(U)HPLC analysis of siRNA under denaturing and non-denaturing conditions using IP-RP and AEX

Small interfering RNA (siRNA) are short oligonucleotides, which effectively reduce gene expression. siRNAs can be analysed by high-performance liquid chromatography (HPLC) as double-stranded duplex under non-denaturing conditions or as two single strands under denaturing conditions. Examination of the siRNA under non-denaturing conditions can be helpful to prove the success of duplex formation and detect the amount of excess single stranded RNA.

The analysis of siRNA is quite challenging due to their negatively charged phosphate backbone. Ion pair reversed phase liquid chromatography (IP-RP) is often the first choice for characterisation of such oligonucleotides. However, anion exchange chromatography (AEX) is also a popular method for oligonucleotide analysis. In this application note, the analysis of denatured and non-denatured siRNA by use of the bioinert YMC-Accura Triart Bio C18 column in IP-RP mode and the BioPro IEX QF column in AEX mode, is presented.

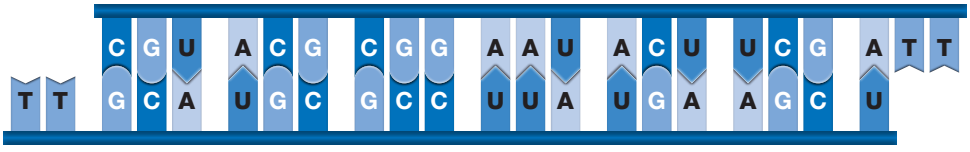

Table 1: Sequence of sense and antisense strands of the siRNA duplex

siRNA	5'-CGU ACG CGG AAU ACU UCG AdTdT-3' 3'-dTdTGCA UGC GCC UUA UGA AGC U-5'	sense strand antisense strand

Analysis of a siRNA duplex by IP-RP

The used YMC-Accura Triart Bio C18 column is ideal for the optimum conditions of pH 8 and temperatures of up to 65 °C, due to its robust hybrid-silica based stationary phase. The column body and frits are further provided with a bioinert surface coating to prevent irreversible adsorption of the charged oligonucleotides to the metal surface of the column. Together with the inert stationary phase a successful analysis of siRNA with excellent peak shapes and high recoveries using either denaturing (figure 1) or non-denaturing conditions (figure 2) was possible. Denaturing conditions were achieved by the use of TEAA at pH 8 and temperatures of 65 °C, while the duplex remained stable applying TEA-HFIP at pH 8 and 25 °C only.

Table 2: Chromatographic IP-RP conditions – denaturing

Column:	YMC-Accura Triart Bio C18 (1.9 µm, 30 nm) 50 x 2.1 mm ID
Part No.:	TA30SP9-05Q1PTC
Eluent:	A) 15 mM TEAA* (pH 8)
	B) methanol
Gradient:	5%–20%B (0–15 min)
Flow rate:	0.42 ml/min
Temperature:	65 °C
Detection:	UV at 260 nm
Injection:	1 µl (5 nmol/ml)
Sample:	siRNA duplex

*triethylammonium acetate

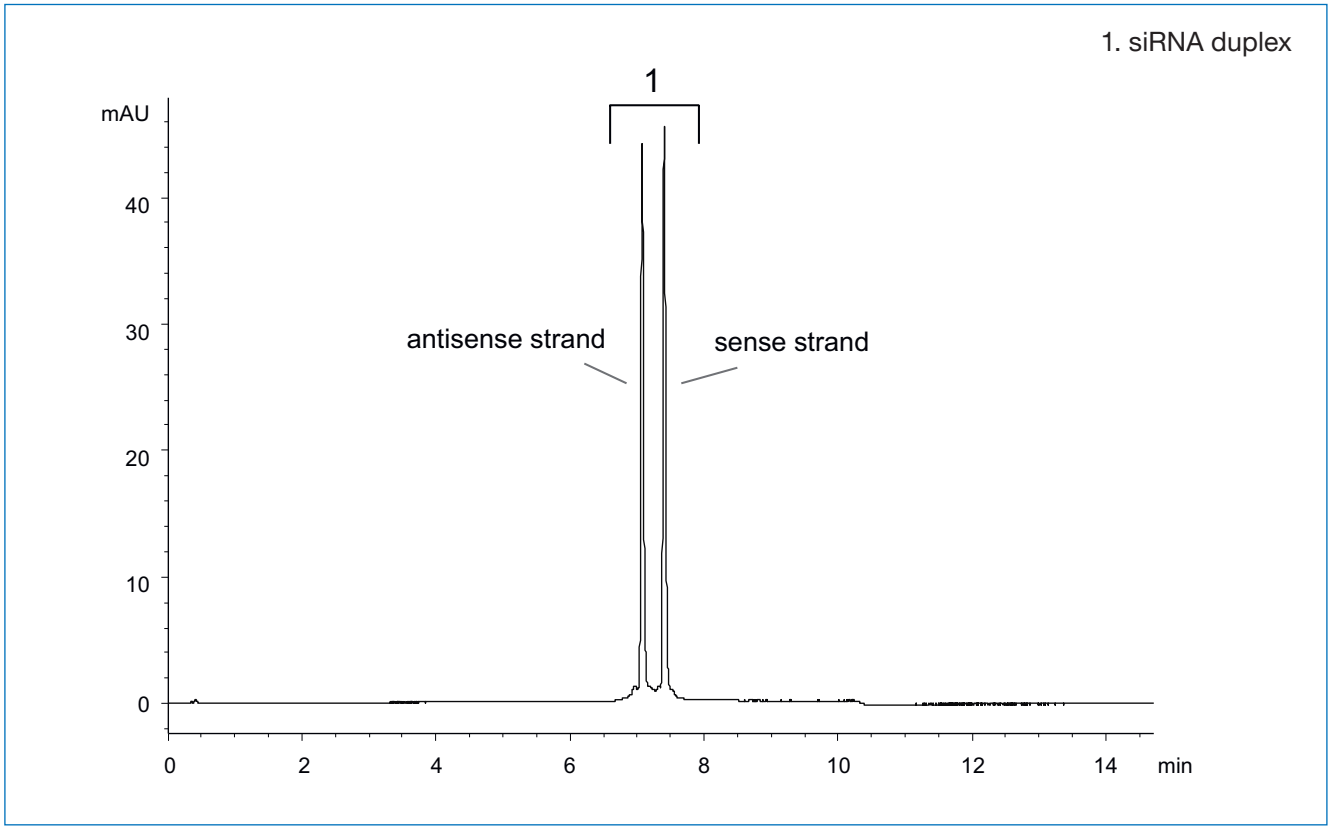

Figure 1: IP-RP analysis of the siRNA duplex under denaturing conditions using a YMC-Accura Triart Bio C18 UHPLC column.

Table 3: Chromatographic IP-RP conditions – non-denaturing

Column:	YMC-Accura Triart Bio C18 (1.9 µm, 30 nm) 50 x 2.1 mm ID
Part No.:	TA30SP9-05Q1PTC
Eluent:	A) 15 mM triethylamine - 400 mM HFIP* (pH 8)
	B) methanol
Gradient:	10%–28%B (0–18 min)
Flow rate:	0.42 ml/min
Temperature:	25 °C
Detection:	UV at 260 nm
Injection:	1 µl (5 nmol/ml)
Sample:	siRNA duplex & single strands

*1,1,1,3,3,3-hexafluoro-2-propanol

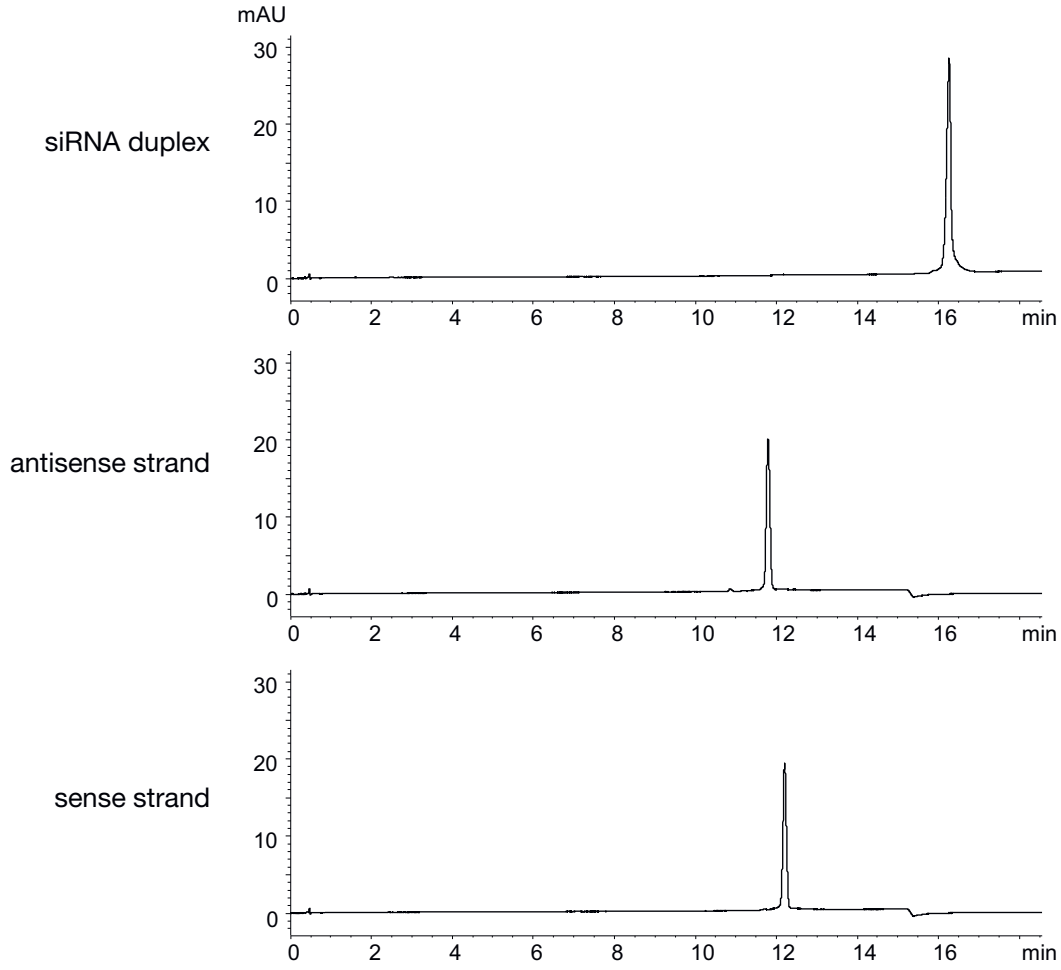

Figure 2: Analysis of the siRNA duplex and its single strands under non-denaturing IP-RP conditions using a YMC-Accura Triart Bio C18 UHPLC column.

Analysis of a siRNA duplex by IEX

The BioPro IEX QF column used in this application note is a strong anion-exchanger with a quaternary amine as functional group and exhibits low nonspecific adsorption. The non-porous column packing guarantees sharp peaks under denaturing (figure 3) and non-denaturing conditions (figure 4). By the use of an eluent containing sodium hydroxide denaturation is achieved. In contrast, non-denaturing conditions include the use of Tris-HCl at pH 8.

Table 4: Chromatographic AEX conditions – denaturing

Column:	BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part number:	QF00S05-1046WP
Eluent:	A) 10 mM NaOH
	B) 10 mM NaOH containing 1 M $NaClO_4$
Gradient:	30%–37%B (0–15 min)
Flow rate:	1.0 ml/min
Temperature:	25 °C
Detection:	UV at 260 nm
Injection:	4 µl (5 nmol/ml)
Sample:	siRNA duplex

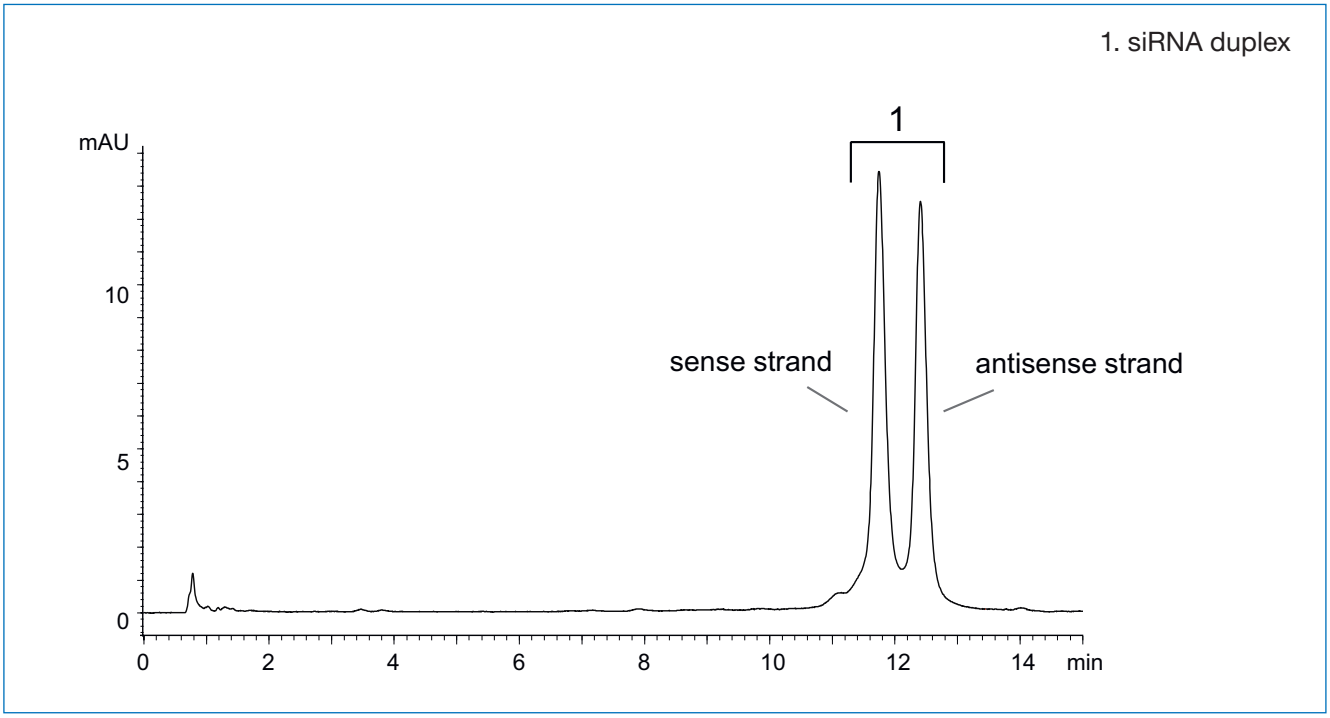

Figure 3: Denaturing AEX analysis of the siRNA duplex using a BioPro IEX QF HPLC column.

Table 5: Chromatographic AEX conditions – non-denaturing

Column:	BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part number:	QF00S05-1046WP
Eluent:	A) 10 mM Tris-HCL (pH 8.1)
	B) 10 mM Tris-HCL (pH 8.1) containing 1 M $NaClO_4$
Gradient:	25%–40%B (0–15 min)
Flow rate:	1.0 ml/min
Temperature:	25 °C
Detection:	UV at 260 nm
Injection:	4 µl (5 nmol/ml)
Sample:	siRNA duplex

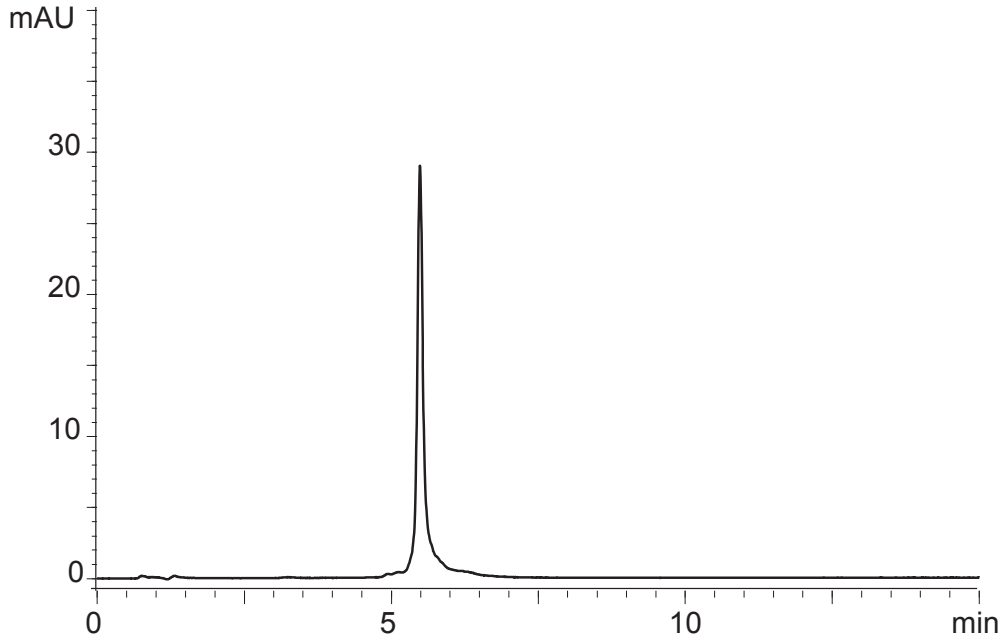

Figure 4: AEX analysis of the siRNA duplex under non-denaturing conditions using a BioPro IEX QF HPLC column.