ferreira, M.M. Diogo, S.C. Ribeiro, J.M.S. Cabral, in: M.R. El-Gewely, Biotechnology Annual Review, Elsevier, Amsterdam, 2001.
- [15] D.M.F. Prazeres, G.N. Ferreira, G.A. Monteiro, C.L. Cooney, J.M. Cabral, Trends Biotechnol. 17 (1999) 169.
- [16] F. Smrekar, A. Podgornik, M. Ciringer, S. Kontrec, P. Raspor, A. Strancar, M. Peterka, Vaccine 28 (2010) 2039.
- [17] S.D. Patil, D.G. Rhodes, D.J. Burgess, AAPS J. 7 (2005) E61.
- [18] J. Stadler, R. Lemmens, T. Nyhammar, J. Gene Med. 6 (2004) S54.
- [19] M.M. Diogo, J.A. Queiroz, D.M. Prazeres, J. Chromatogr. A 1069 (2005) 3.
- [20] S. Iuliano, J.R. Fisher, M. Chen, W.J. Kelly, J. Chromatogr. A 972 (2002) 77.
- [21] R.R. Sinden, DNA Structure and Function, Academic Press, San Diego, CA, 1994.
- [22] P.H. Oliveira, K.J. Prather, D.M. Prazeres, G.A. Monteiro, Trends Biotechnol. 27 (2009) 503.
- [23] F. Sousa, J.A. Queiroz, J. Chromatogr. A 1218 (2011) 124.
- [24] D.K. Summers, The Biology of Plasmids, Blackwell, Oxford, UK, 1996.
- [25] L. Cupillard, V. Juillard, S. Latour, G. Colombet, N. Cachet, S. Richard, S. Blanchard, L. Fischer, Vaccine 23 (2005) 1910.
- [26] J.Y. Cherng, N.M. Schuurmans-Nieuwenbroek, W. Jiskoot, H. Talsma, N.J. Zuidam, W.E. Hennink, D.J. Crommelin, J. Control. Release 60 (1999) 343.
- [27] G.N. Ferreira, G.A. Monteiro, D.M.F. Prazeres, J.M. Cabral, Trends Biotechnol. 18 (2000) 380.
- [28] F. Sousa, D.M.F. Prazeres, J.A. Queiroz, Trends Biotechnol. 26 (9) (2008) 518.

- [29] D.M.F. Prazeres, T. Schluep, C. Cooney, J. Chromatogr. A 806 (1998) 31.
- [30] A. Sousa, F. Sousa, J.A. Queiroz, J. Chromatogr. B 879 (2011) 3507.
- [31] K.J. Prather, S. Sagar, J. Murphy, M. Chartrain, Enzyme Microb. Technol. 33 (2003) 865.
- [32] H.C. Birnboim, J. Doly, Nucleic Acids Res. 7 (1979) 1513.
- [33] D.S. Holmes, M. Quigley, Anal. Biochem. 114 (1981) 193.
- [34] G.N.M. Ferreira, J.M.S. Cabral, D.M.F. Prazeres, Biotechnol. Prog. 15 (1999) 725.
- [35] M. Marquet, N.A. Horn, J.A. Meek, Bio. Pharm. 8 (1995) 26.
- [36] D.M.F. Prazeres, G.N.M. Ferreira, Chem. Eng. Process 43 (2004) 615.
- [37] H.L. Davis, M. Schleef, P. Moritz, M. Mancini, J. Schorr, R.G. Whalen, Biotechniques 21 (1996) 92.
- [38] M. Przybylowski, S. Bartido, O. Borquez-Ojeda, M. Sadelain, I. Riviere, Vaccine 25 (2007) 5013.
- [39] S.G. Quaak, B. Nuijen, J.B. Haanen, J.H. Beijnen, J. Pharm. Biomed. Anal. 49 (2009) 282.
- [40] M. Marquet, N.A. Horn, J.A. Meek, Bio. Pharm. (1997) 42.
- [41] M. Schleef, in: A. Mountain, U. Ney, D. Schomburg (Eds.), Recombinant Proteins, Monoclonal Antibodies and Therapeutic Genes, Wiley-VCH, Weinheim, 1999.
- [42] M. Marquet, J. Meek, Pharm. Technol. April (1996) 28-44.
- [43] J. Sambrook, E. Fritsch, T. Maniatis, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1989.
- [44] T. Schmidt, K. Friehs, E. Flaschel, J. Biotechnol. 49 (1996) 219.
- [45] T. Schmidt, K. Friehs, M. Schleef, C. Voss, E. Flaschel, Anal. Biochem. 274 (1999) 235.
- [46] T. Schmidt, K. Friehs, M. Schleef, C. Voss, E. Flaschel, PACE Setter 4 (2000) 1.
- [47] M. Levy, J. Ward, P. Dunnill, Bioprocess Eng. 20 (1999) 7.
- [48] B.M. Barton, G.P. Harding, A.J. Zuccarelli, Anal. Biochem. 226 (1995) 235.
- [49] N.A. Horn, J.A. Meek, G. Budahazi, M. Marquet, Hum. Gene Ther. 6 (1995) 565.
- [50] M.M. Diogo, J.A. Queiroz, D.M. Prazeres, J. Chromatogr. A 998 (2003) 109.
- [51] M.S. Levy, P. Lotfian, R. O'Kennedy, M.Y. Lo-Yim, P.A. Shamlou, Nucleic Acids Res. 28 (2000) E57.
- [52] C.G. Huber, J. Chromatogr. A 806 (1998) 3.
- [53] G. Ferreira, J. Cabral, D. Prazeres, Pharm. Pharmacol. Commun. 5 (1999) 57.
- [54] Y. Onishi, Y. Azuma, H. Kizaki, Anal. Biochem. 210 (1993) 63.
- [55] J.A. Thompson, Biochromatography 1 (1986) 68.
- [56] S. Colote, C. Ferraz, J.P. Liautard, Anal. Biochem. 154 (1986) 15.
- [57] A.N. Best, D.P. Allison, G.D. Novelli, Anal. Biochem. 114 (1981) 235.
- [58] D.M. Fishman, G.D. Patterson, Biopolymers 38 (1996) 535.
- [59] G. Ferreira, J. Cabral, D. Prazeres, J. Mol. Recognit. 11 (1998) 250.
- [60] M.M. Diogo, S.C. Ribeiro, J.A. Queiroz, G.A. Monteiro, N. Tordo, P. Perrin, D.M.F. Prazeres, J. Gene Med. 3 (2001) 577.

- [61] M.M. Diogo, S. Ribeiro, J.A. Queiroz, G.A. Monteiro, P. Perrin, N. Tordo, D.M.F. Prazeres, *Biotechnol. Lett.* 22 (2000) 1397.
- [62] M. Colpan, D. Riesner, *J. Chromatogr.* 296 (1984) 339.
- [63] K. Branovic, D. Forcic, J. Ivancic, A. Strancar, M. Barut, T. Kosutic Gulija, R. Zgorelec, R. Mazuran, *J. Chromatogr. B* 801 (2004) 331.
- [64] Y.F. Maa, S.C. Lin, C. Horvath, U.C. Yang, D.M. Crothers, *J. Chromatogr.* 508 (1990) 61.
- [65] A. Eon-Duval, G. Burke, *J. Chromatogr. B* 804 (2004) 327.
- [66] H. Yamakawa, K. Higashino, O. Ohara, *Anal. Biochem.* 240 (1996) 242.
- [67] G. Chandra, P. Patel, T.A. Kost, J.G. Gray, *Anal. Biochem.* 203 (1992) 169.
- [68] R.N. Hines, K.C. O'Connor, G. Vella, W. Warren, *Biotechniques* 12 (1992) 430.
- [69] M.J. Molloy, V.S. Hall, S.I. Bailey, K.J. Griffin, J. Faulkner, M. Uden, *Nucleic Acids Res.* 32 (2004) e129.
- [70] C.R. Smith, R.B. DePrince, J. Dackor, D. Weigl, J. Griffith, M. Persmark, *J. Chromatogr. B* 854 (2007) 121.
- [71] A. Strancar, A. Podgornik, M. Barut, R. Necina, *Adv. Biochem. Eng. Biotechnol.* 76 (2002) 49.
- [72] R. Mallik, D.S. Hage, *J. Sep. Sci.* 29 (2006) 1686.
- [73] A. Jungbauer, R. Hahn, *J. Sep. Sci.* 27 (2004) 767.
- [74] D. Josic, A. Buchacher, A. Jungbauer, *J. Chromatogr. B* 752 (2001) 191.
- [75] A. Sousa, D. Bicho, C.T. Tomaz, F. Sousa, J.A. Queiroz, *J. Chromatogr. A* 1218 (2011) 1701.
- [76] J. Urthaler, R. Schlegl, A. Podgornik, A. Strancar, A. Jungbauer, R. Necina, *J. Chromatogr. A* 1065 (2005) 93.
- [77] C.M. Ongkudon, M.K. Danquah, *J. Chromatogr. B* 878 (2010) 2719.
- [78] M.K. Danquah, G.M. Forde, *J. Chromatogr. A* 1188 (2008) 227.
- [79] V. Frankovic, A. Podgornik, N.L. Krajnc, F. Smrekar, P. Krajnc, A. Strancar, *J. Chromatogr. A* 1207 (2008) 84.
- [80] A. Jungbauer, R. Hahn, *J. Chromatogr. A* 1184 (2008) 62.
- [81] L. Urbas, B. Kosir, M. Peterka, B. Pihlar, A. Strancar, M. Barut, *J. Chromatogr. A* 1218 (2011) 2432.
- [82] M.M. Diogo, J.A. Queiroz, G.A. Monteiro, S.A. Martins, G.N. Ferreira, D.M. Prazeres, *Biotechnol. Bioeng.* 68 (2000) 576.
- [83] ICH Steering Committee, Committee for Proprietary Medicinal Products, CPMP/ICH/381/95, European Agency for the Evaluation of Medicinal Products, 2005.
- [84] Food and Drug Administration: U.S. Department of Health and Human Services, Guidance for Industry, Bioanalytical Method Validation, FDA, 2001.
- [85] A.F. Hill, M. Desbruslais, S. Joiner, K.C. Sidle, I. Gowland, J. Collinge, L.J. Doey, P. Lantos, *Nature* 389 (1997) 448.

