Technical Note

YMC

# Analysis of non-denatured and denatured siRNA with anion exchange chromatography (AEX)

Small interfering RNAs (siRNAs) are mainly double stranded RNA molecules with short lengths, which are able to interfere with gene expression. This makes them suitable tools for targeted gene therapy. siRNAs are typically analysed by ion pair reversed phase chromatography (IP-RP) or anion exchange chromatography (AEX). Chromatographic conditions allow analysis of double stranded duplex (non denaturing) or single strands (denaturing conditions). The stability of double stranded siRNAs also depends on external stress such as temperature, salts, organic solvents or other chemical additives.

This technical note summarises various method parameters using a non-porous BioPro IEX QF column. In addition, various method parameters for purification are also described. The siRNA duplex of the firefly luciferase GL 2 gene was used as a model compound (table 1).

*Table 1: Firefly luciferase siRNA*

| | |
|---|---|
| 5'-CGU ACG CGG AAU ACU UCG AdTdT-3' | Sense strand |
| 3'-dTdTGCA UGC GCC UUA UGA AGC U-5' | Antisense strand |

## Method development under denaturing conditions

Denaturing conditions allow sense and antisense strand to be analysed. In order to efficiently denature double stranded oligonucleotides, sodium hydroxide (NaOH) is commonly used. NaOH strongly increases the pH, thereby breaking the hydrogen bonds between the two RNA strands. On-column denaturation of the siRNA duplex can be achieved by using 10 mM NaOH as mobile phase.

*Table 2: Overall chromatographic conditions unless otherwise stated.*

| | |
|---|---|
| Column: | BioPro IEX QF (5 µm) 100 x 4.6 mm ID |
| Part No.: | QF00S05-1046WP |
| Eluent: | A) 10 mM NaOH |
| | B) 10 mM NaOH containing 1.0 M $NaClO_4$ |
| Gradient: | See table 3 |
| Flow rate: | 0.5 ml/min |
| Temperature: | 25 °C |
| Detection: | UV at 260 nm |
| Injection: | 4 µl (5 nmol/ml) |
| Sample: | siRNA duplex, sense strand, antisense stand |

Gradient slopes with different initial composition and enhancement show nicely the effect on the overall elution profile (Table 3, Figure 1). Steeper gradients with a lower initial percentage of mobile phase B (gradient 1 and 2) show sharper peaks but longer retention and less resolution. In contrast, shallower gradients result in a faster and better separation of the two peaks (gradient 3 and 4).

The condition with the best resolution (gradient 4) was used for separating the siRNA duplex as well as sense and antisense strands. A good separation of both strands, sharp peaks and a high resolution were achieved.

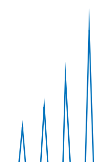
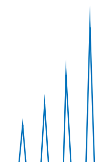


*Table 3: Gradient conditions used for analysis of the denatured siRNA.*

| Gradient | Time [min] | %B | %B/min |
|---|---|---|---|
| 1 | 0–10 | 32–42 | 1.0 |
| 2 | 0–10 | 30–40 | 1.0 |
| 3 | 0–10 | 33–40 | 0.7 |
| 4 | 0–10 | 33–38 | 0.5 |

| Gradient | RT1 [min] | RT2 [min] | Rs (1,2) |
|---|---|---|---|
| 1 | 10.07 | 10.46 | 1.66 |
| 2 | 12.58 | 12.87 | 1.44 |
| 3 | 8.55 | 9.26 | 1.87 |
| 4 | 8.62 | 9.45 | 1.92 |

*Figure 1: Influence of gradient slope on the separation of the denatured siRNA duplex.*



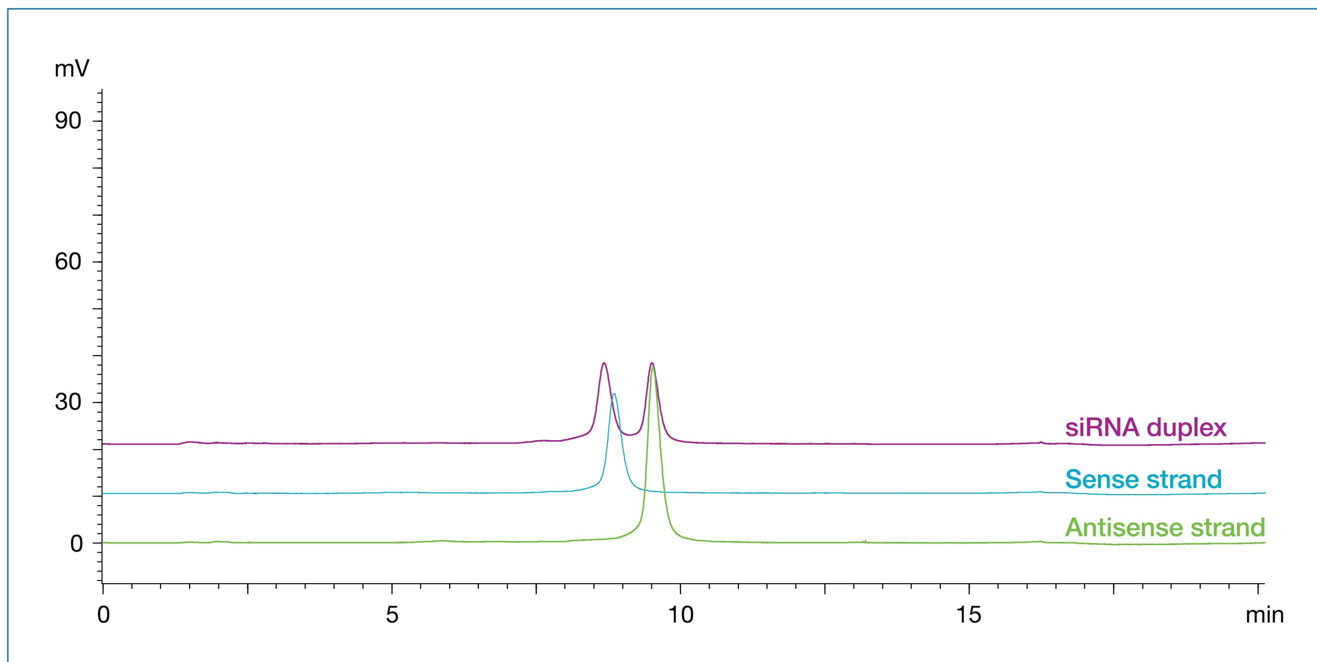



*Figure 2: Analysis of the siRNA duplex, sense strand and antisense strand with optimal gradient slope at denaturing conditions.*

# Method development under non-denaturing conditions

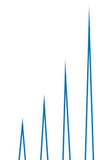
siRNA, sense strand and antisense strand can be analysed in a non-denatured state, for example to detect excessive single strands. The influence of pH, temperature, salts, organic solvents and urea on the analysis of the siRNA duplex and its single strands were investigated under non-denaturing conditions.

*Table 4: Overall chromatographic conditions unless otherwise stated.*

| | |
|---|---|
| Column: | BioPro IEX QF (5 µm) 100 x 4.6 mm ID |
| Part No.: | QF00S05-1046WP |
| Eluent: | A) 20 mM Tris-HCl (pH 7.6, 8.1 or 8.6) |
| | B) 20 mM Tris-HCl (pH 7.6, 8.1 or 8.6) containing 1M NaCl or $NaClO_4$ |
| Gradient: | [NaCl]: 40–90%B (0–25 min), 90%B (25–35 min), 40%B (35–60 min) |
| | [$NaClO_4$]: 25–40%B (0–30 min), 40%B (30–40 min), 25%B (40–65 min) |
| Flow rate: | 0.5 ml/min |
| Temperature: | 25 °C, 40 °C or 60 °C |
| Detection: | UV at 260 nm |
| Injection: | 4 µL (5 nmol/mL) |
| Sample: | sense strand, antisense strand and siRNA duplex |

### 1. Influence of pH, salts and temperature on the analysis of siRNA duplex and it single strands

The siRNA duplex showed acceptable peak shapes at all conditions tested (Figure 3). In contrast, sense and antisense strand were more sensitive to temperature and pH variations. Peak asymmetry could be observed at any temperature and any pH. However, the higher temperature slightly improved the peak shape of the single strands.



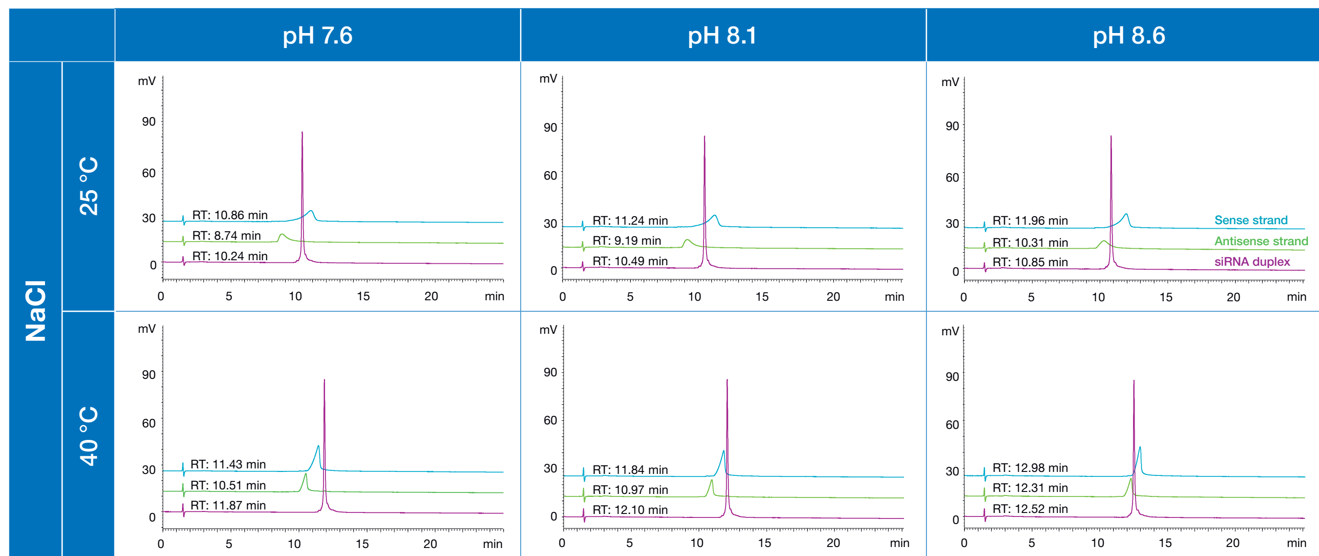



*Figure 3: Effect of temperature and pH on the analysis of the siRNA duplex, sense strand and antisense strand with mobile phase containing NaCl.*

When using $NaClO_4$ instead of NaCl as salt, the siRNA duplex again showed good peak shapes under all conditions tested (Figure 4). The peak shapes for sense and antisense strands improved at 40 °C as shown for the NaCl mobile phase. With a mobile phase containing $NaClO_4$ shorter retention times were achieved for all analytes. The best conditions for single and double stranded siRNA were observed with a mobile phase containing $NaClO_4$ and pH 8.1 at 40 °C. These conditions were used for further method development.

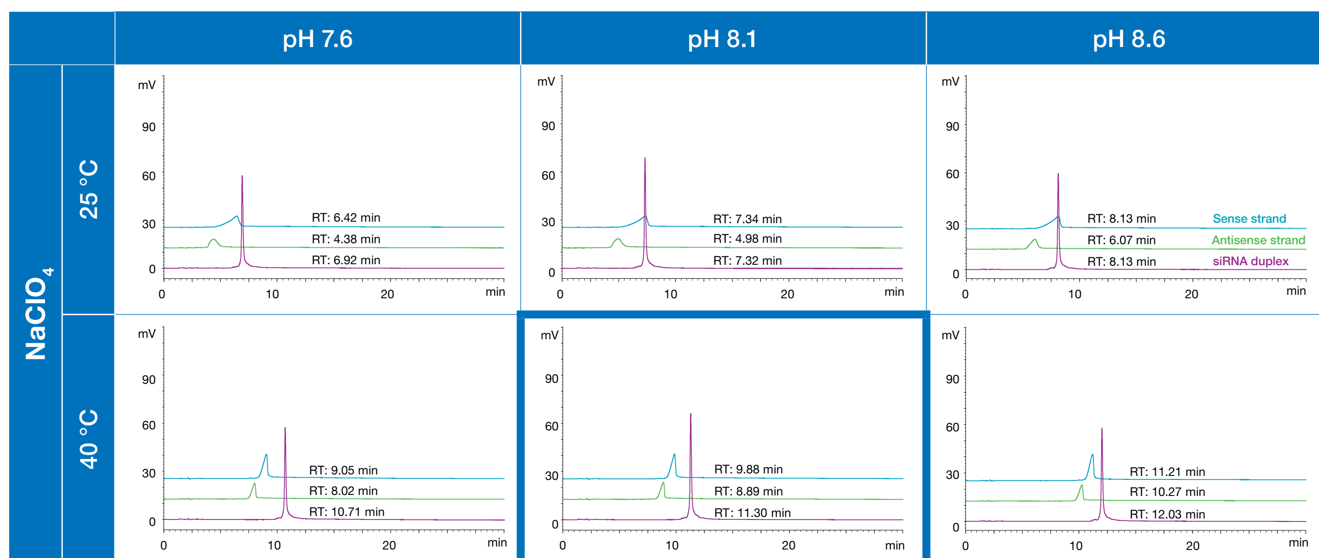



*Figure 4: Effect of temperature and pH on the analysis of the siRNA duplex, sense strand and antisense strand with mobile phase containing $NaClO_4$.*

Because elevated temperatures can improve chromatographic separations, temperatures of 60 °C were tested and compared to the previously determined 40 °C. A higher temperature of 60 °C resulted in better peak shape for sense and antisense strands (Figure 5). However, peak height of the siRNA duplex decreases due to partial denaturation. At 60 °C all three RNAs were retained longer on the stationary phase. Therefore, further experiments were carried out at 40 °C.

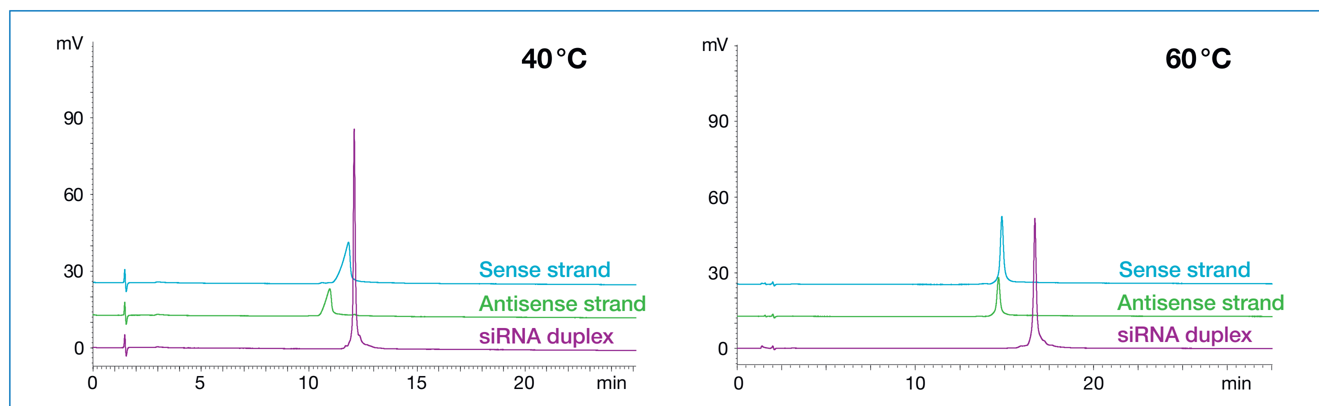



*Figure 5: Effect of elevated temperature on the analysis of siRNA duplex and sense and antisense strands.*

## 2. Influence of organic solvents on the analysis of siRNA duplex and its single strands

The addition of organic solvents changes the hydration of the ions and the ion exchange site within the stationary phase. This affects their selectivity. The influence of organic solvents was tested by adding 30% methanol or acetonitrile to the mobile phase. Addition of 30% methanol or acetonitrile resulted in an increase in the retention of the single strands and the siRNA duplex by approximately 15–20 minutes although the peak shapes of all three analytes remained unaffected. Therefore, addition of solvents did not improve the analysis of the siRNA duplex and sense and antisense strands.

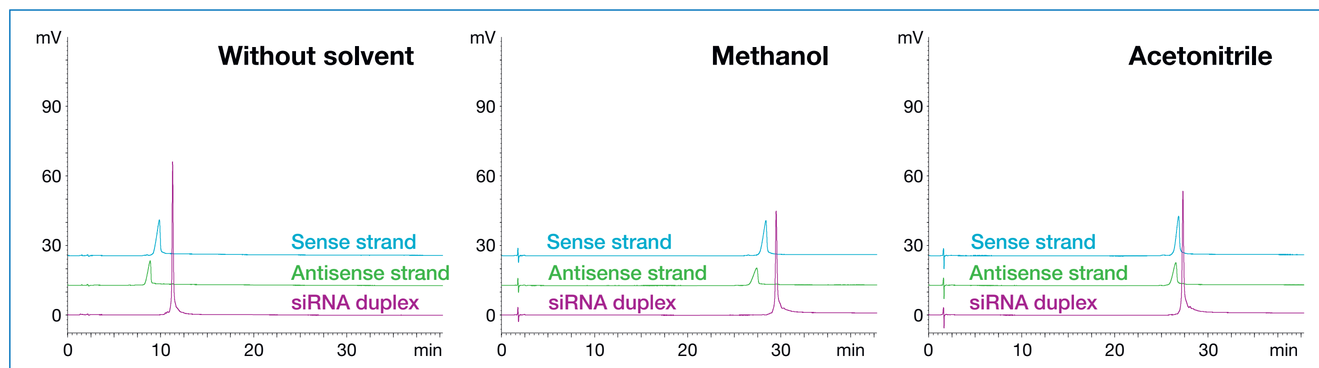



*Figure 6: Effect of organic solvents on the analysis of siRNA duplex and sense and antisense strands.*

## 3. Influence of urea as additive on the analysis of siRNA duplex and it single strands

It has been shown that urea unfolds not only proteins, but also secondary and tertiary structures of RNAs. Therefore, the effect of urea as an additive in AEX analysis of the siRNA duplex and the sense and antisense strands was examined. The addition of 3 M urea to the mobile phase resulted in better peak shapes for both sense and antisense strands and the siRNA duplex. The retention time was reduced for all three analytes and an improvement in the resolution of single stranded and double stranded siRNA was also observed.

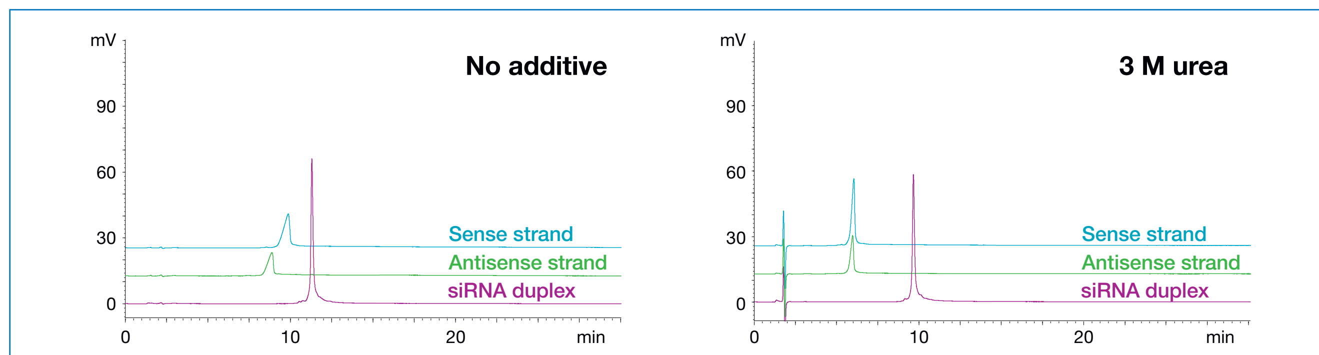



*Figure 7: Effect of urea as additive on the analysis of siRNA duplex and sense and antisense strands.*

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## Method development towards purification of siRNA

The BioPro IEX series enables the scale up from analytical to semipreparative and preparative scale. Therefore, based on the results of the analytical method development, the conditions most suitable for the semipreparative purification of the siRNA were investigated.

*Table 5: Chromatographic conditions.*

| | |
|---|---|
| Column: | BioPro IEX QF (5 µm) 100 x 4.6 mm ID |
| Part No.: | QF00S05–1046WP |
| Eluent: | A) 20 mM Tris–HCl (pH 8.1) containing urea |
| | B) 20 mM Tris–HCl (pH 8.1) containing 1M NaCl + urea |
| Gradient: | 25–40%B (0–30 min), 40%B (30–40 min), 25%B (40–65 min) |
| Flow rate: | 0.5 ml/min |
| Temperature: | 40 °C |
| Detection: | UV at 260 nm |
| Injection: | 4 µl (5 nmol/ml) |
| Sample: | sense strand, antisense strand and siRNA duplex |

Because larger volumes of eluent are used in preparative scale, the non-hazardous and more environmentally friendly NaCl was used as salt. Due to the positive effect of urea on analytical scale analysis, urea concentrations were tested in the mobile phase. On preparative scale, working at elevated temperatures is a major challenge. Therefore, parameters were tested not only at 40 °C but also at 25 °C. Increasing the urea concentration improved the peak shape of siRNA at 40 °C and 25 °C. High concentrations of 6 M urea gave the best results at 25 °C. However, at 40 °C 3 M urea resulted in sharp peaks and fast separation. Therefore 6 M urea was not tested at 40 °C.

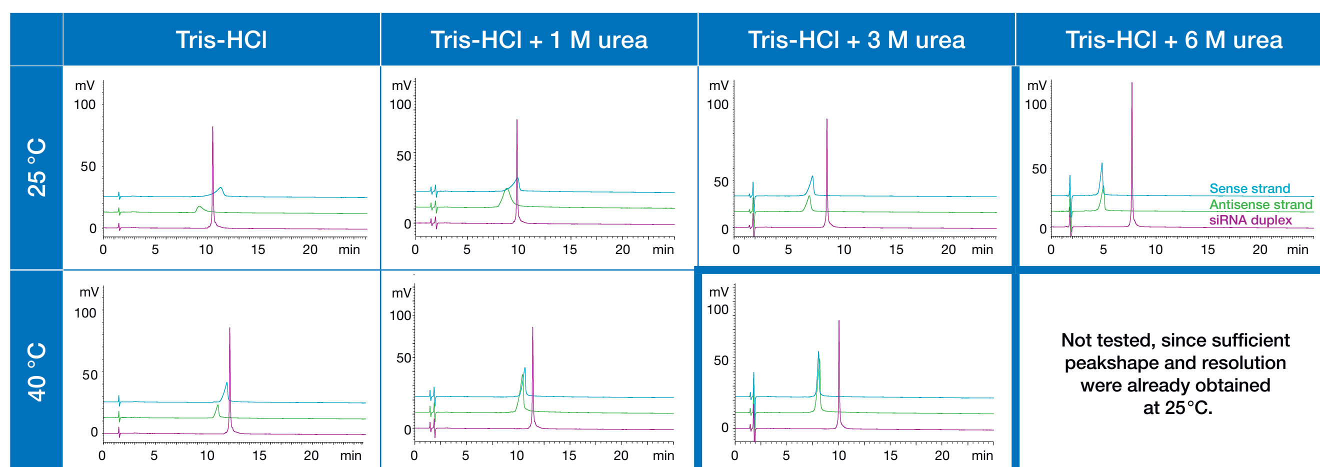


*Figure 8: Effect of urea and temperature on the purification of the siRNA duplex and sense and antisense strands.*

## Summary

Analysis of siRNA duplex and its single strands under **denaturing** conditions revealed that gradient steepness is an important factor in improving retention times and resolution.

When developing a method for **non-denaturing** siRNA duplex analysis, the following parameter should be considered:

- Choice of salt in the mobile phase
- Influence of temperature
- Influence of organic solvents
- Effect of urea as an additive

Method transfer from analytical to **preparative** scale was achieved considering the following factors:

- Hazardousness of mobile phase
- Impact of temperature
- Effect of urea as an additive