Analysis of non-denatured and denatured siRNA with anion exchange chromatography

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Introduction

Small interfering RNAs (siRNAs) are mostly double-stranded RNA molecules with short lengths, which are able to interfere with gene expression. This makes them a suitable tool for targeted gene therapy. siRNAs are typically analysed by ion pair reversed phase chromatography (IP-RP) or anion exchange chromatography (AEX). The chromatographic conditions can be chosen so that siRNAs can be analysed as double-stranded duplex under non-denaturing conditions or as two single strands under denaturing conditions. The stability of double-stranded siRNAs also depends on external stress such as temperature, salts, organic solvents or other chemical additives. In this study, various AEX method parameters to optimise siRNA analyses for denaturing and non-denaturing conditions are examined. The siRNA duplex of the firefly luciferase GL2 gene is used as a model compound.

Model Case Study

- Analytical method development for non-denatured and denatured siRNA
- The influence of salts, temperature, organic solvents or urea as additive is investigated
- BioPro IEX QF, a non-porous strong anion exchange column, is used as the stationary phase

Sample: 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

Conclusions

Non-denaturing conditions

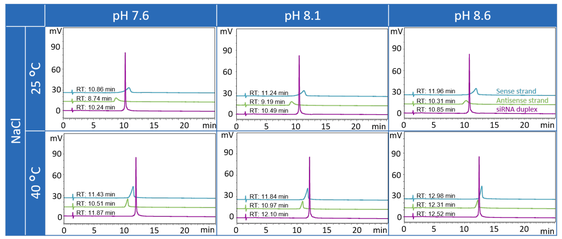
- NaClO4 improves separation of single and double stranded siRNA and also results in reduction of retention time.
- A higher temperature of 60 °C results in better peak shapes of single strands, but leads to partial denaturation of the siRNA duplex. 40 °C is therefore preferential.
- Addition of organic solvents to the mobile phase does not improve siRNA analysis.
- Addition of urea to the mobile phase leads to sharper peaks and an increase in resolution.

Denaturing conditions

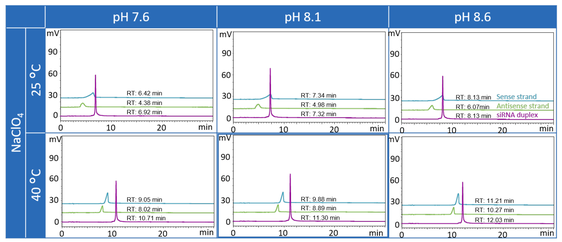
- A shallower gradient results in the reduction of retention times and improved resolution of the denatured siRNA duplex.

Non-denaturing conditions – different salts and pH at different temperatures

Column: BioPro IEX QF (5 µm) 100 x 4.6 mm ID
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 NaClO4
Gradient: [NaCl]: 40-90 %B (0-25 min), 90 %B (25-35 min), 40 %B (35-60 min)
[NaClO4]: 25-40%B (0-30 min), 40%B (30-40 min), 25%B (40-65 min)
Flow rate: 0.5 mL/min
Temperature: 25 or 40 °C
Detection: UV at 260 nm
Injection: 4 µL (each 5 nmol/mL)
Sample: sense strand, antisense strand, siRNA duplex



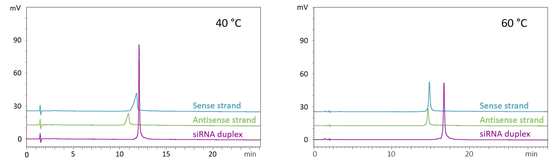
The siRNA duplex shows acceptable peak shapes at all conditions tested. In contrast, sense and antisense strand are more sensitive to variations in temperature and pH. Peak asymmetry can be observed at each temperature and pH. However, higher temperature slightly improves the peak shape of the single strands.



Using NaClO4 instead of NaCl, the siRNA duplex again shows good peak shapes under all conditions tested. The peak shapes of sense and antisense strands improve at 40 °C as demonstrated for the mobile phase with NaCl as salt. With mobile phase containing NaClO4 shorter retention times are achieved for all analytes. Best conditions tested for single and double stranded siRNA are observed with a mobile phase containing NaClO4 and a pH of 8.1 at 40 °C.

Non-denaturing conditions – at elevated temperature

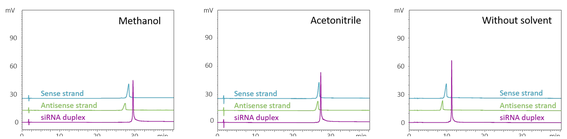
Column: BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Eluent: A) 20 mM Tris-HCl (pH 8.1)
B) 20 mM Tris-HCl (pH 8.1) containing 1M NaClO4
Gradient: 25-40 %B (0-30 min), 40 %B (30-40 min), 25 %B (40-65 min)
Flow rate: 0.5 mL/min
Temperature: 40 or 60 °C
Detection: UV at 260 nm
Injection: 4 µL (each 5 nmol/mL)
Sample: sense strand, antisense strand, siRNA duplex



Higher temperature of 60 °C provided better peak shape of sense and antisense strand. However, peak height of the siRNA duplex decreases due to partial denaturation. At 60 °C all three RNAs retained longer on the stationary phase.

Non-denaturing conditions – use of organic solvents

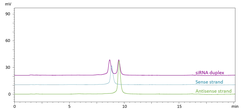
Column: BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Eluent: A) 20 mM Tris-HCl (pH 8.1) or mixed with solvent (70/30)
B) 20 mM Tris-HCl (pH 8.1) containing 1M NaClO4 or mixed with solvent (70/30)
Gradient: 25-40 %B (0-30 min), 40 %B (30-40 min), 25 %B (40-65 min)
Flow rate: 0.5 mL/min
Temperature: 40 °C
Detection: UV at 260 nm
Injection: 4 µL (each 5 nmol/mL)
Sample: sense strand, antisense strand, siRNA duplex



Addition of 30 % methanol or acetonitrile leads to an increase in the retention of the single strands and the siRNA duplex of about 15-20 min. The peak shapes of all three analytes is not improved when organic solvents are added to the mobile phase. Therefore, addition of solvents does not improve the analysis of firefly luciferase GL2 siRNA.

Denaturing conditions – optimum for single and double strands

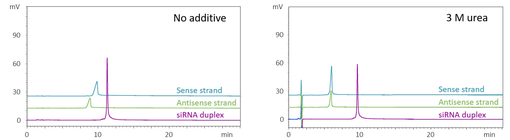
Analysis of all three analytes with the optimised conditions reveal a good separation of sense and antisense strands as well as the siRNA duplex. Sharp peaks and high resolution are achieved by using the shallowest gradient.



Column: BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Eluent: A) 10 mM NaOH
B) 10 mM NaOH containing 1.0 M NaClO4
Gradient: 33-38%B (0-10 min), 100%B (10-20 min)
Flow rate: 0.5 mL/min
Temperature: 25 °C
Detection: UV at 260 nm
Injection: 4 µL (each 5 nmol/mL)
Sample: sense strand, antisense strand, siRNA duplex

Non-denaturing conditions – addition of urea

Column: BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Eluent: A) 20 mM Tris-HCl (pH 8.1) containing 3 M Urea (pH 8.1)
B) 20 mM Tris-HCl (pH 8.1) containing 1M NaClO4
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 (each 5 nmol/mL)
Sample: sense strand, antisense strand, siRNA duplex of the firefly luciferase GLS

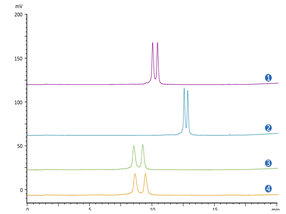


Addition of 3 M urea to the mobile phase results in better peak shapes of both sense and antisense strands as well as the siRNA duplex. The retention time is reduced for all three analytes and an improvement in resolution of the single strands and the double stranded siRNA is also observed.

Denaturing conditions – impact of gradient slope

Column: BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Eluent: A) 10 mM NaOH
B) 10 mM NaOH containing 1.0 M NaClO4
Gradient: see table
Flow rate: 0.5 mL/min
Temperature: 25 °C
Detection: UV at 260 nm
Injection: 4 µL (each 5 nmol/mL)
Sample: siRNA duplex

	Time	%B	%B/min	RT1 [min]	RT2 [min]	Rs 1,2
1	0-10	32-42	1.0	10.07	10.46	1.66
2	0-10	30-40	1.0	12.58	12.87	1.44
3	0-10	33-40	0.7	8.55	9.26	1.87
4	0-10	33-38	0.5	8.62	9.45	1.92



On column denaturation of the siRNA duplex is achieved using 10 mM NaOH as mobile phase and at a temperature of 25 °C. As the gradient has the highest impact, different slopes were tested: Steeper gradients (1 & 2) with a lower initial percentage of mobile phase B show sharper peaks but longer retention and less resolution. In contrast, shallower gradients result in a faster and better separation of the two peaks (3 & 4).