Efficient HIC plasmid isoform analysis

Artificial plasmid DNA (pDNA) can exist in different isoforms: open circular, linear and supercoiled (see Figure 1). Hydrophobic interaction chromatography (HIC) is particularly suitable for separating these forms from each other. The described practical example will help you to ensure that you have all the important parameters for your HIC method development in view. The following aspects are decisive for method development:

- **Influence of salt concentration**
- **Optimum separation conditions for different buffer systems**
- **Influence of temperature**

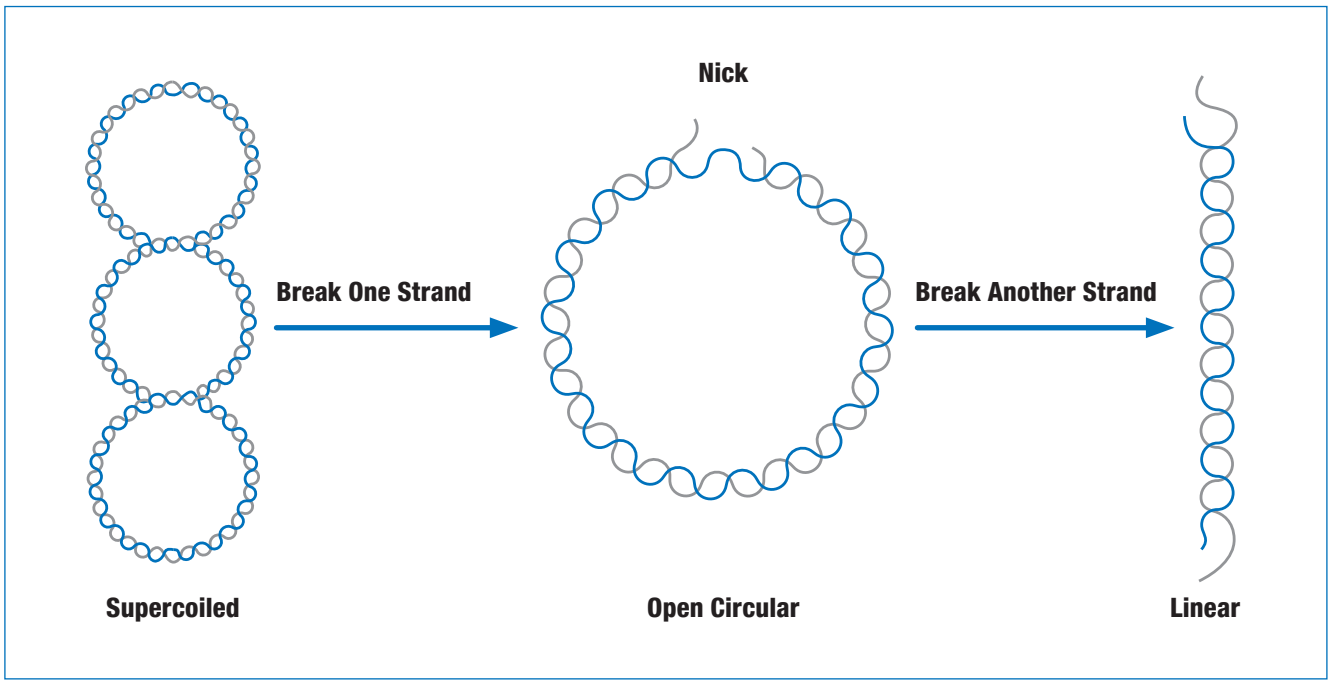

Figure 1: pDNA isoforms

The influence of the initial salt concentration

The composition of the mobile phase significantly impacts HIC analysis, particularly the choice of salt and its concentration, which affect analyte binding. Ammonium sulphate is commonly used for its strong salting-out effect. With higher initial concentrations of ammonium sulphate the analyte is stronger retained, thereby directly influencing the analysis time.

In HIC, the added salt itself as well as its concentration, which have an effect on analyte binding, are decisive for the separation.

To illustrate this, different concentrations of ammonium sulphate were used in this example (see Figures 2 & 3). The gradient of the slope was adjusted accordingly in each case. Initially, a Tris-HCl buffer was used as mobile phase.

However, with 2.2 M ammonium sulphate, the open circular and linear isoforms were not sufficiently retained. As a result, 2.3 M ammonium sulphate was identified as the optimal initial concentration, providing the shortest analysis time while ensuring sufficient analyte retention. Using phosphate buffer as mobile phase and an initial concentration of 2.3 M ammonium sulphate shortened the analysis time. All pDNA isoforms were well separated. In order to monitor the influence of other factors as closely as possible, a concentration of 2.5 M ammonium sulphate was used for further experiments.

Table 1: Chromatographic conditions.

Column:	BioPro HIC BF (4 µm), 100 x 4.6 mm ID
Part No.:	BF00S04-1046WT
Eluents:	A) 50 mM buffer containing different concentrations $(NH_4)_2SO_4$ (see Figures 2 & 3)
	B) 50 mM buffer
Gradient:	X%B (0–3.5 min), X–20%B (gradient slope 0.59%/min), 20%B (7 min after gradient elution)
Flow rate:	0.5 mL/min
Temperature:	25 °C
Detection:	UV at 260 nm
Injection:	10 µL undigested pUC19, 4 µL linear isoform, 4 µL open circular isoform
Sample:	pUC19 plasmid extracted from *E. coli* (dominated with supercoiled isoform, 150 µg/mL)
	Nt.BspQI*1-digested pUC19 (open circular isoform, 150 µg/mL)
	BamHI*2-digested pUC19 (linear isoform, 150 µg/mL)

*1 nicking endonuclease, *2 restriction enzyme

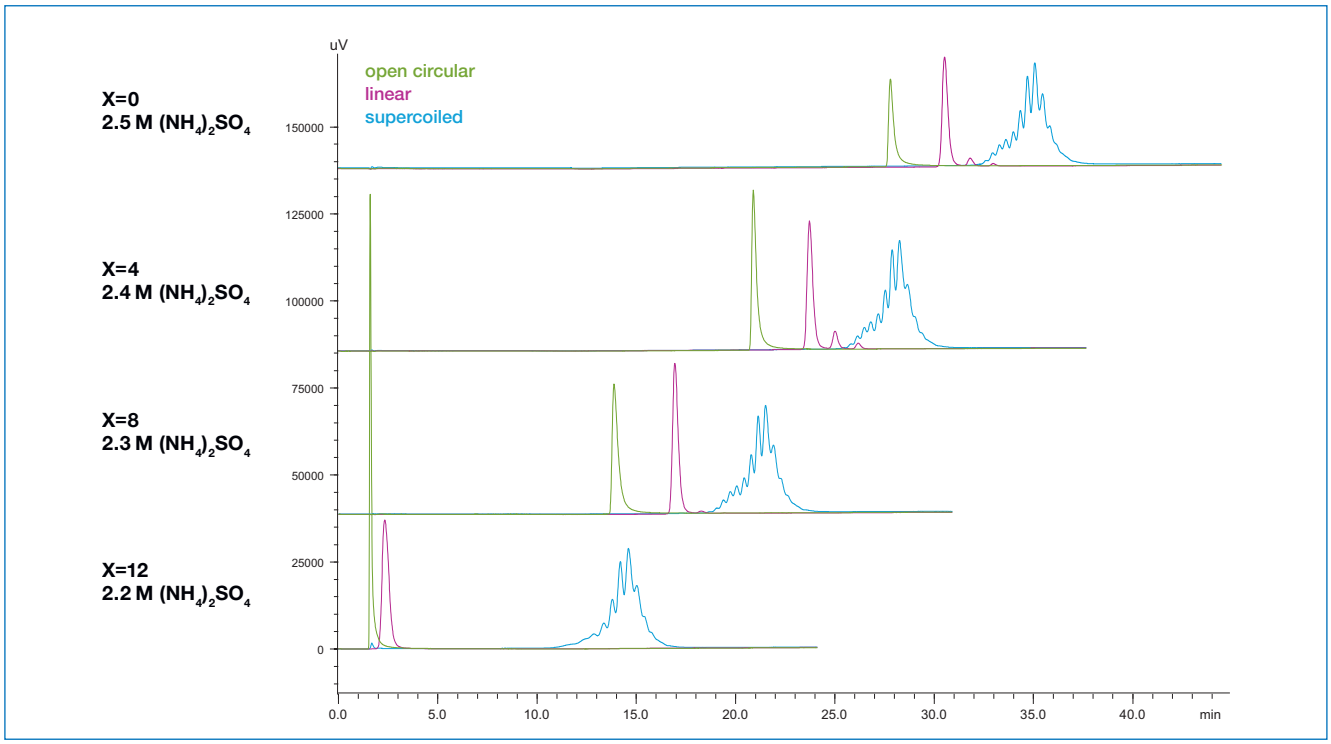

Figure 2: Determination of the optimal initial salt concentration for pDNA isoform analysis using Tris-HCl buffer as mobile phase.

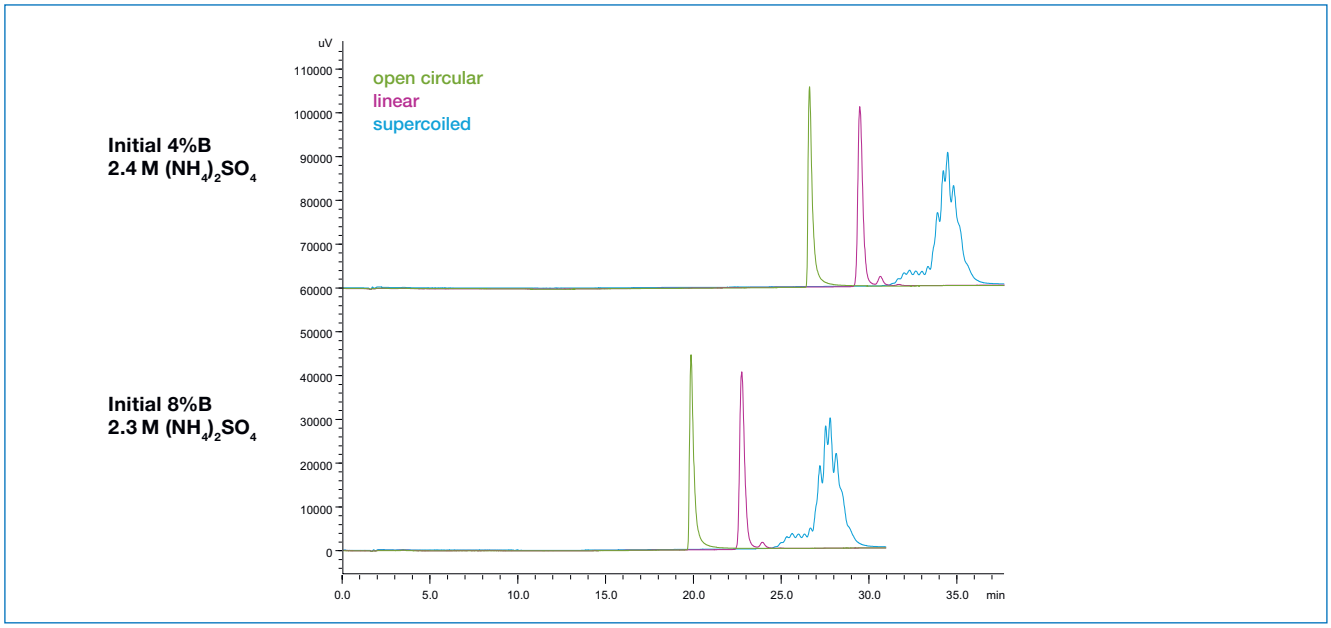

Figure 3: Determination of the optimal initial salt concentration for pDNA isoform analysis using phosphate buffer as mobile phase.

Optimisation of the flow rate

Higher flow rates can reduce analysis time, so determining an optimal flow rate for both buffers is critical. When Tris-HCl buffer was used as mobile phase, increasing the flow rate resulted in increased peak tailing and inadequate separation of all isoforms (see Figure 4).
In contrast, using phosphate buffer, all pDNA isoforms were successfully separated at every flow rate tested (see Figure 5). By increasing the flow rate to 1.0 mL/min, the analysis time was reduced by 50% to 17.5 minutes.

Table 2: Chromatographic conditions.

Column:	BioPro HIC BF (4 µm), 100 x 4.6 mm ID
Part No.:	BF00S04-1046WT
Eluents:	A) 50 mM buffer containing 2.5 M $(NH_4)_2SO_4$
	B) 50 mM buffer
Gradient:	1) 4%B (0–3.5 min), 4–20%B (3.5–30.7 min), 20%B (30.7–37.7 min)
	2) 4%B (0–2.3 min), 4–20%B (2.3–20.5 min), 20%B (20.5–25.0 min)
	3) 4%B (0–1.8 min), 4–20%B (1.8–15.4 min), 20%B (15.4–18.9 min)
Flow rate:	see Figures 4 & 5
Temperature:	25 °C for Tris-HCl buffer
	40 °C for phosphate buffer
Detection:	UV at 260 nm
Injection:	9 µL undigested pUC19, 4 µL linear isoform, 4 µL open circular isoform
Sample:	pUC19 plasmid extracted from *E. coli* (dominated with supercoiled isoform, 83 µg/mL)
	Nt.BspQI*1-digested pUC19 (open circular isoform, 33 µg/mL)
	BamH I*2-digested pUC19 (linear isoform, 33 µg/mL)

*1 nicking endonuclease, *2 restriction enzyme

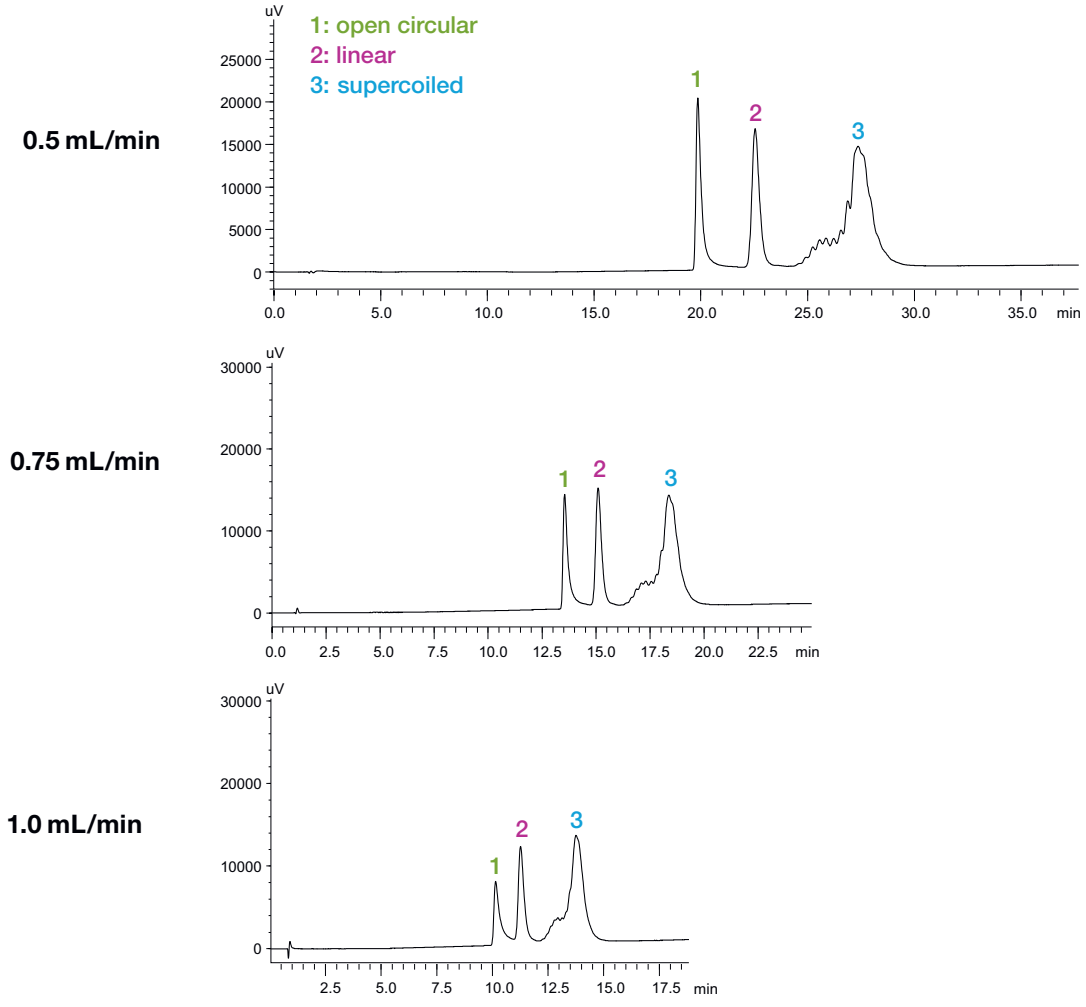

Figure 4: Influence of the flow rate on the separation of all three pDNA isoforms using Tris-HCl buffer as mobile phase.

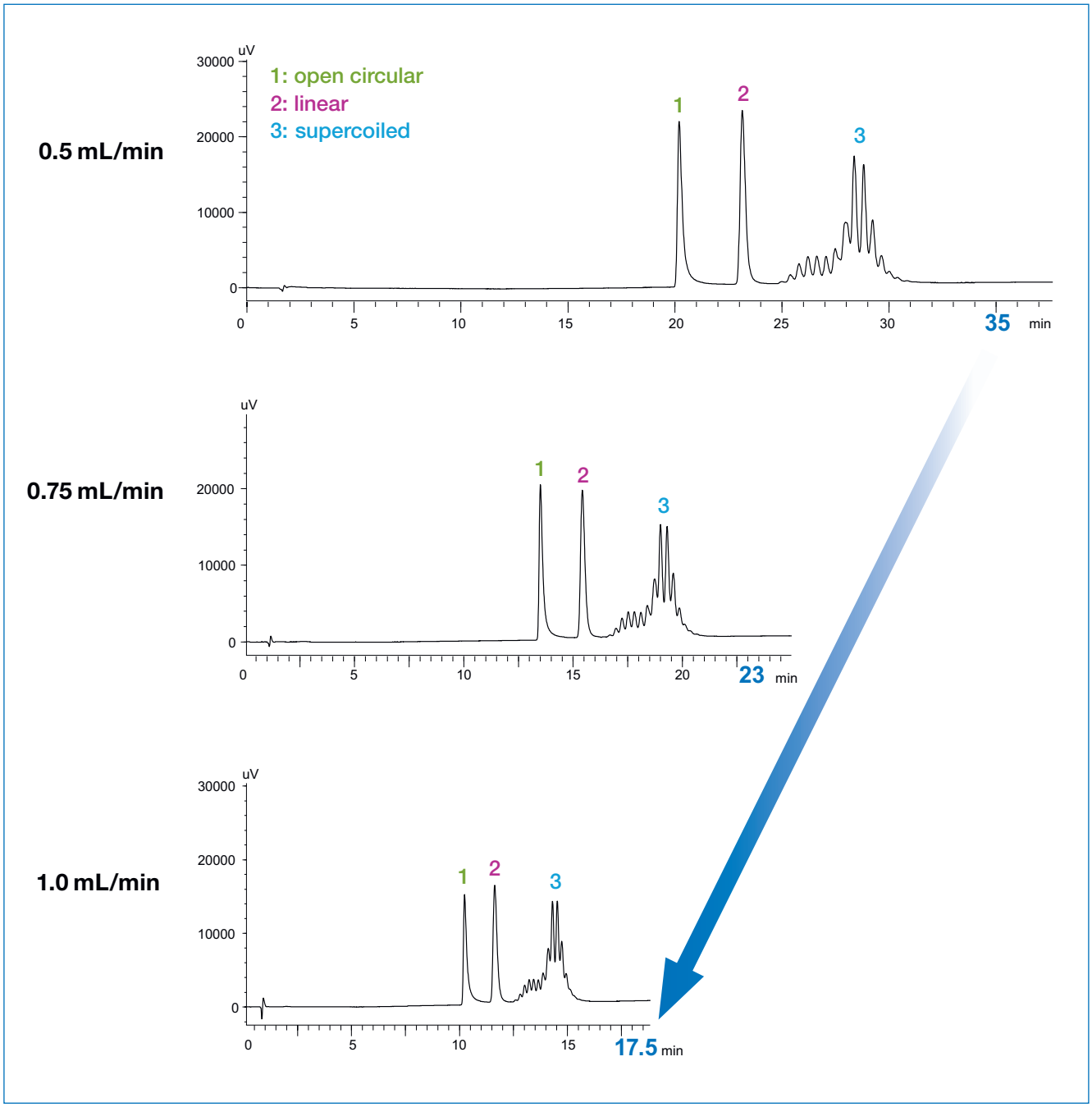

Figure 5: Influence of the flow rate on the separation of all three pDNA isoforms using phosphate buffer as mobile phase.

The influence of temperature on the separation of pDNA isoforms

Column temperature can significantly influence the analysis time and other parameters such as peak shape. This makes it a very valuable tool for optimizing the separation. For the analysis of pDNA, three temperatures were examined using phosphate buffer as mobile phase (see Figure 6). Higher temperatures were accompanied with shorter analysis times and improved peak shapes. However, the analysis was not carried out with Tris-HCl buffer because its pH is highly sensitive to temperature changes.

Table 3: Chromatographic conditions.

Column:	BioPro HIC BF (4 µm), 100 x 4.6 mm ID
Part No.:	BF00S04-1046WT
Eluents:	A) 50 mM NaH_2PO_4-Na_2HPO_4 containing 2.5 M $(NH_4)_2SO_4$ (pH 6.5)
	B) 50 mM NaH_2PO_4-Na_2HPO_4 (pH 6.5)
Gradient:	1) 4%B (0–3.5 min), 4–20%B (3.5–30.7 min) 20%B (30.7–37.7 min)
	2) 4%B (0–3.5 min), 4–30%B (3.5–47.7 min) 30%B (47.7–54.7 min)
Flow rate:	0.5 mL/min
Temperature:	see Figure 6
Detection:	UV at 260 nm
Injection:	10 µL undigested pUC19, 4 µL linear isoform, 4 µL open circular isoform
Sample:	pUC19 plasmid extracted from *E. coli* (dominated with supercoiled isoform, 150 µg/mL)
	Nt.BspQI*1-digested pUC19 (open circular isoform, 150 µg/mL)
	BamHI*2-digested pUC19 (linear isoform,150 µg/mL)

*1 nicking endonuclease, *2 restriction enzyme

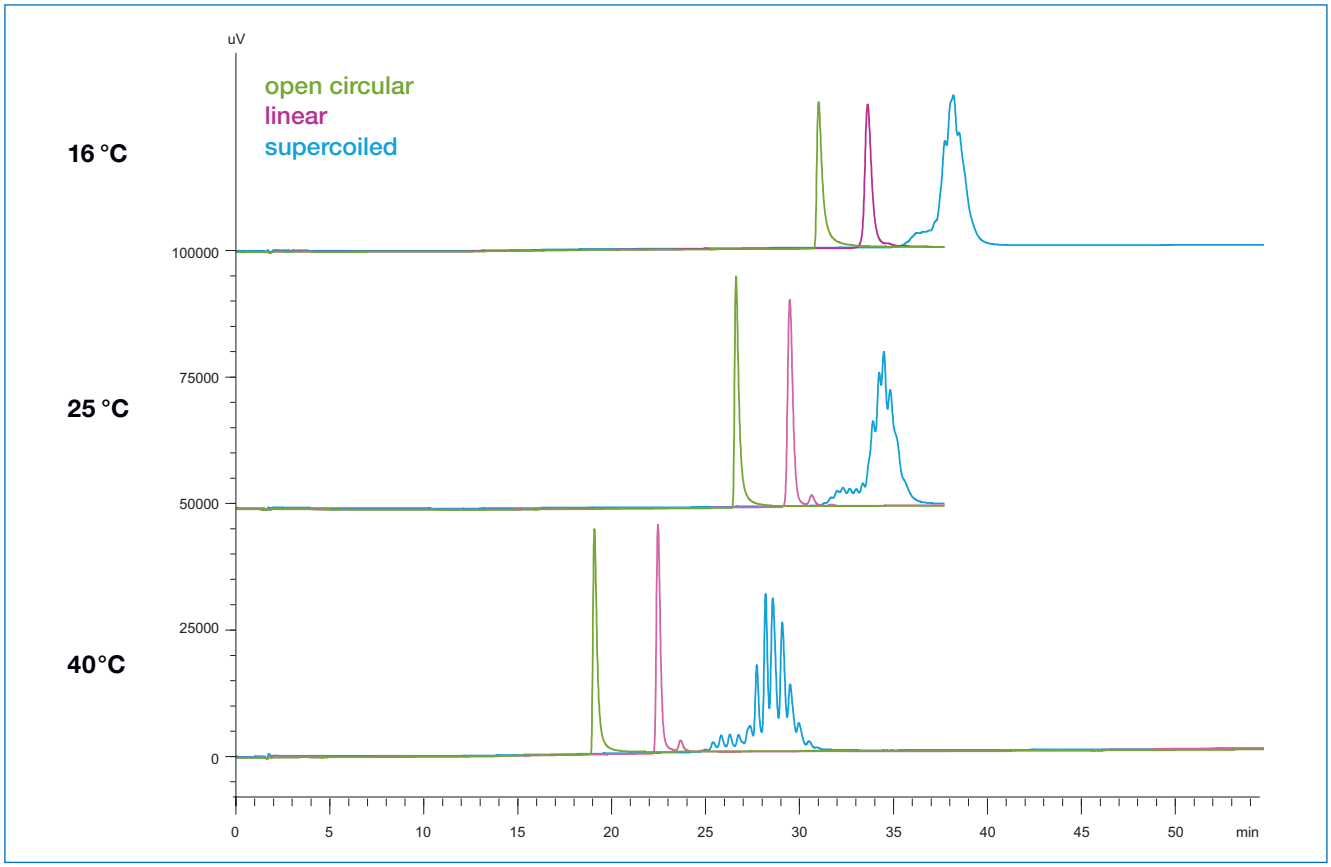

Figure 6: Influence of temperature on the separation of all three pDNA isoforms using phosphate buffer.

Conclusions

With HIC, the choice of salt and its initial concentration are critical for analyte binding. A low concentration is required to achieve short analysis times. In combination with a higher flow rate, the analysis can also be further accelerated. A higher temperature also shortens the analysis time. However, it is important to note that the buffer stability may vary with the temperature.