How to choose the optimum conditions for analysis of denatured or non-denatured siRNA duplexes

Small interfering RNAs (siRNAs) usually occur as oligonucleotide duplexes. They can be analysed under non-denaturing conditions so that duplex formation is assured and excessive single-strands are detected. Alternatively, analysis of siRNAs can be conducted under denaturing conditions so that both sense and antisense strands are monitored. Analysis of siRNA under denaturing and non-denaturing conditions can be achieved by ion pair reversed phase high performance liquid chromatography (IP-RP HPLC). Several parameters influence siRNA separation:

1. Column temperature – key factor for the denaturation of siRNA
2. Ion pair reagents and acids – affect double-strand stability and separation efficiency

Expert tip

The phosphate-rich backbone of siRNA molecules enables the siRNA to adhere to metal surfaces, including column hardware. To prevent metal surface interaction, all experiments were conducted with a bioinert **YMC-Accura Triart Bio C18** column. The bioinert surface coating of column body and frit considerably increases the sensitivity and sample recovery.

This technical note introduces all relevant aspects of denaturing and non-denaturing IP-RP HPLC. As a sample the siRNA duplex targeting the firefly luciferase gene GL2 was used (table 1). The siRNA consists of 19 nucleotides.

Table 1: Oligonucleotide sequence

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

1. Choosing the optimum column temperature

At high temperatures, double-stranded oligonucleotides dissociate. The denaturation temperature of siRNAs depends on their length and composition. On-column denaturation can be observed by a peak shift since oligonucleotide duplexes adhere stronger to the stationary phase so that single-strands elute earlier. Therefore, it is useful to determine the denaturation temperature of the RNA sample. Of course, LC can help to monitor the transition.

In figure 1, the different states of the siRNA at different temperatures are visible. At an elevated temperature of 65 °C, the double-stranded oligonucleotide is separated into two single-strands. The retention corresponds to the two single-strands analysed separately. At 45 °C the siRNA is partially degraded and the retention time increases. At 25 °C non-denatured siRNA is detected, which elutes significantly later from the stationary phase.

Table 2: Overall chromatographic conditions unless otherwise stated.

Column:	YMC-Accura Triart Bio C18 (1.9 µm, 30 nm), 50 x 2.1 mm ID
Part No.:	TA30SP9-05Q1PTC
Eluent:	A) 8 mM triethylamine - 100 mM HFIP* (pH 8)
	B) methanol
Gradient:	1%B/min (initial = 3%B)
Flow rate:	0.42 mL/min
Temperature:	65 °C, 45 °C or 25 °C
Detection:	UV at 260 nm
Injection:	1 µl (5 nmol/mL)
Sample:	siRNA duplex, sense strand, antisense strand

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

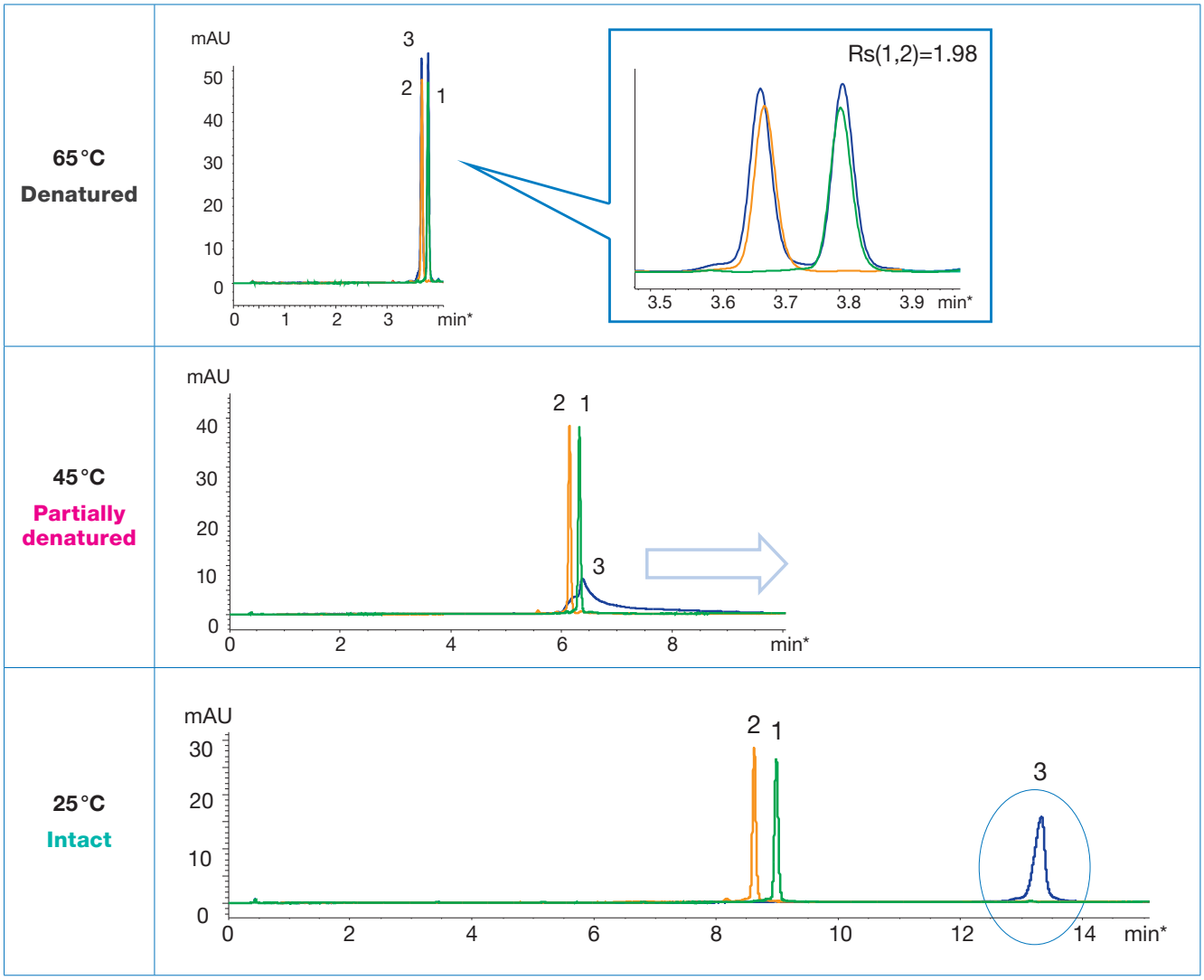


Figure 1: Analysis of the denaturation state of the siRNA duplex at different temperatures.

1. sense strand
2. antisense strand
3. siRNA duplex

2. Selecting the appropriate type and concentration of ion pair reagent

Due to the negatively charged phosphate backbone of siRNAs the retention on hydrophobic stationary phases is low. Therefore, ion pair reagents are needed to neutralise charged siRNAs. This ensures sufficient retention on the stationary phase.

Different ion pair reagents are available:

- *n*-butylamine (BA)
- triethylamine (TEA)
- hexylamine (HA)
- dibutylamine (DBA)

All of them were tested at 8 mM in combination with 100 mM HFIP. The gradient was adjusted individually (figure 2). At 65 °C the siRNA was denatured with all ion pair reagents tested. At 25 °C no denaturation was observed. At 45 °C the siRNA partially denatured with TEA and HA, and completely denatured with DBA. No denaturation was observed with BA. Due to negatively charged phosphate backbones of siRNAs, ion pair reagents are needed in RP to form hydrophobic ion pairs with siRNAs.

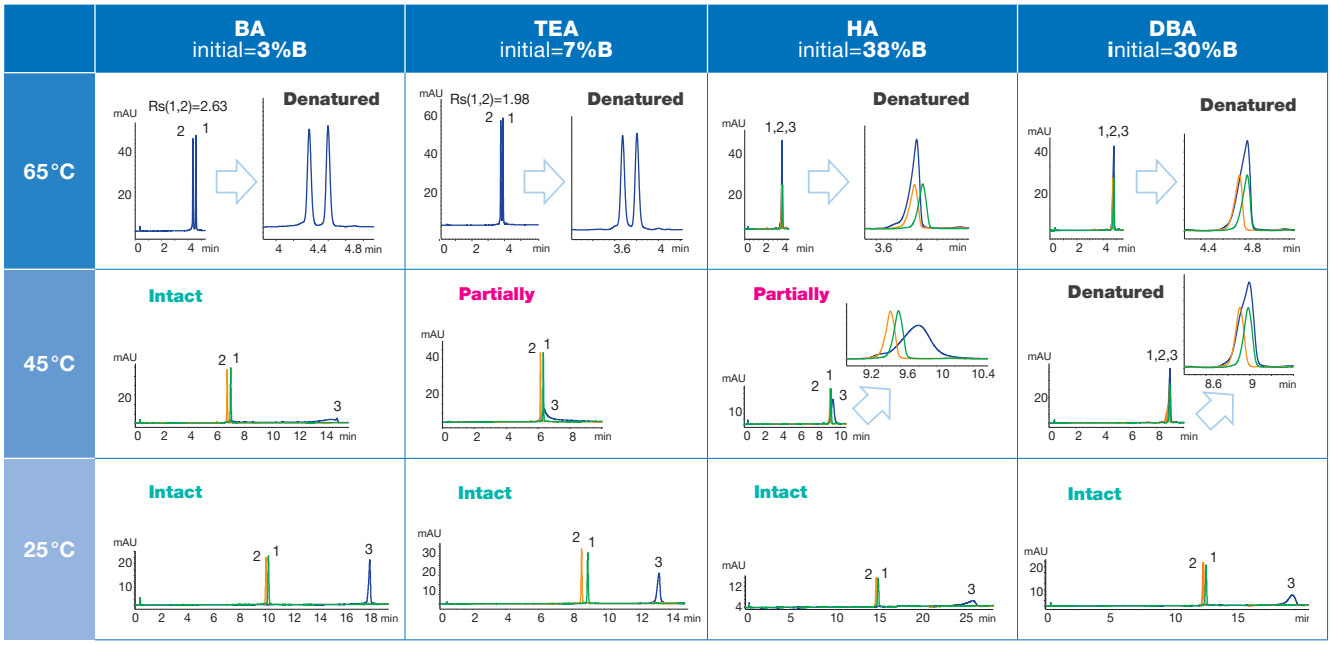


Figure 2: Effect of four different ion pair reagents on the denaturation of a siRNA duplex at different temperatures.

1. sense strand
2. antisense strand
3. siRNA duplex

Mass spectrometry (MS) applications specifically require volatile ion pair reagents. TEA in combination with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) as the acidic counter ion and methanol as organic modifier are a common choice.

Therefore, different concentrations of TEA and HFIP were tested (figure 3). Lower concentrations of TEA and HFIP improved the separation of denatured siRNA marginally. However, the siRNA duplex showed improved peak shape at higher concentrations of TEA and HFIP.

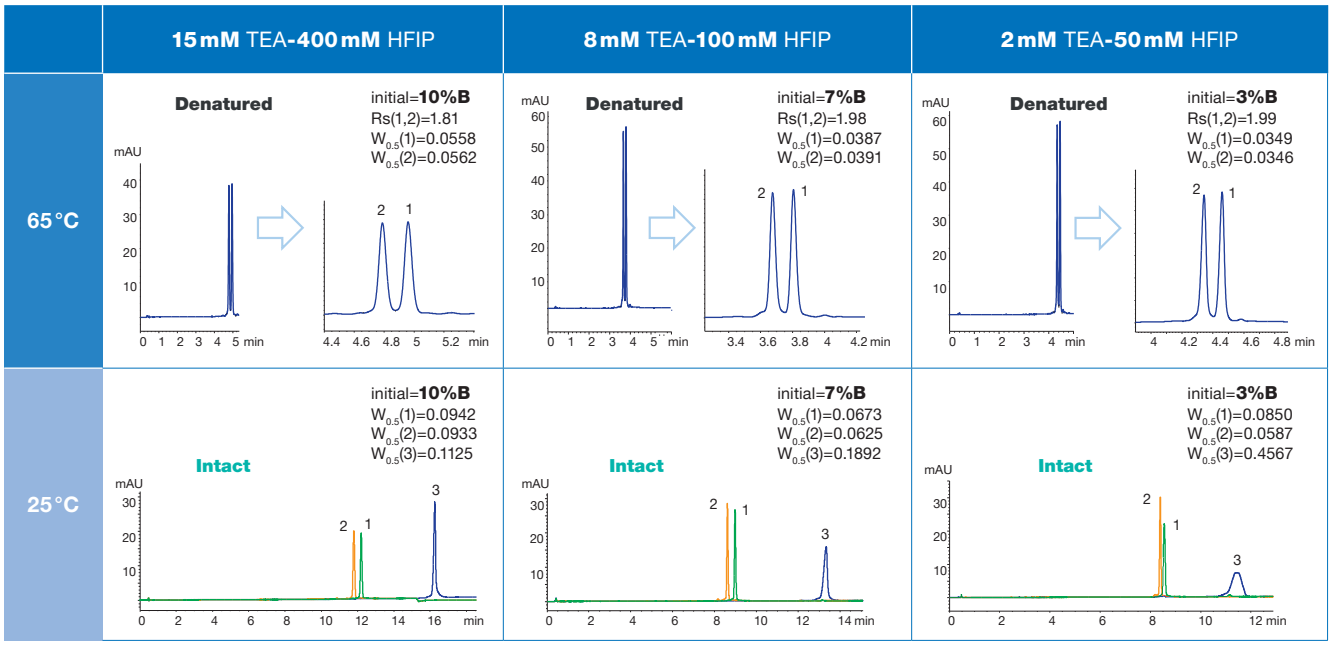
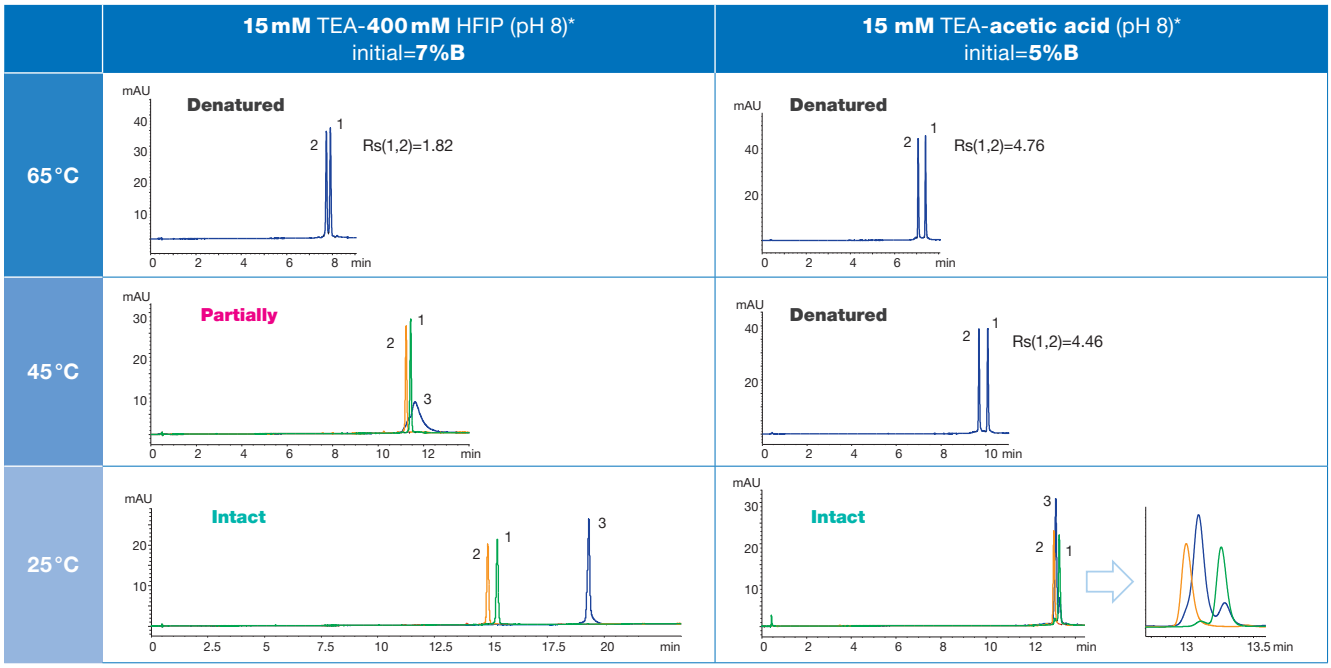


Figure 3: Analysis of a siRNA duplex at non-denaturing and denaturing conditions with different concentrations of TEA and HFIP.

1. sense strand
2. antisense strand
3. siRNA duplex

3. Considering the influence of acidic organic acid as alternative to HFIP

Besides TEA in combination with HFIP, TEAA buffer (TEA and acetic acid) is commonly used as ion-pair reagent. Especially when high-throughput analyses are performed, costs can be reduced by exchanging costly HFIP with another counter ion. The influence of acetic acid on oligonucleotide separation and denaturation was tested by comparing both ion pair reagents (figure 4). Eluent A with 15 mM TEA and 400 mM HFIP or acetic acid (pH 8) were compared. The use of TEAA resulted in better resolution at 65 °C compared to TEA/HFIP. At 4 °C siRNA was still denatured with TEAA but already only partially denatured with TEA/HFIP. However, at 25 °C siRNA was only partially denatured when TEAA was used whereas it was intact at these conditions tested with TEA/HFIP.



* This study was performed at pH 8 to reduce parameters.

Figure 4: Influence of acetic acid on denaturation of a siRNA duplex at different temperatures.

1. sense strand
2. antisense strand
3. siRNA duplex

Other ion pair reagents such as BA, HA, and DBA were tested in combination with acetic acid and compared to the same ion pair reagents with HFIP (figure 5). 15 mM amine with either acetic acid (pH 8) or 400 mM HIFP were used at 65 °C.

The best resolution was again seen with TEA and acetic acid, while BA still provided acceptable results. However, other combinations such as HA and DBA with HFIP as well as acetic acid do not lead to a separation of siRNA or the respective single-strands.

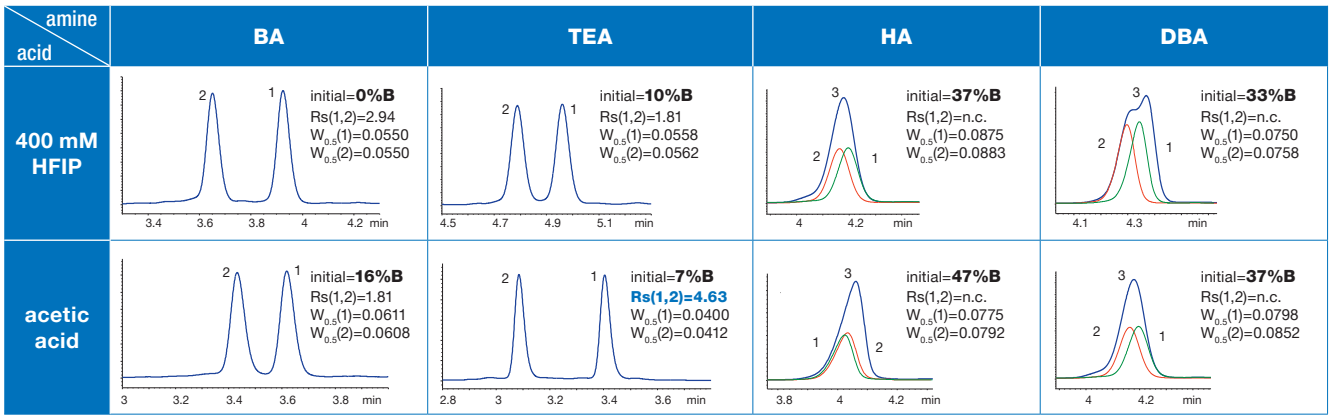


Figure 5: Influence of four ion pair reagents in combination with HFIP or acetic acid on denaturation of a siRNA duplex at 65 °C.

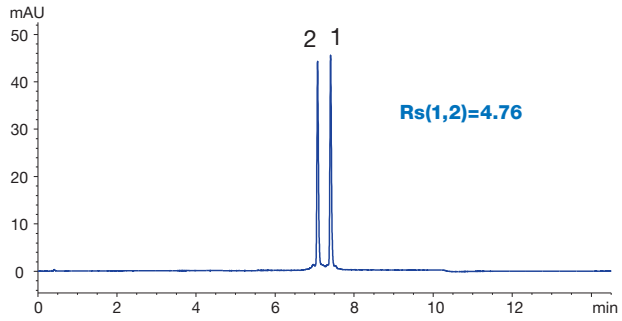
1. sense strand
2. antisense strand
3. siRNA duplex

4. Optimal conditions for analysis of denatured and non-denatured siRNA duplex

For the siRNA tested, optimal conditions for denatured and non-denatured analysis were determined. siRNA was successfully denatured at 65 °C, with 15 mM TEA and acetic acid with methanol as organic modifier (figure 6). In contrast, intact siRNA could be analysed best at 25 °C with 15 mM TEA and 400 mM HFIP with methanol (figure 6). Nevertheless, the analysis of siRNA by IP-RP must always be individually adapted to the properties of the respective siRNA.

Denatured

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 TEA-**acetic acid** (pH 8)
B) methanol
Gradient: 1%B/min (initial=5%B)
Flow rate: 0.42 mL/min
Temperature: **65 °C**



Intact

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 TEA-400 mM HFIP** (pH 8)
B) methanol
Gradient: 1%B/min (initial=10%B)
Flow rate: 0.42 mL/min
Temperature: **25 °C**

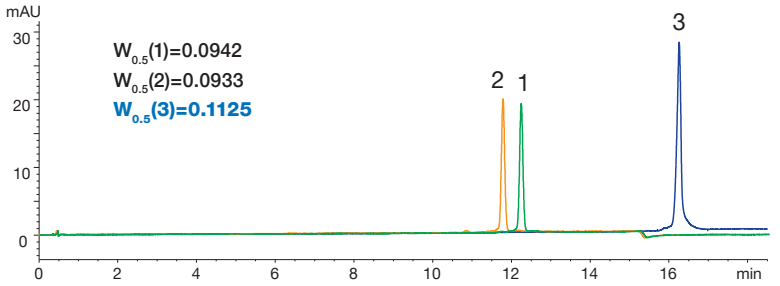


Figure 6: Optimal conditions tested for analysis of a denatured or non-denatured RNA duplex.

1. sense strand
2. antisense strand
3. siRNA duplex

Conclusions

The following factors should be considered during optimisation of siRNA analysis:

- **Column temperature**
- **Choice of ion pair reagent**
- **Influence of acidic component**

siRNA can be analysed by means of IP-RP in a denatured or non-denatured state. IP-RP can be applied with MS detection. Though, ion pair reagents have a strong influence on ionisation efficiency and sensitivity. This limits the choice of suitable eluent modifier. HFIP is well suited and its compatibility with MS has great advantages. But the higher cost is a disadvantage.

Therefore, alternative counter ions, especially for high-throughput analyses, need to be considered. However, counter ions such as acetic acid can increase the denaturation of siRNAs. Nevertheless, the analysis of siRNA by IP-RP must always be individually adjusted to the properties of the respective siRNA.