

Validated chromatographic method for mcDNA quantification in complex samples

Ana I. Valente , João Vasco Valente , Pedro L. Ferreira ,
Augusto Q. Pedro , Ana P.M. Tavares , Fani Sousa ,
Mara G. Freire

PII: S0021-9673(26)00878-2
DOI: <https://doi.org/10.1016/j.chroma.2026.467552>
Reference: CHROMA 467552



To appear in: *Journal of Chromatography A*

Received date: 12 April 2026
Revised date: 14 October 2026
Accepted date: 15 October 2026

Please cite this article as: Ana I. Valente , João Vasco Valente , Pedro L. Ferreira ,
Augusto Q. Pedro , Ana P.M. Tavares , Fani Sousa , Mara G. Freire , Validated chromatographic
method for mcDNA quantification in complex samples, *Journal of Chromatography A* (2026), doi:
<https://doi.org/10.1016/j.chroma.2026.467552>

This is a PDF of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability. This version will undergo additional copyediting, typesetting and review before it is published in its final form. As such, this version is no longer the Accepted Manuscript, but it is not yet the definitive Version of Record; we are providing this early version to give early visibility of the article. Please note that Elsevier's sharing policy for the Published Journal Article applies to this version, see: <https://www.elsevier.com/about/policies-and-standards/sharing#4-published-journal-article>. Please also note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2026 The Author(s). Published by Elsevier B.V.

This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>)

Highlights

- Novel validated anion-exchange chromatographic method for mcDNA quantification
- Separation of supercoiled mcDNA from open circular mcDNA isoforms, PP, and RNA
- The method is selective, linear, accurate, precise, and sensitive over the concentration range of 10-100 $\mu\text{g/mL}$

Journal Pre-proof

Validated chromatographic method for mcDNA quantification in complex samples

Ana I. Valente^{a,†}, João Vasco Valente^{a,†}, Pedro L. Ferreira^b, Augusto Q. Pedro^a, Ana P. M. Tavares^a, Fani Sousa^c, Mara G. Freire^{a,*}

^a CICECO-Instituto de Materiais de Aveiro, Departamento de Química, Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal; anaivalente@ua.pt; valente.joao@ua.pt; apedro@ua.pt; aptavares@ua.pt; maragfreire@ua.pt

^b RISE-Health, Departamento de Química, Faculdade de Ciências, Universidade da Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal; pedroflf@live.com.pt

^c RISE-Health, Departamento de Ciências Médicas, Faculdade de Ciências da Saúde, Universidade da Beira Interior, Av. Infante D. Henrique, 6200-506 Covilhã, Portugal; fani.sousa@fcsaude.ubi.pt

*Corresponding author: maragfreire@ua.pt

[†]These authors contributed equally to this work

Abstract

Minicircle DNA (mcDNA) is a non-viral vector known for its superior therapeutic potential and safety, making it a promising biopharmaceutical platform for gene therapy, DNA vaccination, and molecular biology applications. However, its structural similarity to parental plasmid (PP), the presence of isoforms (e.g., open circular mcDNA and supercoiled mcDNA), and host-derived impurities pose significant challenges for accurate detection and quantification, which are key steps for effective quality control. This work reports, for the first time, the development and validation of an anion-exchange chromatographic method for quantifying p53-mcDNA using a customized TSKgel[®] DNA-NPR column. Chromatographic parameters, including pH, elution gradient, gradient time, and flow rate, were optimized. Real samples from complex *Escherichia coli* lysates containing p53-mcDNA, p53-PP, miniplasmid (mP), and RNA were used, demonstrating applicability for process monitoring. The method demonstrated consistent linearity ($R^2 = 0.9994$) over the 10–100 µg/mL range for total DNA, with accuracy meeting FDA guidelines. The method was validated in terms of selectivity, linearity, accuracy, precision, and sensitivity over the concentration range of 10–100 µg/mL, with the lower limit of quantification (LLOQ) and detection (LOD) established at 10 µg/mL and 1 µg/mL, respectively. Intra- and inter-day precision studies confirmed reproducibility, with coefficients of variation below 6%. Overall, the chromatographic method here developed and proposed effectively separates p53-mcDNA from its parental plasmid and may support process monitoring during upstream and downstream operations.

Keywords: Biopharmaceuticals; minicircle DNA; Anion-exchange Chromatography; Analytical Method; Quality control.

1. Introduction

The accurate quantification of minicircle DNA (mcDNA) is crucial in various applications, including gene therapy, DNA vaccination, and molecular biology research [1,2]. mcDNA, a small circular DNA molecule devoid of bacterial sequences, exhibits enhanced safety, higher transfection efficiency, and prolonged expression in host cells compared to plasmid DNA (pDNA). These advantages make it a highly attractive vector for non-viral gene delivery [3,4]. The absence of prokaryotic sequences is associated with the production process based on *in vivo* recombination. However, in addition to mcDNA, other similar DNA species are generated, increasing the complexity of mcDNA sample and posing challenges for its purification, characterization, and dosage control [3–5].

Developing a robust analytical method for mcDNA quantification is essential to accurately assess both product purity and batch-to-batch consistency during the manufacturing process. This is particularly relevant for ensuring compliance with the established impurity criteria for DNA-based biopharmaceuticals, as required by the regulatory agency Food and Drug Administration (FDA) [6]. Despite the increasing therapeutic relevance of mcDNA, robust and specific quantification methods remain limited. Indeed, most available techniques either estimate total DNA concentration, or infer mcDNA levels based on purity ratio, or indirect methods such as parental plasmid (PP) quantification, which can be unreliable in complex mixtures where mcDNA coexists with the PP, miniplasmid (mP), RNA, and genomic DNA (gDNA) [3,7,8].

Chromatography, known for its high-resolution, reproducibility, and quantitative capabilities, has proven effective in separating, analysing, and quantifying nucleic acids. Different techniques, including size-exclusion chromatography (SEC) [9], ion pairing reversed-phase chromatography (IP-RP) [9,10], anion-exchange chromatography (AEX) [11], slalom chromatography [12], affinity chromatography [13], and hydrophilic interaction chromatography (HILIC) [14] have been applied at various stages of oligonucleotide and larger nucleic acid analysis, purification, and quantification. Indeed, HILIC has been revealed for the analysis of large RNA molecules and mRNA-containing lipid nanoparticles [14], whereas IP-RP have been extended beyond short oligonucleotides to the separation and characterization of larger RNA species [9]. In addition, capillary gel electrophoresis is a high-resolution complementary technique that separates nucleic acids based on electrophoretic mobility and molecular size [15]. In contrast, hydrophobic interaction chromatography (HIC) has been used as a tool for plasmid DNA purification. Indeed, this technique has shown to efficiently remove not only proteins, RNA, and endotoxin impurities, but also to separate open circular (oc) pDNA from the desired supercoiled (sc) pDNA isoform [16,17]. However, the direct and specific quantification of mcDNA in complex matrices containing related DNA impurities remains challenging.

In fact, supercoiled pDNA has superior therapeutic activity and is characterized by enhanced *in vivo* delivery, increased transcriptional efficiency, and robust antigen expression. In contrast to oc pDNA, the use of high-purity sc pDNA is essential for achieving a potent and durable immune response and improved protection against infectious diseases [18,19]. However, HIC presents some weaknesses, namely a very high salt load (e.g. 3 M ammonium sulfate), which in turn means extensive salt consumption and, respectively, high conductivity throughout the column [16]. This inevitably reduces the column's operational lifetime and increases the probability of equipment damage if proper maintenance is not provided. Moreover, the need for an additional lengthy desalting step will significantly increase the total operational time of the process. In turn, using slalom chromatography, the separation of plasmid DNA from its linear form is demonstrated by the isolation and quantification of these latter species considered as impurities [12]. Although slalom chromatography provides high resolving performance for both linear and supercoiled/circular/nicked dsDNA, its lower applicability limit is approximately 2-2.5 kbp. Because p53-mcDNA is a flexible circular structure of relatively small size (~3 kbp), it lies close to this lower limit, which may constrain chain extension and relaxation dynamics as well as narrow the effective selectivity window, ultimately leading to the loss of chromatographic resolution and overall performance [12]. Also, Almeida *et al.* [20] employed a cadaverine-modified monolith within a chromatographic method to quantify sc mcDNA. The method exhibited validated linearity, precision, accuracy, and a lower limit of quantification of 1 µg/mL, demonstrating that mcDNA-specific quantification is feasible [20]. Nevertheless, this method's reliance on monolith modification with the ligand cadaverine represents a critical limitation, as this modification can introduce batch-to-batch variability in the material, potentially leading to inconsistencies and affecting analytical reproducibility. In another work, Almeida *et al.*, (2020) [21] developed a size-exclusion chromatography method for sc mcDNA purification using Sephacryl S-1000 SF resin. This allowed the fractionation of 5 kbp mcDNA from the 8.2 kbp PP. While a mcDNA recovery of 66.7% and a purity of 98.1% were achieved, chromatographic selectivity and peak resolution were not optimal. This limits the analysis and underscores the need for new approaches suitable for broader samples in which other isoforms may be present.

In this work, the p53-mcDNA vector, which encodes the tumour suppressor p53 protein, is used to explore anion-exchange chromatography (AEX-HPLC) as a strategy for mcDNA quantification. This technique, well established for nucleic acids separation based on charge differences, is particularly suitable for highly negative molecules like p53-mcDNA, which forms strong interactions with positively charged ligands within the column [22,23]. Its high resolving power and compatibility with aqueous mobile phases make it ideal for quantifying

mcDNA [22]; however, a dedicated application for mcDNA has not yet been fully realized. Successful implementation demands careful optimization of chromatographic parameters, including resin selection, gradient profile, and detection settings, to ensure specificity, sensitivity, and reproducibility [24]. To establish and validate the method under realistic conditions, pre-purified samples containing p53-mcDNA, p53-PP, and mP were used to mimic typical sample complexities, and further, a second more complex lysate, containing RNA was employed to evaluate the method's applicability to highly complex samples. In summary, these data establish the newly developed and optimized AEX-HPLC method for the direct, and reliable quantification of p53-mcDNA, providing a valuable tool for routine quality control and supporting the development of mcDNA therapeutics.

2. Experimental

2.1 Chemicals

The NZYMaxiprep kit (NZYtech, Portugal) was used for plasmid extraction from *Escherichia coli* (*E. coli*). Tris(hydroxymethyl)aminomethane (Tris, PA grade) was obtained from PRONALAB (Portugal). Hydrochloric acid (HCl, 37 wt%) was from Honeywell (USA), ammonium sulfate (99% pure) was sourced from Scharlau (Spain), and isopropanol (HPLC grade) was acquired from Normapur (France). GRS Agarose LE (molecular biology grade) was purchased from Grisp (Portugal). Sodium hydroxide (NaOH, 98% pure pellets) and sodium chloride (NaCl, 99.5% purity) were obtained from Thermo Scientific (USA). Potassium phosphate (KH_2PO_4 , 97% pure) was from Alfa Aesar (USA), and di-potassium hydrogen phosphate (K_2HPO_4 , PA grade) was from Panreac (Spain). Kanamycin disulfate salt (for research use) was purchased from Sigma-Aldrich (USA). Tryptone USP and yeast extract were from Biokar (France), while Agar (for bacteriological use) was from Liofilchem (Italy). Glycerol (analytical reagent) was purchased from Biochem - Frilabo (Mexico), and Luria Bertani (LB) Broth (Lennox) was from Sigma-Aldrich (USA). L-Arabinose (L-Ara, for microbiology) was from Merck Milipore (Germany). Terrific Broth (TB) medium was prepared in the laboratory using 20 g/L tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 0.017 M KH_2PO_4 , and 0.072 M K_2HPO_4 . Milli-Q water used was obtained from a HALIOS 12 ultrapure water system (NEPTec GmbH, Germany).

2.2 *E. coli* culture and p53-mcDNA production

Prior to culturing *E. coli* in liquid medium, an LB-agar plate (25 g/L LB, 15 g/L agar, 50 µg/mL kanamycin) was inoculated with 100 µL of the genetically modified *E. coli* ZYCY10P3S2T strain from an existing cell bank. After being evenly spread across the plate, it was incubated overnight at 37 °C in a Memmert Oven (model UN55). The cell bank was prepared by transferring a colony from the plate to LB medium, and *E. coli*

was grown under agitation using an IKA Orbital Shaker model KS 4000 IC control (Germany). Cell growth was monitored until an optical density (OD) at 600 nm (OD_{600}) of approximately 0.7 was reached, determined by a Shimadzu UV-1800 spectrophotometer (Japan). NaCl 0.8% (w/v) was used for blanking and sample dilution. Subsequently, the resulting cell suspension was prepared in 1 mL aliquots, containing 30% v/v of glycerol as a cryoprotectant, and long-term stored in VWR freezer at -80°C . All the culture procedures were handled in a class II laminar flux chamber BIOAIR SAFEMATE ECO PLUS (Italy).

To produce p53-mcDNA, p53-PP amplification was first carried out as previously described by Ventura and co-workers [25]. *E. coli* strain ZYCY10P3S2T was cultivated in TB medium supplemented with 50 $\mu\text{g/mL}$ kanamycin, and incubated under stirring on IKA Orbital Shaker model KS 4000 IC control (Germany) at 42°C and 250 rpm. After reaching an OD_{600} of 5.0, the *E. coli* cells were harvested by centrifugation ($2000 \times g$, 10 min, 25°C ; Heraeus Megafuge 16R, Thermo Scientific). The resulting pellet was resuspended in LB medium containing 0.01% (w/v) L-Ara (to initiate recombination and produce p53-mcDNA), along with 0.04 M NaOH. Subsequently, induction was performed by incubating the suspensions for 2 h with agitation on an IKA Orbital Shaker (Germany) at 32°C and 250 rpm. Thereafter, bacterial cells were recovered by centrifugation ($2000 \times g$, 10 min, 4°C) and stored at -20°C .

2.3 Preparation of *Escherichia coli* lysates

Three different samples were used throughout this work: pre-purified samples, p53-PP-enriched sample, and the complex lysates. Each sample type was prepared to address a specific stage of method development and validation. Pre-purified samples were primarily used for method optimization and for linearity, accuracy, and precision studies. The p53-PP-enriched samples were employed as chromatographic references to facilitate peak identification during selectivity assessment, and complex lysates were used to evaluate the applicability of the method to process-derived samples containing multiple nucleic acid species and host-cell impurities. Details on the composition of the different samples are provided in Table 1.

The pre-purified samples consisted predominantly of p53-mcDNA, with traces of p53-PP and mP generated during the recombination process. These samples were obtained using the NZYMaxiprep kit (NZYtech, Portugal) following the manufacturer's protocol. Then, isopropanol was added to the supernatant to precipitate the plasmids, which were recovered by centrifugation ($15,000 \times g$, 30 min, 4°C ; MPW-380R, MPW Med. Instruments), and finally resuspended in 10 mM Tris-HCl buffer (pH 8.0).

To facilitate peak assignment during selectivity studies, a p53-PP-enriched sample was prepared as a reference material expected to contain predominantly parental plasmid and no p53-mcDNA, since the recombination step required for minicircle generation was omitted. This involved the culture of the same *E. coli* strain, using a similar procedure, but without performing the induction step, thereby ensuring that only p53-PP was amplified. These samples were prepared using the same extraction protocol followed for pre-purified samples.

To obtain complex lysates, typical *E. coli* fermentations were carried out, the lysis solutions from the NZYMaxiprep kit were used without RNase I, and subsequent column purification steps were omitted. This ensured the presence of all nucleic acid species (p53-mcDNA, p53-PP, mP, RNA, and gDNA) and host cell proteins in the lysate. To precipitate excess RNA and proteins, 2.0 M ammonium sulfate was added to the sample and incubated at 4 °C for 15 min. Then, the supernatant was recovered at 16 000 ×g for 30 min at 4 °C in the MPW-380R centrifuge from MPW Med. Instruments. Following this, ammonium sulfate was removed from the sample using an ultrafiltration device with a membrane of 10 000 MWCO PES (Vivaspin® 6 from Sartorius). For this purpose, the sample was centrifuged using an MPW-380R centrifuge (MPW Med. Instruments), at 2000 ×g and 4 °C. The ammonium sulfate solution was replaced with 10 mM Tris-HCl, pH 8. This sample type was specifically designed to mimic upstream process streams and therefore contained a higher diversity of nucleic acid species and host-cell impurities than the pre-purified preparations. Consequently, it was used to evaluate the applicability of the developed method to complex samples and to investigate whether matrix components interfered with p53-mcDNA detection and quantification (as discussed in section 3.6).

Table 1. Detailed description of the different sample types used for the method development and validation.

	p53-mcDNA	p53-PP	mP	gDNA	RNA
Pre-purified sample	✓	✓	trace	-	-
p53-PP-enriched sample	-	✓	-	-	-
Complex lysate sample	✓	✓	trace	✓	✓

✓, component detected; –, not detected; Trace, detected at trace levels.

The nominal concentrations of plasmid samples were determined using a Nabi™ Ultraviolet-Visible Nano Spectrophotometer from MicroDigital (South Korea). Those values were directly measured by applying a 1 µL aliquot of the sample to the equipment and recording the absorbance at 260 nm. The DNA concentration was

calculated using the Beer-Lambert law and the dsDNA conversion factor, where an absorbance of 1.0 at 260 nm corresponds to 50 ng/ μ L. Also, absorbance ratios of 260/280 nm and 260/230 nm were automatically given by the instrument software. For DNA samples, the accepted purity parameters are typically around 1.8 for A_{260}/A_{280} and about 2.0 to 2.2 for A_{260}/A_{230} . The samples used throughout this work presented $A_{260}/A_{280} / A_{260}/A_{230}$ ratios within the accepted ranges. Nonetheless, the A_{260} quantification does not distinguish mcDNA from PP or mP. Therefore, for pre-purified sample, the reported nominal concentration represents the total UV-derived DNA concentration of mcDNA, PP, and mP mixture rather than a specific mcDNA mass concentration. Consequently, quantitative parameters based on the nominal A_{260} should be interpreted with this limitation in mind.

2.4 Optimization of the AEX-HPLC method

For the initial optimization of p53-mcDNA quantification, an ÄKTA Pure™ preparative chromatography system (Cytiva, USA) equipped with a binary pump, a variable multi-wavelength UV-visible spectrometer set to 260 nm, and UNICORN Software (Cytiva, USA) was used. These experiments were performed using an anion-exchange TSKgel® DNA-NPR analytical column (75 mm $\times$ 4.6 mm, 2.5 μ m particle size) from TOSOH Bioscience (Germany). The optimization details are described in Section S1 in the Supplementary Materials (SM). Initially, several 10-minute linear elution gradients were evaluated; however, none of the tested conditions (50-65 %, 55-65 % and 57-65 %) enabled the effective separation of the mcDNA and PP peaks (Figure S1). Notably, a slightly higher resolution was observed with the 50-65% gradient, as indicated by a closer baseline intersection between the peaks. Furthermore, different mobile-phase compositions and pH values were assessed (Figure S2). Comparison of the chromatographic profiles obtained using 57-65 % gradients at pH 5.5, 7.0 and 9.0 revealed no noticeable differences in peak shape or resolution. Since pH 7 is close to the physiological range, it represents the most biologically relevant condition to guarantee the stability of biomolecules and should therefore be preferred for this application. Additionally, gradient modifications were tested, either by applying a linear gradient followed by an isocratic step or using two isocratic steps (Figure S3). Different gradient durations were also assessed. However, these conditions did not improve peak resolution; instead, they resulted in increased peak broadening. Distinct flow rates were also explored (Figure S4), and co-elution of different species was observed, further indicating insufficient resolution. Even when different gradient durations (10 min and 20 min) were tested, no improvement in separation was observed (Figure S5). To further optimize the method, various conditions, including pH, elution gradients, mobile phase composition, and gradient duration, were systematically combined to maximize the information obtained (Figure S6). By collecting this data, it was expected to assess potential interactions between the column's matrix and the injected

species, thereby enabling more efficient separation and improved peak resolution. Since none of the previous approaches yielded satisfactory results, a new strategy was adopted by acquiring a customized TSKgel DNA-NPR analytical column (120 mm × 4.6 mm, 2.5 µm particle size) from TOSOH Bioscience (Germany). Nonetheless, these preliminary experiments provided valuable insights, enabling the identification of promising conditions for mcDNA and PP separation, namely a mobile phase consisting of Tris buffer at pH 7, an elution gradient ranging from 50% to 65% over 10min, using a flow rate of 0.5 mL/min. The Chromaster HPLC system (VWR Hitachi, Poland) was used to carry out this work due to ÄKTA Pure™ system's back-pressure limitations. The system was equipped with a binary pump, column oven (operating at 40 °C, within the range 0 – 60 °C indicated by the manufacturer), temperature-controlled autosampler (10 °C), diode array detector (DAD) set to 260 nm, and was operated using OpenLAB Software (Agilent Technologies, USA). Resolution (Rs) was calculated using the conventional baseline-width equation, with peak widths at the baseline, as follows (1):

$$Rs = \frac{2(t_{R2} - t_{R1})}{W_{b1} + W_{b2}} \quad (\text{Eq. 1})$$

Where t_{R2} and t_{R1} corresponds to retention times of peak 2 and 1, respectively, and W_{b1} and W_{b2} the baseline width of peak 1 and 2.

The mobile phase selected for the HPLC experiments consisted of 20 mM Tris-HCl buffer (pH 7.0) (referred to as mobile phase A) and 1.0 M NaCl in 20 mM Tris-HCl buffer (pH 7.0) (referred to as mobile phase B). The optimized chromatographic conditions used to evaluate the method in terms of selectivity, linearity, accuracy, precision, and p53-mcDNA assessment in complex solutions followed the gradient profile: 0-1 min, 50 % of mobile phase A; 1-11 min, linear decrease of mobile phase A from 50% to 35 %; 11-32 min, 35 % of mobile phase A; 32-35 min, linear increase of mobile phase A from 35 % to 50 %; and 35-40 min, 50 % of mobile phase A. A flow rate of 0.7 mL/min was applied, and 25 µL of each sample was injected. Sample concentrations ranged from 1 to 100 µg/mL and were diluted in mobile phase A when necessary. Before use, both mobile phases were filtered through 0.45 µm regenerated cellulose filter (Sartorius, Germany) and degassed in an ultrasonic bath (Elma Schmidbauer GmbH, Germany).

2.4.1. mcDNA selectivity

For the selectivity assays, two different samples were injected, with a concentration of 52 µg/mL, namely the pre-purified sample and the p53-PP-enriched sample. Additionally, a blank composed of 20 mM Tris-HCl (pH 7.0), the mobile phase A, was also analyzed.

2.4.2. Linearity assessment

For the linearity assay, seven pre-purified samples (mainly containing p53-mcDNA and p53-PP) with known concentrations (1, 2, 10, 20, 50, 75, and 100 µg/mL), were prepared by suitable dilution with mobile phase A (20 mM Tris-HCl buffer (pH 7.0)). Each concentration was analyzed in triplicate ($n = 3$). With the respective areas of the peaks and the known concentrations, an equation was determined (Eq. 2: $Y = 0.4853X - 0.6859$, being Y the area of all peaks, and X the total DNA concentration (µg/mL)) by linear regression between 1 and 100 µg/mL.

2.4.3. Evaluation of the method's accuracy

The method's accuracy was evaluated by assessing the pre-purified samples' concentrations through the equation determined in the linearity assay (Eq. 2). Seven samples with known concentrations were analyzed in triplicate ($n = 3$). The concentrations used were the same as the linearity assay (1, 2, 10, 20, 50, 75, and 100 µg/mL), together with an additional intermediate concentration of 40 µg/mL, to validate the method's performance. The samples were prepared by diluting the pre-purified sample in the mobile phase A (20 mM Tris-HCl buffer (pH 7.0)).

2.4.4. Limit of detection and limit of quantification of total DNA

To determine the method's capacity to detect and quantify small amounts of an analyte, i.e. sensitivity, the lower limit of quantification (LLOQ) and the lower limit of detection (LOD) were determined. The LLOQ indicates the lowest DNA concentration that can be assessed with suitable precision and adequate accuracy ($\pm 20\%$ of the nominal concentration) [6] and LOD is the lowest amount of DNA in a sample that can be detected, but not necessarily quantified, being typically established by a signal-to-noise ratio of 3:1 between the measured signals of known low-level DNA samples and the blanks. The chromatographic analysis was arranged from low to high concentration to minimize the impact of potential carryover on low-level samples. Moreover, autosampler washing between sets ensured the needle/seat/loop cleaning, and a 20 min hold at the strongest elution composition was kept to guarantee the clearance of the retained species within the column.

2.4.5. Evaluation of the method's precision

To evaluate precision, reproducibility, and repeatability of the analytical method, three pre-purified samples with known concentrations of 20, 45, and 75 µg/mL were injected on the same day (intra-day) and on different days (inter-days). Each concentration was analyzed in triplicate ($n = 3$).

2.4.6. p53-mcDNA quantification in complex samples

The applicability of the method to quantify p53-mcDNA in a complex sample was assessed using a lysate mainly containing p53-mcDNA, p53-PP, and RNA at a known concentration of 297.43 µg/mL (higher than previous experiments to achieve unambiguously distinguishable species), which was diluted from the complex lysate with the mobile phase (20 mM Tris-HCl buffer, pH 7.0). In this assay, the sample profile was evaluated to verify if the RNA peak interferes with the elution of the plasmids and to validate the quantification of sc-mcDNA from more complex samples.

3. Results and discussion

The establishment of a satisfactory analytical method for the quality assessment of biopharmaceuticals such as mcDNA requires the validation of multiple parameters, such as selectivity, linearity, specificity, precision, accuracy, limits of detection and quantification, and reproducibility [6]. This rigorous validation guarantees that analytical performance and regulatory requirements for the reliable quality control of mcDNA-based biopharmaceuticals are met, thereby ensuring process effectiveness and reliability.

For the initial optimization of p53-mcDNA quantification, an ÄKTA Pure™ preparative chromatography system was employed, using an anion-exchange TSKgel DNA-NPR analytical column (75 mm × 4.6 mm, 2.5 µm particle size). This nonporous, high-efficiency column is composed of hydrophilic polymer beads with a polymethacrylate-based matrix, enabling effective DNA separation under ion-exchange conditions [26]. Targeted optimization assays were performed by varying the mobile phase gradient, flow rate, buffer pH, and run time.

None of the tested conditions allowed complete resolution between p53-mcDNA and its parental plasmid (p53-PP). However, these trials provided valuable information for subsequent experiments, namely the general gradient conditions that promote nucleic acid binding/elution and the range that enables partial separation between the two DNA species. The chromatographic profile also suggested that a column with increased length and, consequently more theoretical plates, could improve resolution and mcDNA isolation. Thus, a new customized TSKgel DNA-NPR analytical column, with 120 mm in length, was acquired to further enhance separation efficiency. The increased column length was anticipated to enhance method development and enable the near-complete separation of p53-mcDNA and p53-PP, consistent with findings reported in other chromatographic studies [27,28]. However, because the longer column generated high backpressure (~190 bar), which exceeded the operational limits of the ÄKTA Pure™ system, all subsequent optimization experiments were performed using a Chromaster HPLC system, which is capable of operating at pressures up to 400 bar. Indeed, chromatographic resolution and sensitivity are critical for potential applications regarding p53-mcDNA

quantification. In this regard, a precise balance between the baseline separation of p53-mcDNA and p53-PP, which is mandatory for quantification, and sensitivity needs to be carefully evaluated to ensure efficient method validation. It should be considered that while increased column length improves separation, it also increases backpressure. Moreover, optimisation of experimental parameters, particularly the flow rate, may influence the sensitivity of method.

Figure 1 presents the chromatograms resulting from the investigation of different elution gradients, gradient times, and flow rates after loading the pre-purified sample.

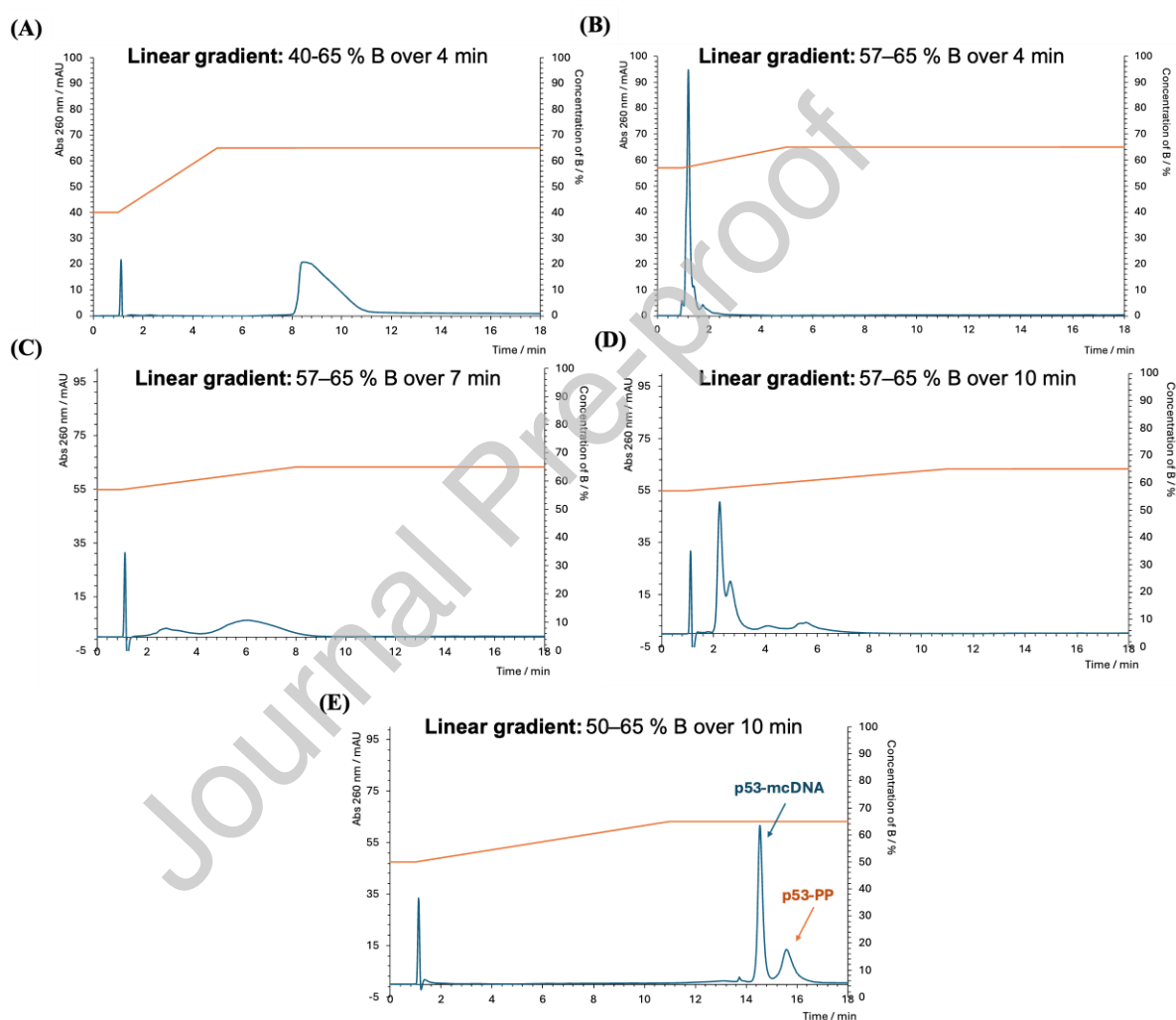


Figure 1. Evaluation of different gradient conditions for p53-mcDNA and p53-PP separation using the TSKgel DNA-NPR column. A - Linear gradient from 40 to 65 % mobile phase B over 4 min (total run time: 24 min); B - linear gradient from 57 to 65 % B over 4 min (total run time: 24 min); C - linear gradient from 57 to 65 % B over 7 min (total run time: 27 min); D - linear gradient from 57 to 65 % B over 10 min (total run time: 30 min); E - optimized gradient profile: 50 % B (0-1 min), 50-65 % B (1-11 min), hold at 65 % B (11-32 min), re-equilibration to 50 % B (32-35 min), and hold at 50 % B (35-40 min); total run time: 40 min. The reported gradient times correspond to the elution window, whereas the total

chromatographic run also included column washing and re-equilibration steps. Chromatographic runs were performed using 20 mM Tris-HCl (pH 7.0) and 1.0 M NaCl in 20 mM Tris-HCl (pH 7.0), at a flow rate of 0.7 mL/min.

The purpose of this optimization was to separate the different species present in the sample (mainly mcDNA, and PP). As shown in Figure 1, the evaluated conditions were unable to resolve the distinct peaks, with the important exception of Figure 1E. With the gradient of 40-65% during 24 min (Figure 1A), a single peak was observed after sample injection, with a retention time between 7 and 10 min, suggesting that a co-elution of the biomolecules (PP and mcDNA) occurred. For the gradient 57-65%, different gradient times (24, 27, and 30 min) were evaluated (Figure 1B, C, and D, respectively). With a 24-min gradient time, the samples eluted, at the beginning of the chromatographic run (single peak), while with a 27-min gradient time, two broad peaks were obtained with retention times of 3.0 and 6.0 min. Moreover, the use of 30 min of gradient time, resulted in several peaks in the chromatogram, where two peaks co-eluted at the beginning of the assay (retention times of 2.1 min and 2.6 min) and then two wider peaks at 4 and 6 min. Lastly, the chromatographic conditions that provided the best separation performance for mcDNA and p53-PP, were achieved with the following gradient profile: 0-1 min, 50% of mobile phase A; 1-11 min, linear decrease of mobile phase A from 50% to 35%; 11-32 min, 35% of mobile phase A; 32-35 min, linear increase of mobile phase A from 35% to 50%; and 35-40 min, 50% of mobile phase A (Figure 1E). These were confirmed by performing resolution calculations using equation (1) described in section 2.4 of Materials and Methods. Among the tested conditions, chromatograms in Figure 1C and Figure 1E exhibit clearly superior resolution with R_s values of 3.84 and 2.93, respectively, which are well above the usual baseline target of 1.5-2 (Section S1.6 – Table S1 – in the Supplementary Information). In the case of Figure 1C, the very large retention time gap between the two evaluated peaks and the broader peak profile suggest they may not represent the true critical pair, and there is a risk that closely eluting isoforms or variants could still be co-eluting within that interval. The condition from Figure 1E offers the ideal balance, providing very good separation between closely eluting neighbourhood peaks with adequate baseline resolution. Considering that mcDNA is a product of a PP recombination, the peak with a higher intensity is expected to correspond to the mcDNA, and the peak with a lower intensity could be attributed to PP. Also, further experiments concerning the injection of the p53-PP-enriched sample proved its elution occurs after 15 minutes, whereas the p53-mcDNA elutes earlier. Consequently, the aforementioned conditions were applied in subsequent experiments to validate the method for mcDNA quantification.

3.1. mcDNA selectivity

Selectivity was the first parameter assessed, since it is mandatory for a precise identification and quantification of the target p53-mcDNA. The separation of the different species present in the sample is facilitated by electrostatic interactions between the negatively charged phosphate groups of pDNA and the positively charged functional groups present on the surface of the column's hydrophilic polymer beads [29]. Adjusting the pH and ionic strength of the mobile phase is critical for controlling nucleic acid binding and elution. Consequently, the TSKgel DNA-NPR column is expected to effectively distinguish p53-mcDNA from impurities in the pre-purified samples containing p53-mcDNA, PP and mP.

The injected sample had a concentration of 52 µg/mL, being the resulting chromatograms displayed in Figure 2.

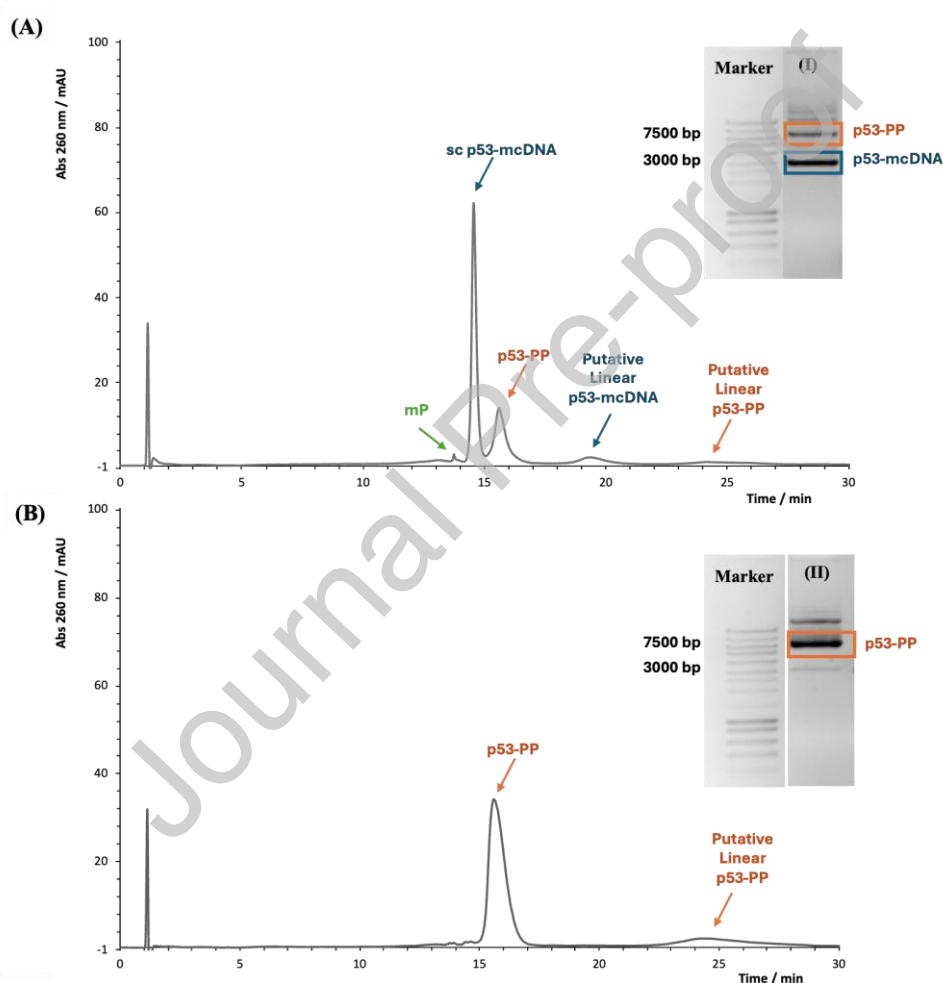


Figure 2. Chromatographic profiles of p53-mcDNA and p53-PP-enriched samples obtained under the optimized chromatographic conditions using a customized TSKgel DNA-NPR column (120 mm in length). A – Chromatographic profile of a pre-purified sample with a concentration of 52 µg/mL of total DNA and agarose gel electrophoresis of the injected sample ((I) pre-purified sample). B – Chromatographic profile of p53-PP-enriched sample with the concentration of 52 µg/mL of total DNA and agarose gel electrophoresis of the injected sample ((II) p53-PP-enriched sample). Major peaks were attributed to p53-mcDNA and p53-PP based on comparative analysis of the enriched samples and agarose gel electrophoresis. Additional late-eluting peaks were tentatively assigned to linear plasmid isoforms. Chromatographic assays were performed with a linear gradient of 20 mM Tris-HCl (pH 7.0), and 1.0 M NaCl in 20 mM Tris-HCl (pH 7.0) and a 0.7

mL/min flow rate. The respective electrophoresis was carried out with 0.8% of agarose using NZYDNA Ladder III as a molecular weight marker.

By analyzing the chromatograms, it is evident a minor peak (~1.00–1.20 min) in both samples and the blank (SI), which is attributed to a disturbance from the buffer transition [20,30], as this peak appears at the moment the linear gradient is initiated in the method. In the pre-purified sample (Figure 2A), two main peaks appeared at retention times (RT) of 14.60 min and 15.65 min, presumed to correspond to mc p53-mcDNA and p53-PP, respectively. A smaller peak at 13.80 min was also detected, which is supposed to be mP. Additionally, broad peaks around 20 and 25 min may represent the linear isoforms of p53-mcDNA and p53-PP, which, due to increased exposure of phosphate groups, interact more strongly with the column matrix [23,31]. In contrast, the p53-PP-enriched sample (Figure 2B) displayed only the 15.65 min peak, confirming the absence of p53-mcDNA. Electrophoresis (Figure 2 I, II) further corroborated these findings: in the p53-mcDNA lane, both p53-mcDNA and a weaker p53-PP band were present, whereas in the p53-PP-enriched sample lane, a faint p53-mcDNA band was present. This should be considered a residual recombination. Even without the addition of the inductor (arabinose), incomplete repression of the recombination promoter or intrinsic changes related with *E. coli* growth may still allow low level recombination, ultimately leading to the generation of p53-mcDNA [32,33]. In both cases, no visible mP band was detected, consistent with the chromatograms, which showed only a residual mP peak. These suggests that the endonucleases effectively cleaved, fragmented and degraded the majority of mP isoforms.

The elution profile of the pre-purified sample, with the p53-mcDNA peak eluting before the p53-PP peak, can be explained by differences in the physicochemical properties of the two molecules. p53-mcDNA is a smaller molecule with a lower overall negative charge compared to the larger parental plasmid [3,34]. Thus, more negatively charged phosphate groups, as occurs in p53-PP, exhibit stronger interaction with the anion-exchange column's ligands, leading to higher retention times. In contrast, the smaller size of the p53-mcDNA results in less phosphate groups and therefore a weaker interaction and faster elution from the column. This chromatographic behavior aligns with findings from previous studies employing ion-exchange matrices for plasmid and p53-mcDNA separation [35,36]. Even though, the more compact structure of mcDNA can be responsible for a higher charge density, which will also contribute to strengthening the interaction with the ligands. This explains the similar retention profile and justifies the challenge of separating all the species, when different phenomena are contributing to the overall retention in the column. Also, the later elution peaks (20 and 25 min.) could suggest slalom chromatography effects, in which flow-induced deformation drives the flexible

linear mcDNA to a more stretched and compact conformation. Under these conditions, the molecules are subjected to elongational and shear forces and follow a more tortuous flow path, such that hydrodynamic effects become dominant over anion-exchange interactions and lead to increased retention times [37].

3.2. Linearity assessment

After confirming the selectivity of the method for p53-mcDNA in relation to p53-PP, the evaluation of linearity is critical to verify whether the analytical response is directly proportional to p53-mcDNA concentration across the working range. A linear method ensures an satisfactory and reliable quantification for both low- and high-concentration samples, a key requirement for its application in quality control and process development.

Figure 3 illustrates the chromatographic profiles obtained for each p53-mcDNA pre-purified sample ranging from 1 to 100 µg/mL. Although the retention times for PP and mcDNA slightly differ from those determined from Fig. 1 and Fig. 2, these variations may be attributed to differences in matrix composition and the ionic environment resulting from sample preparation. The peak corresponding to sc p53-mcDNA elutes at 13.15 min, and an additional peak, attributed to the open circular (oc) mcDNA isoform, is observed at 12.49 min. As expected, the p53-mcDNA peak area increases with the increase of mcDNA concentration. The p53-PP peak was obtained at 13.70 min. In addition, the chromatographic profile suggests partial separation of distinct topological isoforms (relaxed and supercoiled) for p53-mcDNA. Topology and conformation (sc, relaxed, open circular or linear isoforms) modulate the effective charge density and hydrodynamic behavior, which in turn govern how molecules interact with the positively charged ligands on the stationary phase [37]. In the supercoiled form, the plasmid can wrap and overlap, most often in a clockwise crossover, orienting the molecule into a negatively charged state [38]. Coupled with the reduced hydrodynamic radius of this conformation, a higher effective charge density per molecule is achieved when compared with the more relaxed isoforms [38]. Consequently, the combination of such characteristics promotes stronger interactions and facilitates deeper access into the stationary phase positively charged ligands and therefore exhibit increased retention and later elution times.

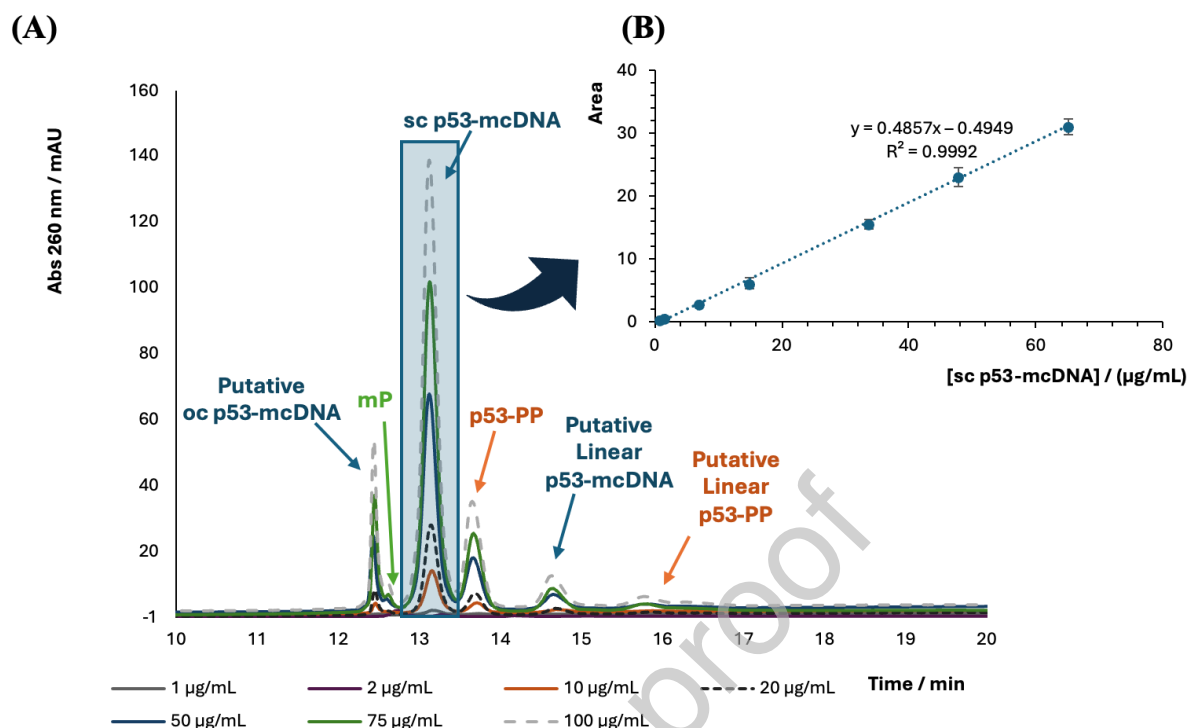


Figure 3. A - Linearity assessment obtained for pre-purified samples with the concentration of 1, 2, 10, 20, 50, 75 and 100 µg/mL of total DNA. The chromatographic runs were performed with 20 mM Tris-HCl (pH 7.0) and 1.0 M NaCl in 20 mM Tris-HCl (pH 7.0) as mobile phases, at a flow rate of 0.7 mL/min. B – Calibration curve for the determination of sc p53-mcDNA.

Because the mcDNA pre-purified sample still contains residual PP and different isoforms may arise from sample manipulation, two different possibilities were explored: the first to develop an analytical method to quantify total DNA in the sample, and the second to quantify only mcDNA species. These strategies were implemented to address the limitation of not having pure mcDNA available to use as a standard for generating the calibration curve. The commercially available mcDNA materials are functional constructs for cellular transfection but are not certified for analytical use. Thus, the calibration curve for total DNA was determined by summing the areas of all peaks present in each chromatogram (raw data available in Table S1, SM, section S2). The resulting equation (Eq. 2), $Y = 0.4853X - 0.6859$, where Y is the area of all peaks, and X is the total DNA concentration in µg/mL, with replicate measurements at each level ($n = 3$) showed a correlation coefficient (R^2) of 0.9994 and mean peak areas with low standard deviations in the range of 1 – 100 µg/mL (curve available in Figure S8, section S2 of the SM). Residual values, obtained as the difference between observed and predicted peak areas, were small and randomly distributed around zero, with no visible curvature or systematic trend, supporting the suitability of a linear model. Also, back-calculated concentrations were used to estimate the relative error for the different levels of concentrations, revealing higher deviations at 1 and 2 µg/mL, whereas

concentrations from 10 – 100 ug/mL showed relative error values around 10%, thereby demonstrating acceptable agreement between measured peak absorbance and the mcDNA concentration in good agreement with literature guidelines [39].

To determine the concentration of sc p53-mcDNA, another calibration curve based on this single peak was determined with replicate measurements at each level ($n = 3$). For this, the sc p53-mcDNA peak area was considered in each sample to calculate the concentration of the sc p53-mcDNA (Figure 3B). The calibration curve for sc p53-mcDNA presents the following equation (Eq. 3), $Y' = 0.4857X' - 0.4949$, where Y' is the area of sc p53-mcDNA peak and X' is sc p53-mcDNA concentration in $\mu\text{g/mL}$, correlation coefficient (R^2) of 0.9992. Again, residuals were examined and found to be of low magnitude and randomly distributed around zero. Back-calculated concentrations were also used to estimate the relative error at each concentration level, revealing larger deviations for 0.815 and 1.478 ug/mL, with relative error values around or above 30%, thus indicating unacceptable agreement between measured peak absorbance and mcDNA concentration. However, concentrations from 7.027 – 65.001 ug/mL demonstrated relative error values below 10%, which demonstrates acceptable agreement between measured peak absorbance and the mcDNA concentration as established by literature guidelines [43].

Since Eq. 2 considers all the species (p53-mcDNA, p53-PP, and others) present in the pre-purified sample and an acceptable agreement between measured peak absorbance and mcDNA concentration, the analysis of accuracy and precision were conducted considering this total DNA equation (Eq. 2) and by integrating the area of all the detected peaks.

3.3. Evaluation of the method's accuracy

This parameter is critical to confirm that the method provides reliable and unbiased quantification of p53-mcDNA, ensuring confidence in future dosage determination and quality control of nucleic acid-based therapeutics. The results are summarized in Table 1, which provides the mean calculated concentrations, standard deviations, coefficients of variation (CV), and mean relative errors for all standards ($n = 3$). It should be emphasized that the nominal concentrations used were determined by A_{260} measurements and therefore represent total DNA content, expressed as dsDNA equivalents, of the pre-purified pDNA samples, rather than independently determined, species-specific absolute p53-mcDNA mass concentrations.

Table 2. Accuracy for the assessment of pre-purified pDNA samples containing p53-mcDNA, and p53-PP in the range of 1–100 µg/mL (n = 3).

Nominal concentration (ug/mL)	1.000	2.000	10.000	20.000	40.000	50.000	75.000	100.00
Mean concentration (ug/mL)	2.059	2.917	8.965	19.234	40.567	49.819	77.072	102.572
Standard deviation	0.067	0.013	0.152	0.792	3.126	4.047	2.118	5.764
Coefficient of variation (%)	3.269	0.451	1.696	4.118	7.706	8.123	2.748	5.620
Mean relative error (%)	105.930	45.846	10.353	3.830	1.417	0.363	2.763	2.572

As shown in Table 1, the lowest nominal concentration (1 µg/mL) resulted in the highest mean relative error (105.93%), indicating low accuracy at this concentration level. Conversely, the lowest mean relative error (0.36%) was observed at 50 µg/mL, although this concentration also exhibited the highest coefficient of variation (8.12%), suggesting some variability in replicate measurements. The lowest coefficient of variation (0.45%) was observed at 2 µg/mL, indicating excellent precision among three replicates at that concentration. However, a relatively high mean relative error (45.85%) was observed, indicating low accuracy at this concentration.

Overall, the method here developed demonstrated satisfactory accuracy within the range of 10–100 µg/mL, where all values fall well within the $\pm 15\%$ threshold recommended by FDA regulatory guidelines [6]. The method performance is comparable to previous reports; for instance, Sousa *et al.* [36] developed a quantification method using a CIM-DEAE monolithic disk for sc pDNA, reporting coefficient of variations between 0.36% and 10.02%, and relative errors from 0.14% to 10.19%. Although the lowest concentration in the present study showed poorer performance, accuracy at higher concentrations was comparable to or better than that reported in the literature. Sousa *et al.* [36] were able to calculate pDNA concentrations low as 2 µg/mL, until concentrations up to 200 µg/mL. Similar trends were observed in other studies, such as that by Quaak *et al.* [40], who also employed the TSKgel DNA-NPR column for pDNA quantification, being able to quantify between 5 and 150 µg/mL. Additionally, in contrast to other published studies that validated only up to 25 µg/mL, this study extends the method validation up to 100 µg/mL. For instance, Diogo *et al.* [41] established a calibration curve up to 20 µg/mL for their hydrophobic interaction chromatographic method for pDNA quantification, while

Almeida *et al.* [20] achieved quantification up to 25 µg/mL using an affinity chromatographic approach for p53-mcDNA. The present method demonstrates applicability and versatility across a broader concentration range, which is particularly relevant for downstream applications, where achieving highly concentrated p53-mcDNA solutions remains a key objective.

3.4. Limit of detection and limit of quantification of total DNA

The data shown in Table 1 also allow inference of the method's sensitivity, which represents its capacity to detect and quantify small amounts of an analyte. The LLOQ, a key parameter in assessing sensitivity, is presented in Table 1. Considering that the typical concentrations of lysate complex samples containing p53 mcDNA are about 100 µg/mL, as determined by UV absorbance at 260 nm, the LLOQ was defined in the low µg/mL range (1 µg/mL), *i.e.*, at least one order of magnitude below the typical sample concentration, to ensure reliable quantification across normal process variability. However, according to the results, a LLOQ of 10 µg/mL was established for the method. At this concentration, the method exhibited a coefficient variation of 1.696% and a mean relative error of 10.353%, both of which lower than the 20% limit range accepted by the FDA reference guidelines [6]. The established LOD, defined as a signal-to-noise ratio of 3:1 between the measured signals of known DNA samples and the blanks, is presented in Figure S7. Analysis of this chromatogram showed no peaks within the elution windows of any of the studied plasmids and therefore and LOD of 1 µg/mL was assigned for this method. Previous studies have reported LLOQ values ranging from 1 to 5 µg/mL. To mention some examples, 1 µg/mL was defined for a hydrophobic interaction method developed for pDNA [41] and for an affinity-based method for p53-mcDNA [20], 2 µg/mL was established for both ion-exchange and affinity chromatography methods applied to pDNA [36,42], and 5 µg/mL for an anion-exchange chromatographic method developed for pDNA with the TSKgel DNA-NPR (75 mm) [40]. In all these studies, the LOD was equivalent to the reported LLOQ.

Among these previously reported works, the method proposed by Quaak *et al.* [40] exhibited the higher LLOQ value (5 µg/mL). Since their chromatographic column differs from the one used in this work only in length (75 mm vs 125 mm), it is reasonable to attribute the higher LLOQ value observed here to column-related effects. A longer column generally increases the residence time and the number of theoretical plates, which enhances resolution but may also broaden the peaks, leading to a lower signal-to-noise ratio for low-concentration samples [28]. Consequently, this can reduce the method's sensitivity and increase the LLOQ threshold. Thus, the higher LLOQ value observed in this work likely reflects a trade-off between improved resolution and detection sensitivity inherent to the use of a longer analytical column.

Considering the LOD of 1 µg/mL and the LLOQ of 10 µg/mL, a regression curve within the working calibration range, 10 µg/mL to 100 µg/mL for total DNA and 7 µg/mL to 65 µg/mL for sc mcDNA, were determined, according to Figures S8 and S9 in section S2 of the SM, respectively, for total DNA and sc-p53mcDNA. The resulting equation for total DNA determination (Eq. 4) was $Y = 0.4930X - 1.2582$ (range of 10 – 100 µg/mL), where Y is the area of all peaks, and X is the total DNA concentration in µg/mL, with a correlation coefficient (R^2) of 0.9997. In contrast, the calibration curve obtained for sc p53-mcDNA (Eq. 5) was $Y' = 0.4946X' - 0.9164$ (range of 10 – 100 µg/mL), where Y' is the area of sc p53-mcDNA peak and X' is sc p53-mcDNA concentration in µg/mL, with a correlation coefficient (R^2) of 0.9995.

3.5. Evaluation of the method's precision

To evaluate the precision of this chromatographic method, reproducibility and repeatability were studied for three DNA pre-purified samples, containing p53-mcDNA, and p53-PP, injected on the same day (intra-day) and different days (inter-day) with replicate measurements at each level ($n = 3$). This parameter is essential for demonstrating that the method yields reproducible p53-mcDNA quantification results, thereby ensuring its reliability. The repeatability (or intra-day) assessment for p53-mcDNA quantification is shown in Table 2.

Table 3. Intra-day precision for the assessment of pre-purified pDNA samples containing p53-mcDNA and p53-PP ($n = 3$).

Nominal concentration (ug/mL)	20.000	45.000	75.000
Mean concentration (ug/mL)	19.527	43.840	69.614
Standard deviation	0.924	0.974	0.601
Coefficient of variation (%)	4.734	2.222	0.864
Mean relative error (%)	2.365	2.578	7.181

As presented in Table 2, the known sample with the highest mean relative error was 75 µg/mL (7.181 %) and the standard with the highest variation was 20 µg/mL (4.734 %). Both values remain below the 15% range accepted by the FDA guidelines, consequently highlighting the repeatability of this analytical method [6]. In Table 3, the method's reproducibility (inter-day) is shown.

Table 4. Inter-day precision for the assessment of pre-purified pDNA samples containing p53-mcDNA and p53-PP ($n = 3$).

Nominal concentration (ug/mL)	20.000	45.000	75.000
-------------------------------	--------	--------	--------

Mean concentration (ug/mL)	19.008	40.992	70.993
Standard deviation	0.405	2.414	1.655
Coefficient of variation (%)	2.131	5.890	2.331
Mean relative error (%)	4.962	8.907	5.343

The inter-day results (Table 3) show that the 45 $\mu\text{g/mL}$ sample exhibits the highest mean relative error (8.907 %) and the highest coefficient of variation (5.890 %). Again, these values remain below the accepted 15% range, suggesting the analytical method's reproducibility [6]. Thus, both reproducibility and repeatability studies unequivocally demonstrate the precision and accuracy of this method for p53-mcDNA quantification.

3.6. p53-mcDNA assessment in complex samples

After completing the validation of the proposed chromatographic strategy through the required parameters, its applicability to more complex samples containing RNA was further investigated. The method must demonstrate a consistent performance across samples within higher complexity. To verify this, a more complex lysate containing p53-mcDNA, p53-PP, mP, and RNA was prepared and injected at a known concentration (297.43 $\mu\text{g/mL}$). This sample differs from the pre-purified sample in that, in the first extraction step, RNase solution is not used, and the excess RNA is precipitated with 2.0 M ammonium sulfate. The respective chromatogram is presented in Figure 4.

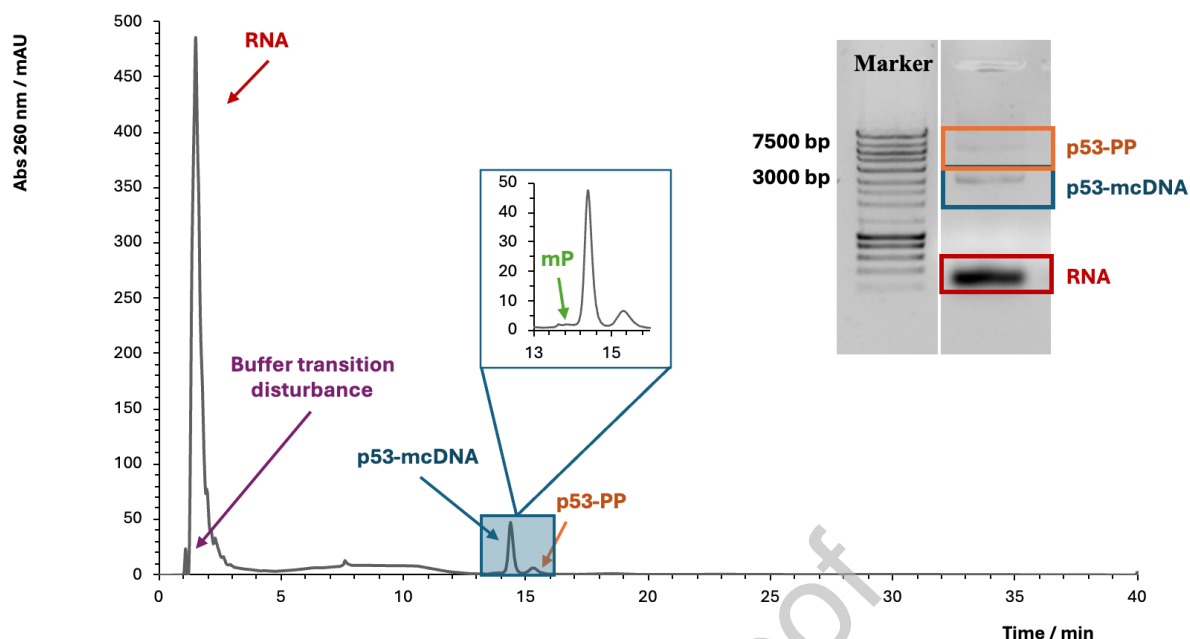


Figure 4. Chromatographic profile of complex lysate containing p53-mcDNA, p53-PP and RNA. Chromatographic conditions performed with a linear gradient of 20 mM Tris-HCl (pH 7.0), and 1.0 M NaCl in 20 mM Tris-HCl (pH 7.0) at 0.7 mL/min flow rate. The inset represents the electrophoresis of the complex lysate injected, with 0.8% of agarose gel using NZYDNA Ladder III as a molecular weight marker.

As shown in Figure 4, and by comparison with previous results, it is observed that RNA elutes within the first 2 min, followed by mP at 13.80 min, p53-mcDNA at 14.60 min, and p53-PP at 15.70 min. The chromatographic profile, namely peak intensity, was corroborated by electrophoresis, which revealed a strong RNA band and only faint bands for p53-mcDNA and p53-PP. Overall, this experiment confirmed the presence of RNA, p53-mcDNA, and p53-PP in a more complex sample, while also demonstrating that RNA does not interfere with the selectivity or quantification of sc p53-mcDNA established by the analytical method. The content of sc p53-mcDNA, calculated from two independent experiments using Eq. 4, was determined to be $23.96 \pm 1.21 \mu\text{g/mL}$, which corresponds to approximately 10% of the nucleic acids present in the sample. Nonetheless, an important limitation should be taken into consideration, the nominal concentration used to establish the calibration were assigned by absorbance at 260 nm. Therefore, these concentrations represent total UV-derived DNA content (p53-mcDNA, p53-PP, and mP) expressed as dsDNA equivalents and not independently determined plasmid mass. Although the chromatographic method described here can distinguish resolved peaks, calibration against these mixtures does not, by itself, establish the absolute concentration of p53-mcDNA. This is particularly important when interpreting results from the more complex RNA-containing sample, in which proportions of

p53-mcDNA, p53-PP, and mP may differ. The separation obtained underlines the potential of this method to quantify p53-mcDNA by resorting to a commercial column and the chromatographic method herein developed. This technique demonstrated potential for monitoring the levels of p53-mcDNA and related nucleic-acid components in process samples. To quantify the amount of p53-mcDNA present in the sample, it can be employed the calibration curve previously obtained. However, these values should be reported as apparent concentration relative to the A_{260} quantification, rather than as absolute p53-mcDNA mass concentrations. The percentage of the p53-mcDNA peak area relative to the total integrated peak area can provide a comparative measure of the chromatographic profile, but not be interpreted as an absolute purity percentage, particularly in samples containing RNA. The high-resolution of p53-mcDNA from the p53-PP under the tested conditions supports further evaluation of the method for process monitoring. Nonetheless, its utilization across different sample types and laboratories would require validation of peak identity, matrix effects, calibration strategy, and method transferability.

4. Conclusions

In this work, a new chromatographic method for p53-mcDNA quantification and analysis is proposed, resorting to AEX-HPLC. Linearity, precision and accuracy were in good agreement with international guidelines for the range of concentrations of total DNA within 10–100 $\mu\text{g}/\text{m}$, being the LLOQ and LOD established as 10 and 1 $\mu\text{g}/\text{mL}$, respectively. Overall, the results confirm that the developed chromatographic method provides reliable accuracy across the working range of DNA concentrations, with potential relevance to the processing of DNA-based products. It was also demonstrated that this method is suitable for p53-mcDNA quantification in more complex mixtures containing RNA, as the species elute separately and do not interfere with the detection or quantification of the other plasmid molecules. This study presents, for the first time, the development of a method employing a tailored commercially available anion-exchange column for the separation and quantitative assessment of p53-mcDNA samples. Further validation using species-specific reference materials and formal robustness and transfer studies will be required before broader implementation. However, the method enabled high-resolution separation of p53-mcDNA from its parental plasmid under the tested conditions and may support process monitoring during upstream and downstream operations.

CRediT authorship contribution statement

Ana I. Valente: Writing – original draft, Writing – review & editing, methodology, data curation, formal analysis, investigation, validation. **João V. Valente:** Writing – original draft; Writing – review & editing, methodology, data curation, investigation, validation. **Pedro L. Ferreira:** Writing – review, methodology, data curation, investigation. **Augusto Q. Pedro:** Writing – review & editing, conceptualization, supervision, resources. **Ana P.M. Tavares:** Writing – review & editing, conceptualization, supervision, resources. **Fani Sousa:** Writing – review & editing, conceptualization, supervision, resources, funding acquisition, Project administration. **Mara G. Freire:** Writing – review & editing, conceptualization, supervision, resources, funding acquisition, Project administration.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was developed within the scope of the project CICECO-Aveiro Institute of Materials, UID/50011/2025 (DOI 10.54499/UID/50011/2025) & LA/P/0006/2020 (DOI 10.54499/LA/P/0006/2020) & UID/PRR/50011/2025 (DOI 10.54499/UID/PRR/50011/2025), financed by national funds through the FCT/MCTES (PIDDAC). UBI project DOI:10.54499/UIDP/00709/2020, financed by national funds through the FCT/MCTES also helped to develop this work. This work was additionally developed within the scope of the project PureDNA (2022.03394.PTDC, DOI 10.54499/2022.03394.PTDC), financially supported by national funds (OE), through FCT/MCTES. Ana I. Valente, João V. Valente and Pedro L. Ferreira acknowledge FCT, respectively, for the PhD grants DOI:10.54499/2021.08352.BD, 2023.04779.BDANA and DOI:10.54499/2022.13803.BD. Augusto Q. Pedro and Ana P. M. Tavares acknowledges FCT for the research contracts under the Scientific Stimulus – Individual Call, DOI:10.54499/2020.02599.CEECIND/CP1589/CT0023.

Data availability

Data will be made available on request.

References

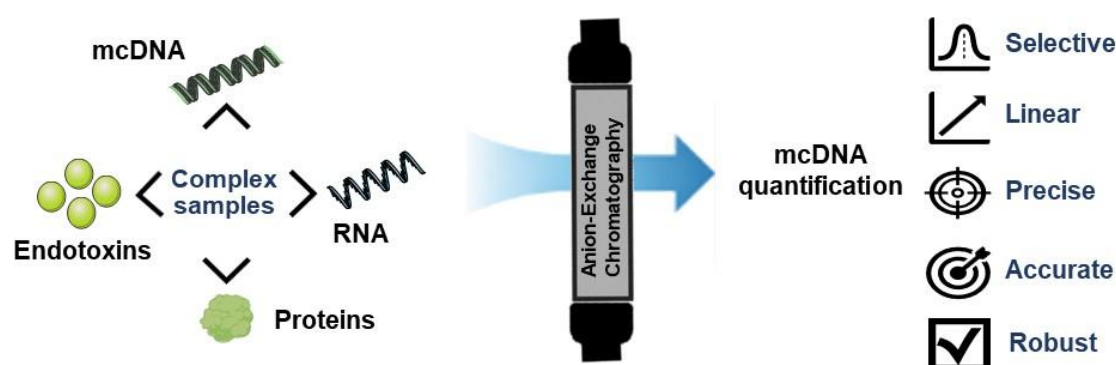
- [1] M. Foldvari, D.W. Chen, N. Nafissi, D. Calderon, L. Narsineni, A. Rafiee, Non-viral gene therapy: Gains and challenges of non-invasive administration methods, *J. Control. Release* 240 (2016) 165–190. <https://doi.org/10.1016/j.jconrel.2015.12.012>.

- [2] D. Scherman, *Advanced Textbook on Gene Transfer, Gene Therapy and Genetic Pharmacology: Principles, Delivery and Pharmacological and Biomedical Applications of Nucleotide-based Therapies*, 2nd Ed., World Scientific, Singapore, 2020.
- [3] P. Mayrhofer, M. Schleef, W. Jechlinger, Use of minicircle plasmids for gene therapy, in: *Methods in Molecular Biology*, vol. 542, 2009, pp. 87–104. https://doi.org/10.1007/978-1-59745-561-9_4.
- [4] M. Grund, M. Schleef, Minicircle Patents: A Short IP Overview of Optimizing Nonviral DNA Vectors, in: M. Schleef (Ed.), *Minicircle and Miniplasmid DNA Vectors: The Future of Nonviral and Viral Gene Transfer*, Wiley-VCH, Weinheim, 2013, pp. 1–6. <https://doi.org/10.1002/9783527670420.ch1>.
- [5] M.A. Kay, C.-Y. He, Z.-Y. Chen, A robust system for production of minicircle DNA vectors, *Nat. Biotechnol.* 28 (2010) 1287–1289. <https://doi.org/10.1038/nbt.1708>.
- [6] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine, *Bioanalytical Method Validation Guidance for Industry*, May 2018. <https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry>.
- [7] A.-M. Darquet, R. Rangara, P. Kreiss, B. Schwartz, S. Naimi, P. Delaère, J. Crouzet, D. Scherman, Minicircle: an improved DNA molecule for in vitro and in vivo gene transfer, *Gene Ther.* 6 (1999) 209–218. <https://doi.org/10.1038/sj.gt.3300816>.
- [8] V.M. Gaspar, C.J. Maia, J.A. Queiroz, C. Pichon, I.J. Correia, F. Sousa, Improved Minicircle DNA Biosynthesis for Gene Therapy Applications, *Hum. Gene Ther. Methods* 25 (2014) 93–105. <https://doi.org/10.1089/hgtb.2013.020>.
- [9] J. Maurer, C. Malburet, M. François-Heude, D. Guilleme, Evaluation of ion pairing reversed-phase liquid chromatography for the separation of large RNA molecules, *J. Chromatogr. A* 1740 (2025) 465574. <https://doi.org/10.1016/j.chroma.2024.465574>.
- [10] A.L.J. Webb, E.N. Welbourne, T.C. Minshall, K.A. Loveday, P. Bora, Z. Kis, G.A. Nilsson, A. Murthy, E. Ørnskov, M.J. Dickman, Analysis of mRNA multimerisation (aggregation) using non-denaturing ion-pair reversed-phase liquid chromatography, *J. Chromatogr. A* 1769 (2026) 466653. <https://doi.org/10.1016/j.chroma.2025.466653>.
- [11] E.N. Welbourne, K.A. Loveday, A. Nair, E. Nourafkan, J. Qu, K. Cook, Z. Kis, M.J. Dickman, Anion exchange HPLC monitoring of mRNA in vitro transcription reactions to support mRNA manufacturing process development, *Front. Mol. Biosci.* 11 (2024) 1250833. <https://doi.org/10.3389/fmolb.2024.1250833>.
- [12] F. Gritti, Ultra-high pressure slalom chromatography: Application to the characterization of large DNA and RNA samples relevant in cell and gene therapy, *J. Chromatogr. A* 1738 (2024) 465487. <https://doi.org/10.1016/j.chroma.2024.465487>.
- [13] L.M. Sandberg, Å. Bjurling, P. Busson, J. Vasi, R. Lemmens, Thiophilic interaction chromatography for supercoiled plasmid DNA purification, *J. Biotechnol.* 109 (2004) 193–199. <https://doi.org/10.1016/j.jbiotec.2003.10.036>.
- [14] J. Maurer, M.A. Lauber, S. Fekete, M. Imiolek, C. Malburet, M. François-Heude, D. Guilleme, Proof of Concept Application of Hydrophilic Interaction Chromatography for Direct Online Disruption of Lipid Nanoparticles, Intact mRNA Analysis, and Measure of Encapsulation Efficiency, *Anal. Chem.* 97 (2025) 7627–7632. <https://doi.org/10.1021/acs.analchem.5c00565>.
- [15] J.P. Tran, J. Gao, C. Lansdell, B. Lorbetskie, M.J.W. Johnston, L. Wang, X. Li, H. Lu, A Comprehensive Evaluation of Analytical Method Parameters Critical to the Reliable Assessment of Therapeutic mRNA Integrity by Capillary Gel Electrophoresis, *Electrophoresis* 46 (2025) 365–375. <https://doi.org/10.1002/elps.8123>.
- [16] K. Božič, A. Sedlar, Š. Kralj, U. Černigoj, A. Štrancar, R. Sekirnik, Selective hydrophobic interaction chromatography for high purity of supercoiled DNA plasmids, *Biotechnol. Bioeng.* 121 (2024) 1739–1749. <https://doi.org/10.1002/bit.28667>.
- [17] H. Bo, J. Wang, Q. Chen, H. Shen, F. Wu, H. Shao, S. Huang, Using a single hydrophobic-interaction chromatography to purify pharmaceutical-grade supercoiled plasmid DNA from other isoforms, *Pharm. Biol.* 51 (2013) 42–48. <https://doi.org/10.3109/13880209.2012.703678>.

- [18] L. Cupillard, V. Juillard, S. Latour, G. Colombet, N. Cachet, S. Richard, S. Blanchard, L. Fischer, Impact of plasmid supercoiling on the efficacy of a rabies DNA vaccine to protect cats, *Vaccine* 23 (2005) 1910–1916. <https://doi.org/10.1016/j.vaccine.2004.10.018>.
- [19] V.B. Pillai, M. Hellerstein, T. Yu, R.R. Amara, H.L. Robinson, Comparative studies on in vitro expression and in vivo immunogenicity of supercoiled and open circular forms of plasmid DNA vaccines, *Vaccine* 26 (2008) 1136–1141. <https://doi.org/10.1016/j.vaccine.2007.10.023>.
- [20] A.M. Almeida, U. Černigoj, J.A. Queiroz, F. Sousa, A. Sousa, Quality assessment of supercoiled minicircle DNA by cadaverine-modified analytical chromatographic monolith, *J. Pharm. Biomed. Anal.* 180 (2020) 113037. <https://doi.org/10.1016/j.jpba.2019.113037>.
- [21] A.M. Almeida, D. Eusébio, J.A. Queiroz, F. Sousa, A. Sousa, The use of size-exclusion chromatography in the isolation of supercoiled minicircle DNA from *Escherichia coli* lysate, *J. Chromatogr. A* 1609 (2020) 460444. <https://doi.org/10.1016/j.chroma.2019.460444>.
- [22] M. Imiolek, S. Fekete, S. Rudaz, D. Guillarme, Ion exchange chromatography of biotherapeutics: Fundamental principles and advanced approaches, *J. Chromatogr. A* 1742 (2025) 465672. <https://doi.org/10.1016/j.chroma.2025.465672>.
- [23] N. Pavlin, U. Černigoj, M. Bavčar, T. Plesničar, J. Mavri, M. Zidar, M. Bone, U. Kralj Savič, T. Sever, A. Štrancar, Analytical separation of plasmid DNA isoforms using anion exchanging chromatographic monoliths with 6 μm channels, *Electrophoresis* 44 (2023) 1967–1977. <https://doi.org/10.1002/elps.202300031>.
- [24] Y. Chen, H. Lu, R. Wang, G. Sun, X. Zhang, J. Liang, A. Jungbauer, S. Yao, D. Lin, Standardized approach for accurate and reliable model development of ion-exchange chromatography based on parameter-by-parameter method and consideration of extra-column effects, *Biotechnol. J.* 19 (2024) e2300687. <https://doi.org/10.1002/biot.202300687>.
- [25] C. Ventura, D. Eusébio, A.M. Gonçalves, J. Barroca-Ferreira, D. Costa, Z. Cui, L.A. Passarinha, Â. Sousa, Maximization of the Minicircle DNA Vaccine Production Expressing SARS-CoV-2 RBD, *Biomedicines* 10 (2022) 990. <https://doi.org/10.3390/biomedicines10050990>.
- [26] Tosoh Bioscience, Rapid Analysis of Plasmid Topoisomers by Anion Exchange Chromatography, available at <https://default.assets-tosoh-prod.roccommercecloud.com/assets%2Fc419d7d537ca95b8e0b8be002049b7fe/an121.pdf> (accessed October 7, 2025).
- [27] M.C. Asare, J.A. Kubde, R.L. Bakal, P.R. Hatwar, V.S. Kalamb, P.K. Tambakhe, Ion exchange chromatography: A comprehensive review, *GSC Biol. Pharm. Sci.* 31 (2025) 026–037. <https://doi.org/10.30574/gscbps.2025.31.1.0127>.
- [28] A. Jones, S. Pravadali-Cekic, G.R. Dennis, R.A. Shalliker, Post column derivatisation analyses review. Is post-column derivatisation incompatible with modern HPLC columns?, *Anal. Chim. Acta* 889 (2015) 58–70. <https://doi.org/10.1016/j.aca.2015.07.003>.
- [29] Tosoh Bioscience, TSKgel U/HPLC Columns 2019 Product Guide - AIEC Columns, available at https://default.assets-tosoh-prod.roccommercecloud.com/assets%2F539b19118fc6e8a7a866381ad1a29813/LCAT02A_IEX.pdf (accessed August 19, 2025).
- [30] A. Sousa, C.T. Tomaz, F. Sousa, J.A. Queiroz, Successful application of monolithic innovative technology using a carbonyldiimidazole disk to purify supercoiled plasmid DNA suitable for pharmaceutical applications, *J. Chromatogr. A* 1218 (2011) 8333–8343. <https://doi.org/10.1016/j.chroma.2011.09.033>.
- [31] C.R. Smith, R.B. DePrince, J. Dackor, D. Weigl, J. Griffith, M. Persmark, Separation of topological forms of plasmid DNA by anion-exchange HPLC: Shifts in elution order of linear DNA, *J. Chromatogr. B* 854 (2007) 121–127. <https://doi.org/10.1016/j.jchromb.2007.04.005>.
- [32] M. Simcikova, K.L.J. Prather, D.M.F. Prazeres, G.A. Monteiro, On the dual effect of glucose during production of pBAD/AraC-based minicircles, *Vaccine* 32 (2014) 2843–2846. <https://doi.org/10.1016/j.vaccine.2014.02.035>.

- [33] M. Šimčíková, C.P.A. Alves, L. Brito, K.L.J. Prather, D.M.F. Prazeres, G.A. Monteiro, Improvement of DNA minicircle production by optimization of the secondary structure of the 5'-UTR of ParA resolvase, *Appl. Microbiol. Biotechnol.* 100 (2016) 6725–6737. <https://doi.org/10.1007/s00253-016-7565-x>.
- [34] A.-M. Darquet, B. Cameron, P. Wils, D. Scherman, J. Crouzet, A new DNA vehicle for nonviral gene delivery: supercoiled minicircle, *Gene Ther.* 4 (1997) 1341–1349. <https://doi.org/10.1038/sj.gt.3300540>.
- [35] F. Sousa, C. Cruz, J.A. Queiroz, Amino acids–nucleotides biomolecular recognition: from biological occurrence to affinity chromatography, *J. Mol. Recognit.* 23 (2010) 505–518. <https://doi.org/10.1002/jmr.1053>.
- [36] É. Mota, Â. Sousa, U. Černigoj, J.A. Queiroz, C.T. Tomaz, F. Sousa, Rapid quantification of supercoiled plasmid deoxyribonucleic acid using a monolithic ion exchanger, *J. Chromatogr. A* 1291 (2013) 114–121. <https://doi.org/10.1016/j.chroma.2013.03.070>.
- [37] F. Gritti, S. Fekete, A. Finny, Dual retention mechanisms in DNA separation: Relevance of slalom chromatography in anion-exchange gradients, *J. Chromatogr. A* 1763 (2025) 466457. <https://doi.org/10.1016/j.chroma.2025.466457>.
- [38] J.B. Schwartzman, A. Stasiak, A topological view of the replicon, *The EMBO Rep.* 5 (2004) 256–261. <https://doi.org/10.1038/sj.embor.7400101>.
- [39] J.M. Jurado, A. Alcázar, R. Muñoz-Valencia, S.G. Ceballos-Magaña, F. Raposo, Some practical considerations for linearity assessment of calibration curves as function of concentration levels according to the fitness-for-purpose approach, *Talanta* 172 (2017) 221–229. <https://doi.org/10.1016/j.talanta.2017.05.049>.
- [40] S.G.L. Quaak, B. Nuijen, J.B.A.G. Haanen, J.H. Beijnen, Development and validation of an anion-exchange LC-UV method for the quantification and purity determination of the DNA plasmid pDERMATT, *J. Pharm. Biomed. Anal.* 49 (2009) 282–288. <https://doi.org/10.1016/j.jpba.2008.11.011>.
- [41] M.M. Diogo, J.A. Queiroz, D.M.F. Prazeres, Assessment of purity and quantification of plasmid DNA in process solutions using high-performance hydrophobic interaction chromatography, *J. Chromatogr. A* 998 (2003) 109–117. [https://doi.org/10.1016/S0021-9673\(03\)00618-6](https://doi.org/10.1016/S0021-9673(03)00618-6).
- [42] F. Sousa, J.A. Queiroz, Supercoiled plasmid quality assessment by analytical arginine-affinity chromatography, *J. Chromatogr. A* 1218 (2011) 124–129. <https://doi.org/10.1016/j.chroma.2010.11.002>.

Graphical abstract



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: