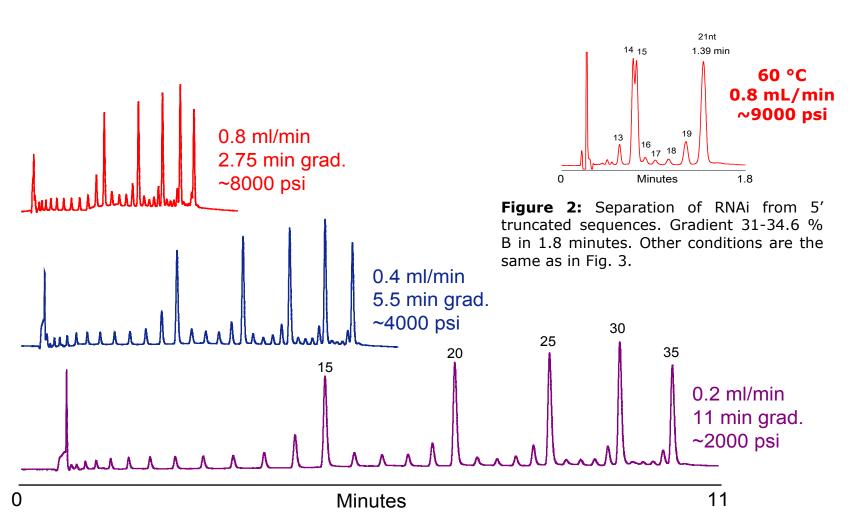
Analysis of RNAi and RNAi duplexes with UPLC-MS

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- Method development for fast oligonucleotide separation with Ultra Performance Liquid Chromatography (UPLC) is discussed.
- 1-2 minute separation of 21nt RNAi with N-1 resolution.
- RNAi characterization with LC-MS.
- Confirmatory MS/MS sequencing of 21nt RNAi.
- Non-denaturing mobile phases for LC-MS duplex RNA analysis.
- Resolution of duplexes from single stranded RNAi oligos.

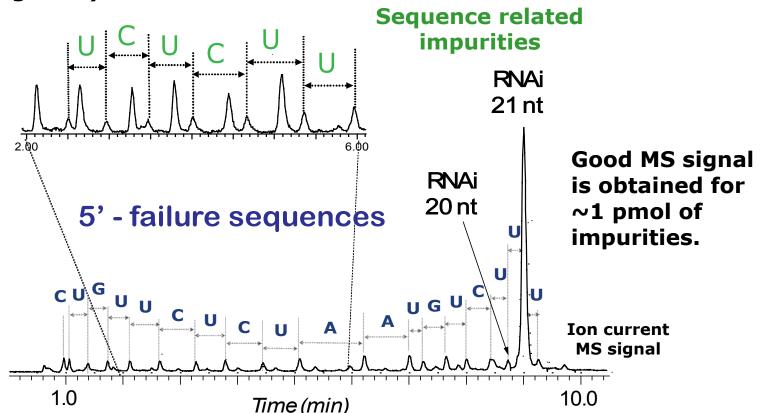
FAST UPLC OLIGONUCLEOTIDE SEPARATIONS

- Use 2.1 x 50 mm, 1.7 µm ACQUITY OST C₁₈ column with 0.2 mL/ min mobile phase flow rate (FR) at 60 °C.
- Select an appropriate initial mobile phase strength (% of MeCN) and gradient slope to achieve a desirable resolution.
- Increase FR while decreasing gradient time proportionally (Figure 1). Gradient volume remains constant, analysis time decreases with minor loss of resolution. Resolution of RNAi from the n-x sequences within 1-2 minutes is possible (Figure 2).

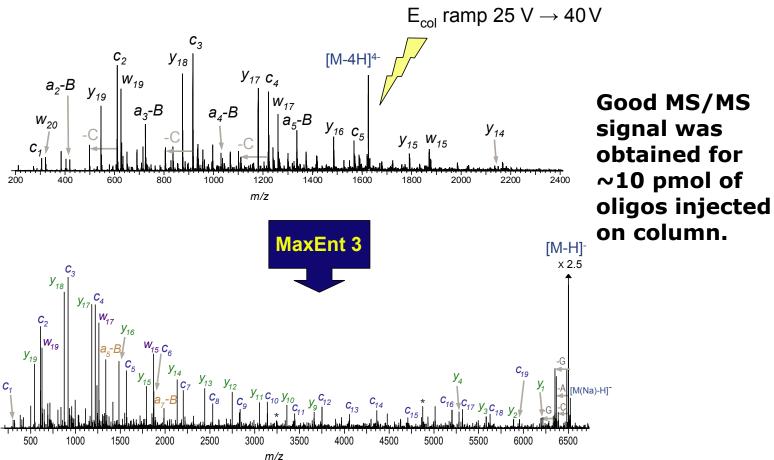


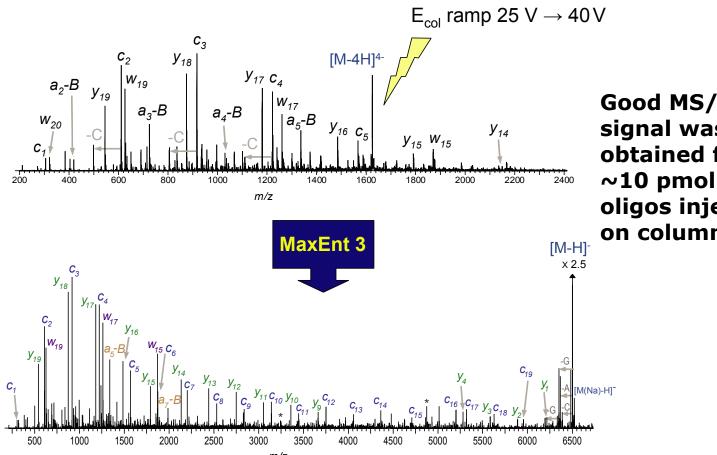
OVERVIEW

- **HFIP** ion-pairing buffer.
- (Figure 4).



50% m.p. A, 50% MeOH. Gradient 20-40 % B in 10 minutes, 0.2 mL/min.





conditions see Fig. 3.

Figure 1: Separation of 2-35nt oligodeoxythymidine standard (PN 186004135) Waters ACQUITY UPLC[®] System and ACQUITY UPLC[®] OST C18, 1.7µm, 2.1x50 mm column, 60 °C, UV 260 nm. Mobile phase A: 100 mM Hexylammonium acetate, pH 7, B: 50% m.p. A, 50% MeCN. Gradient was 56-80.2% B for all separations.

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RNAI CHARACTERIZATION WITH LC-MS

• Use LC-MS compatible mobile phases, such as 15mM TEA, 400 mM

• Sum mass spectra under the peaks of interest, deconvolute mass with MaxEnt1, calculate \triangle mass between adjacent peaks, assign failed sequences based on \triangle mass (Figure 3).

• MS/MS sequencing of 21nt RNA was performed with Synapt MS

Figure 3: UPLC analysis of crude 21nt RNAi, UUC UGU AAU CUC UUG UCU ATT (5'-3'), ACQUITY UPLC® OST C18, 1.7µm, 2.1x50 mm column, 60 °C, UV 260 nm. M.p. A: 15mM TEA, 400 mM HFIP, pH 7.9, B

Figure 4: LC-MS/MS sequencing of 21nt RNAi, UUC UGU AAU CUC UUG UCU ATT (5'-3'), for other

IMPACT OF LC CONDITIONS ON DUPLEX STABLITY

- LC analysis of short RNA duplex (~19 base pairs, bp) should be performed at 10-20 °C.
- Triethylammonium acetate (TEAA) mobile phases are nondenaturing. Duplexes can be analyzed without on-column melting.
- TEA-HFIP mobile phases are denaturing. Some duplexes can melt on-column even at low separation temperature. Alternative non-denaturing ion-pairing mobile phase are recommended for LC-MS analysis of duplexes.

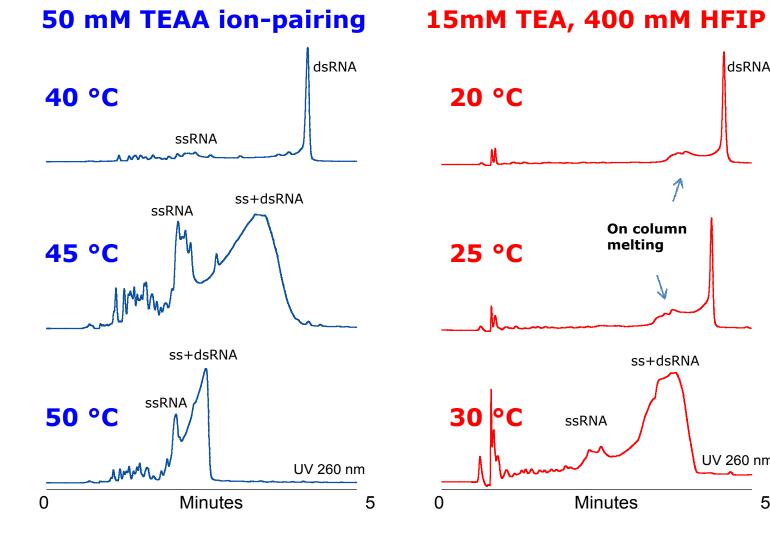


Figure 5: UPLC analysis of crude RNA duplex. 21mer complementary RNAi were annealed and injected on column. T_m of 19 bp region, ~49 °C. ACQUITY UPLC[®] OST C18, 1.7 μ m, 2.1x50 mm column, UV 260 nm. Ion-pairing systems and separation temperature are indicated in the picture, 0.2 mL/min.

- Figure 5 shows that ssRNAi are less retained than duplex.
- Peak broadening indicates on-column duplex melting.
- TEA-HFIP system melts duplexes at lower temperatures than TEAA. Some melting occurs even at low separation temperature.
- 8.6 mM TEA, 100 mM HFIP system is an alternative, less denaturing mobile phase. Hexylammonium acetate could be also used (Figure 6)

LC-UV-MS ANALYSIS OF RNA DUPLEXES

- Figure 6 illustrates the separation of ssRNAi and dsRNA.
- Incomplete duplexes (upper N + lower N-1 strands, etc.) are resolved from full length duplex and elute in order of decreasing length.
- Single quadrupole MS instrument was used for MS analysis. The MS spectrum of duplex contains the signal for both ssRNAi complements (Figure 6).
- Partial duplexes and ssRNAi were identified by their mass.

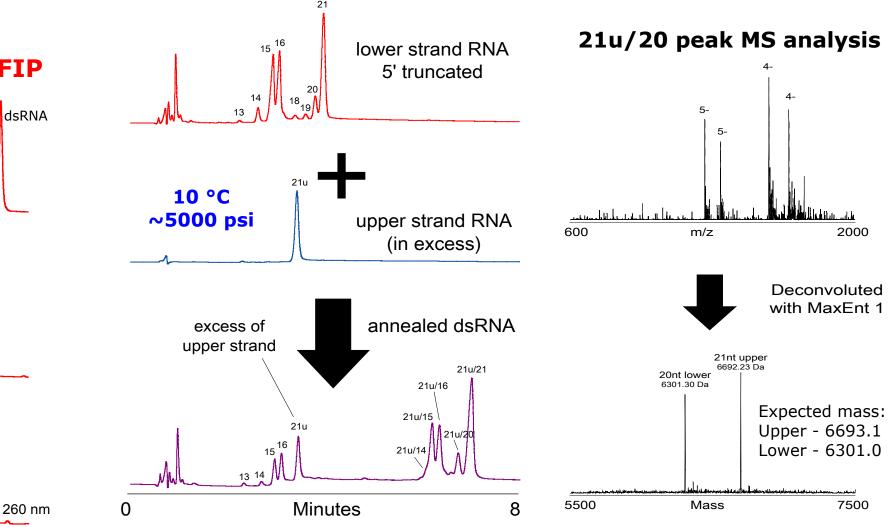


Figure 6: UPLC MS analysis of RNA duplexes. Partially 5' end truncated 21nt RNAi lower strand was annealed with excess of upper 21nt strand (21u). ACQUITY UPLC[®] OST C18, 1.7µm, 2.1x50 mm, UV 260 nm. A: 25mM hexylammonium acetate, B: MeCN, 0.2 mL/min. Grad. 30-40% B in 10 min, 10 °C.

- CONCLUSIONS
- ACQUITY UPLC system and OST columns enable fast oligonucleotide separation, dramatically enhancing the speed of conventional LC analysis (25-60 minutes).
- Both ssRNA and its duplexes can be analyzed with LC-UV-MS. MS is an essential tool for oligonucleotide therapeutics characterization.
- Synapt MS allows for a routine sequencing of ~ 21 nt oligonuclelotides from ~10 pmole sample injection.

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