APPLICATION NOTE 22137

Separating different plasmid DNA forms on a pellicular anion exchange column

Authors: Ankitkumar Patel, Sachin Pandey, Thermo Fisher Scientific, India CoE Lab, Ahmedabad, India

Stacy Tremintin, Thermo Fisher Scientific, Sunnyvale CA, USA

Ken Cook, Thermo Fisher Scientific, Hemel Hempstead, UK

Keywords: Plasmid, DNAPac PA200, biopharma, ion exchange, HPLC

Goal

To showcase the ability of the Thermo Scientific™ DNAPac™ PA200 oligonucleotide HPLC column for separation and quantification of different forms of plasmid DNA from RNA and protein.

Introduction

Multiple gene-therapy-based therapeutic models have been developed to treat life-threatening diseases. Gene therapy involves the insertion of genetic material into a human cell as a therapeutic agent to treat a disease. The concept behind the therapy is to fix a genetic problem at its source. For instance, in an (usually recessively) inherited disease, a mutation in a gene can result in the production of a dysfunctional protein. Gene therapy could be used to deliver a copy of this gene that does not contain the deleterious mutation, and thereby produce a functional protein.[1]

The recent developments in non-viral gene therapy and DNA vaccination have led to advancements in an efficient plasmid DNA (pDNA) purification process.[2,3] The number of plasmid DNA studies have dramatically increased and have contributed to almost 14.7% of all gene therapy clinical trials, after adenoviruses (17.4%) and retroviruses (16.4%).[4] Non-viral vector-like naked pDNA are considered much safer, but they are less effective.

During plasmid production, supercoiled and other forms of product-related plasmid impurities are formed. Separation and quantification of these different plasmid DNA forms from RNA and proteins defines the purity of the plasmid sample. Typically accomplished by anion-exchange or ion-pair reversed-phase chromatography, both approaches enjoy wide popularity and deliver high resolution and sample throughput. Both techniques use ionic interactions between the analytes, either directly with the stationary phase (i.e., anion exchange) or with the ion-pair reagent, which in turn interacts with the stationary phase. This application note focuses on a pellicular anion-exchanger designed for high-resolution separation of different forms of plasmid DNA.[5,6]

ThermoFisher SCIENTIFIC

Experimental

Recommended consumables

- UHPLC-MS grade water, Fisher Scientific™ Optima™ (P/N W8-1)
- Deionized water, 18.2 MΩ·cm resistivity
- DNAPac PA200 Oligonucleotide HPLC column, 2.0 x 250 mm, 8 µm (P/N 063425)
- Thermo Scientific™ National™ Surestop™ vial, clear 2 mL kit with septa and cap (P/N C5000-595W)
- Tris buffer, Qualigens™ ExcelaR™ (P/N Q15964)
- Sodium perchlorate, ACROS Organics™ (P/N 197122500)

Sample pretreatment / sample preparation

A) *Supercoiled plasmid sample:* A supercoiled plasmid sample with a concentration of 1.0 mg/mL is diluted to 0.1 mg/mL using laboratory-grade water.

B) *Sample incubation with Nde1 enzyme:* 5 µL of sample is mixed with 38 µL of water and 5 µL of reaction buffer, then 1.5 µL of enzyme sample is incubated at 37 °C for 90 minutes.

C) *RNA and protein sample*: RNA and protein sample were injected as such without any dilution.

D) *Linear plasmid spike sample preparation:* Sample was spiked at 50% and 5% by mixing 25 µL of supercoiled plasmid sample (sample A) into 25 µL of Nde1 enzyme treated sample (sample B), and similarly for the 5% spike level, 2.5 µL of enzyme treated sample (sample B) were added to 47.5 µL of supercoiled plasmid sample (sample A).

E) *RNA and protein spike sample preparation:* 2 µL of RNA and protein sample were spiked into 15 µL of spiked linear plasmid sample (Sample D).

Instrumentation

Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC system equipped with:

- SRD-3600 Solvent racks with degasser (P/N 5035.9230)
- DGP-3600RS Rapid Separation Pump (P/N 5040.0066)
- WPS-3000TRS Rapid Separation Thermostatted Autosampler (P/N 5841.0020)
- TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)
- DAD-3000RS Rapid Separation Diode Array Detector (P/N 5082.0020)

Separation conditions

Mobile phase A: Water
Mobile phase B: 0.25 M Tris, pH 8.0
Mobile phase C: 0.35 M sodium perchlorate
Flow rate: 0.4 mL/min
Column temperature: 30 °C
Injection details: 5 µL
Injection wash solvent: 50% acetonitrile in water
Detection wavelengths: 260 nm and 280 nm
Gradient: Table 1

Table 1. LC gradient conditions

Time (min)	A%	B%	C%	Curve
0.0	88.0	10.0	2.0	5
0.1	47.0	10.0	43.0	5
26.10	30.0	10.0	60.0	3
27.0	0.0	0.0	100.0	9
32.0	0.0	0.0	100.0	5
33.0	88.0	10.0	2.0	5
45.0	88.0	10.0	2.0	5

Data processing

The Thermo Scientific™ Chromeleon™ 7.2 Chromatography Data System was used for data acquisition and analysis.

Result and discussion

At constant pH, a linear gradient of salt will elute plasmid DNA in order of increasing complexity of topoisomeric forms. Since the relative charge variance between different forms of plasmid DNA is comparatively high, they can be effectively separated using an ion exchange column. When separated by strong anion exchange, a plasmid DNA sample was found to contain two resolved peaks. The larger, later eluting peak is assumed to be the supercoiled plasmid DNA, while the smaller of the two (approximately 0.5% of the major peak) is assumed to be the linear form of the plasmid (Figure 1). Figure 2 overlays this plasmid sample with a diluent injection, confirming the smaller peak is related to the plasmid. Supercoiled plasmid demonstrates higher retentivity on a strong anion exchange (SAX) stationary phase compared to linear forms, with baseline separation.

ND1 enzyme is known to linearize plasmid DNA from its supercoiled form. A supercoiled plasmid sample incubated with ND1 enzyme showed two major peaks (Figure 3). The first peak is linearized plasmid, the second is supercoiled plasmid that was not linearized during treatment. This was confirmed by spiking linear plasmid sample into the supercoiled plasmid sample at 50% (Figure 4) and at 5% (Figure 5) just before injection to demonstrate the ratio change of linear plasmid to supercoiled plasmid at different concentrations.

The chromatographic profile of a second sample, containing RNA and protein, but no plasmid, (Figure 6) analyzed with the same chromatographic conditions shows two major peaks, both with retention times less compared to the plasmid related peak. These peaks are labeled as RNA and protein peak based on a comparison of the absorbance for both peaks at different wavelengths. Absorbance for the protein is relatively higher at 210 nm compared to 260 nm; for the RNA related peak, the absorbance value is higher at 260 nm. Protein absorbance was also confirmed at 280 nm.

Lastly, to demonstrate the separation of different forms of plasmid in the presence of RNA and protein, Figure 7 shows that the anion exchange column is able to separate these impurities without interfering with the different forms of plasmid.

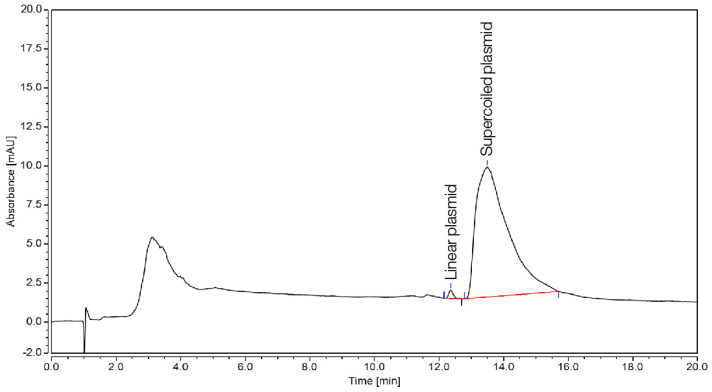

Figure 1. Chromatogram of supercoiled plasmid sample

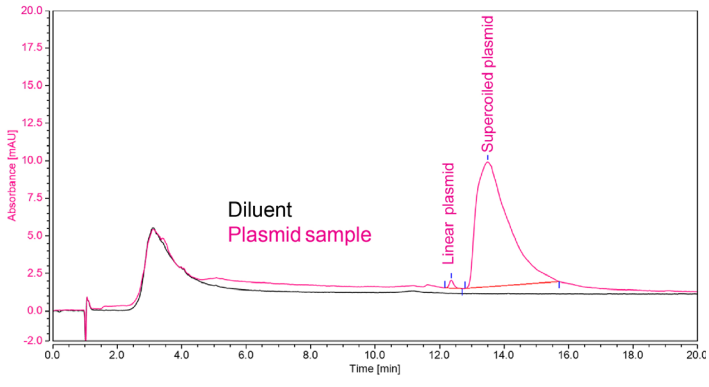

Figure 2. Overlay of supercoiled plasmid sample and diluent

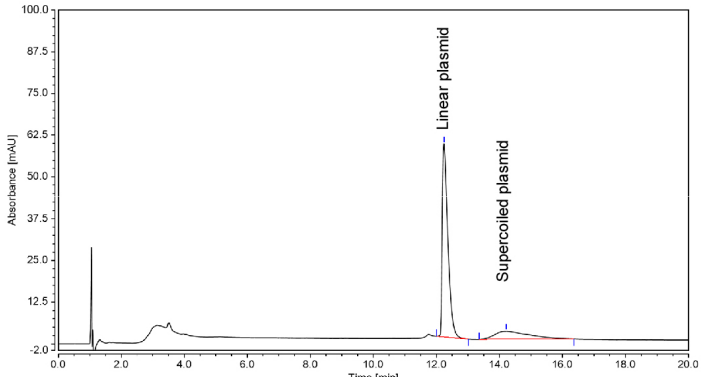

Figure 3. Chromatogram of supercoiled plasmid sample after incubation with ND1 enzyme

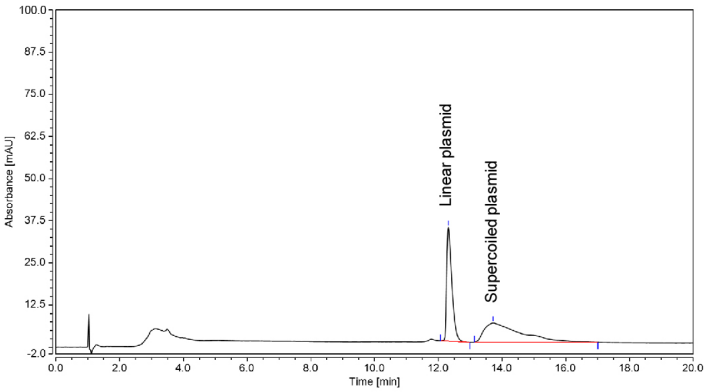

Figure 4. Chromatogram of supercoiled plasmid sample spiked with linear form of plasmid at 50% level

thermo scientific

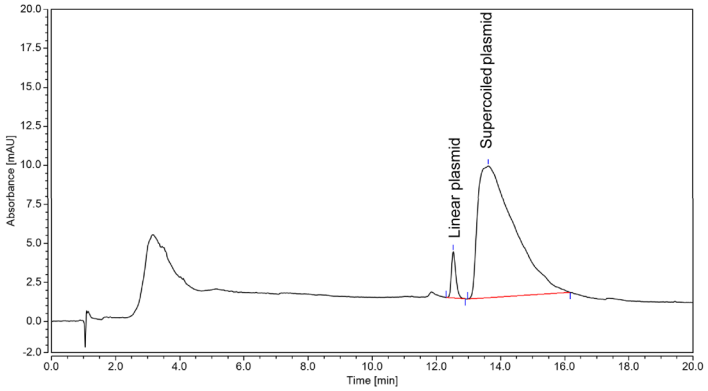

Figure 5. Chromatogram of supercoiled plasmid sample spiked with linear form of plasmid at 5% level

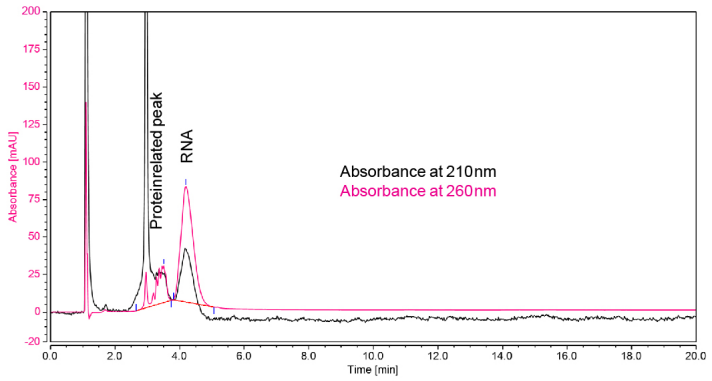

Figure 6. Overlay of RNA and protein sample at different wavelengths

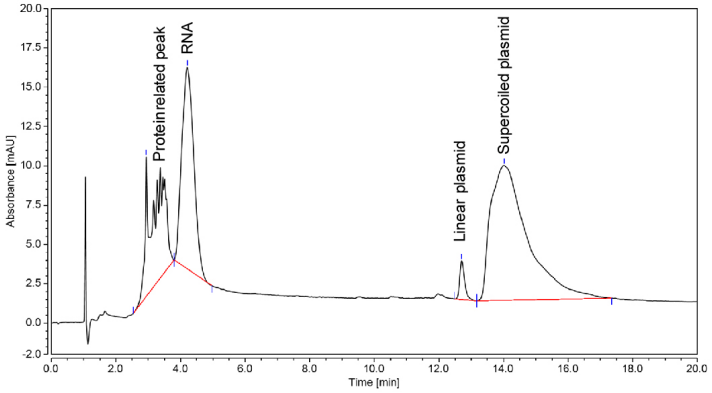

Figure 7. Chromatogram showing separation of different form of plasmid in presence of protein and RNA

Conclusion

Anion-exchange chromatography on the DNAPac PA200 column readily separates different forms of plasmid that are likely to be generated during a production stage. UV absorbance detection provides ample sensitivity and easy monitoring for plasmid samples.

The DNAPac PA200 column withstands the column cleanup step necessary to prevent fouling after multiple injections. The method performs robustly for inter-day and intra-day analysis. The developed method shows capabilities of separation for linear plasmid ranging from 5% to 50% in supercoiled plasmid sample. The DNAPac PA200 column is a modern pelicular resin format that uses surface ion exchange sites. The surface exchange allows for high resolution and fast mass transport but is also ideal for the very large DNA plasmids that would have difficulty entering the pores of a porous resin structure.

Though the separation of different forms of plasmid can be determined by RP HPLC, the ion exchange approach provides an orthogonal and better separation method for different forms of plasmid.

References

1. Thiel, K.W., and Giangrande, P. H. Therapeutic applications of DNA and RNA Aptamers. Oligonculeotides 2009, 19(3), 209-222
2. Diogo, M.M. et al. Chromatography of plasmid DNA, Journal of Chromatography A, 2005, 1069, 3–22.
3. Benčina, M et al. Journal of Separation Science, 2004, 27(10-11), 801-810.
4. Gene Therapy Clinical Trials Worldwide, The Journal of Gene Medicine, 2021. https://a873679.fmphost.com/fmi/webd/GTCT
5. Huangjjn Li, Huabebo and shulin huang, separation of supercoiled from open circular forms of plasmid DNA, and biological activity detection volume 63, pages7–12 (2011) cyto technology 63, pages7–12(2011)
6. High resolution nucleic acid separation by high performance liquid chromatography by James R Thayer, Randy M. Mccormick and Nebojsa Avdalovic.

Find out more at
thermofisher.com/chromatographyconsumables

ThermoFisher SCIENTIFIC