

Agilent
Trusted Answers

Results and Discussion

Synthetic oligonucleotides, including small interfering RNA (siRNA), antisense oligonucleotides (ASO), guide RNA (gRNA) for CRISPR/Cas, and aptamers, have been emerging as therapeutics against a wide range of disease conditions. Comprehensive characterization of oligonucleotide samples can be challenging and, in some cases, time-consuming, as it not only requires the profiling of the target oligonucleotide but also identifying and quantifying all related impurities. This work focuses on developing a comprehensive software ecosystem that enables end-to-end analysis of siRNA for sequence confirmation and impurity profiling. The results demonstrate the software's ease of use and its capability to provide a complete solution for analyzing synthetic oligonucleotides.

Experimental

Two siRNA molecules, inclisiran and givosiran were used to evaluate the software's performance (Table 1). They were dissolved in water without further purification. Ion-pair reversed-phase liquid chromatography coupled with time-of-flight mass spectrometry, along with novel inert column hardware, was employed to acquire data for both denatured and non-denatured forms. The software was implemented

in a client-server architecture to enable end-to-end data acquisition, analysis, and reporting. The LC and Q-TOF conditions are presented in Table 2.

Table 1. siRNA sample sequences.

sRNA name	Length	Sequence
Incisrian	21 S	mCmUmUmAmGmAmCmCmU/GmUdTmUmUmGmCmUmUmUmUmGmU/L96/
	23 AS	mA ⁴ C ⁴ mAfAfAfAmGfCmAfAfAmAfAmCfAmGfCmUfUmUmAmAmAmAmA
Gwosrian	mCm ⁴ mAmAmAmAfAmGfAmUfGmUfCmUfCmUmUmUmUmUmUmUmU/L96/	
	23 AS	mUmGmUmUmCfUmUmUfUfCmUfCfAmCfAmGfAmGfAmUfGfAfAfAmU

Code	Description	Code	Description	Code	Description	Code	Description	Code	Description
L/96	Phosphorothioate bond	mC	2'-O-methyl A	mU	2'-O-methyl G	IA	2-fluoroadenosine	UA	2-fluorouridine
	CaH ₂ Na ₂ O ₃	mC	2'-O-methyl A	mU	2'-O-methyl G	IC	2-fluorocytidine	IU	2-fluorouridine

Table 2. LC and Q-TOF conditions

Target plus impurities				Sequence confirmation
	denatured	non-denatured		
Column	Agilent Altura Oligo HPH-C18, 2.1 x 50 mm, 2.7 μ m			
Flow rate	0.4 mL/min			
Column temperature	65 °C	25 °C		65 °C
Injection volume	1 μ L			
Mobile phase	A: 15 mM TEA and 50 mM HFIP in water B: Methanol			
Gradient	Time (min)	%B	Time (min)	%B
	0	10	0	10
	7	40	1	10
	7.5	90	7	40
	8	90	7.5	90
			8	90
Stop time	8 min			
Exit time	1.5 min			
LUV detection	260 nm with 4 nm bandwidth	-		
MS instrument	6545XT			
Drying gas temperature	360 °C	250/350 °C	350 °C	
Drying gas flow	12 L/min			
Nebulizer	45 psi			
Sheath gas temperature	120 °C	250/400 °C	400 °C	
Sheath gas flow	12 L/min			
Capillary voltage	3500 V			
Nebulizer voltage	2000 V			
Fragmentor	175 V			
Skimmer	65 V			
Instrument mode	System tune			
Acquisition mode	MS	Targeted MS/MS		
Mass range	m/z 40-3200	m/z 40-3200		
Acquisition rate	1 spectra/s	4 spectra/s		
MS/MS mass range	-	m/z 50-2000		
MS/MS acquisition rate	-	2 spectra/s		
Collision energy	-	12 V, 15 V, 18 V, 20 V		
Targeted m/z	-	Calculated monoisotopic mass of the charged ion		
Delta retention time	-	0.5 min		
Isotope width	-	Medium (<4 m/z)		

Seamless sample processing: from data acquisition through data analysis to reporting

A combination of task actions, wait actions, and custom sample parameters enables the sample sequence to run without human intervention, potentially for several days (see Table 3). Unattended processing allows samples to be processed automatically immediately after data acquisition is complete, using predefined data analysis and reporting methods.

Table 3. A sequence for automated data acquisition, processing, reporting, and instrument control.

					Sample Custom Parameters	
Action	Sample name	Inj/Vial	Acquisition method	Processing + Reporting method	BioConfirm Sequence	BioConfirm Modification Profiles, and more...
Inject	Water	3	Oligo_TPI.amx	Oligo_TPI.bcpmx		
Inject	Inclisiran	3	Oligo_TPI.amx	Oligo_TPI.bcpmx	Inclisiran S, Inclisiran AS	
Inject	Inclisiran	5	Oligo_SC.amx	Oligo_SC.bcpmx	Inclisiran S	
Inject	Inclisiran	5	Oligo_SC.amx	Oligo_SC.bcpmx	Inclisiran AS	
Inject	Water	3	Oligo_TPI.amx	Oligo_TPI.bcpmx		
Inject	Givosiran	3	Oligo_TPI.amx	Oligo_TPI.bcpmx	Givosiran S, Givosiran AS	
Inject	Givosiran	5	Oligo_SC.amx	Oligo_SC.bcpmx	Givosiran S	
Inject	Givosiran	5	Oligo_SC.amx	Oligo_SC.bcpmx	Givosiran AS	
Inject	Water	3	Oligo_TPI.amx	Oligo_SC.bcpmx		
Task	Turn off the LC modules, switch MS to Eco Standby					
Wait	Wait until next morning, e.g. 8:00					
Task	Turn on all instruments, run mass calibration for MS					

Analysis of denatured and non-denatured siRNA

Both inclisiran and givosiran were analyzed in their denatured and non-denatured forms using ion-pairing HPLC coupled with DAD and Q-TOF detection. The results are shown in Figures 2 and 3.

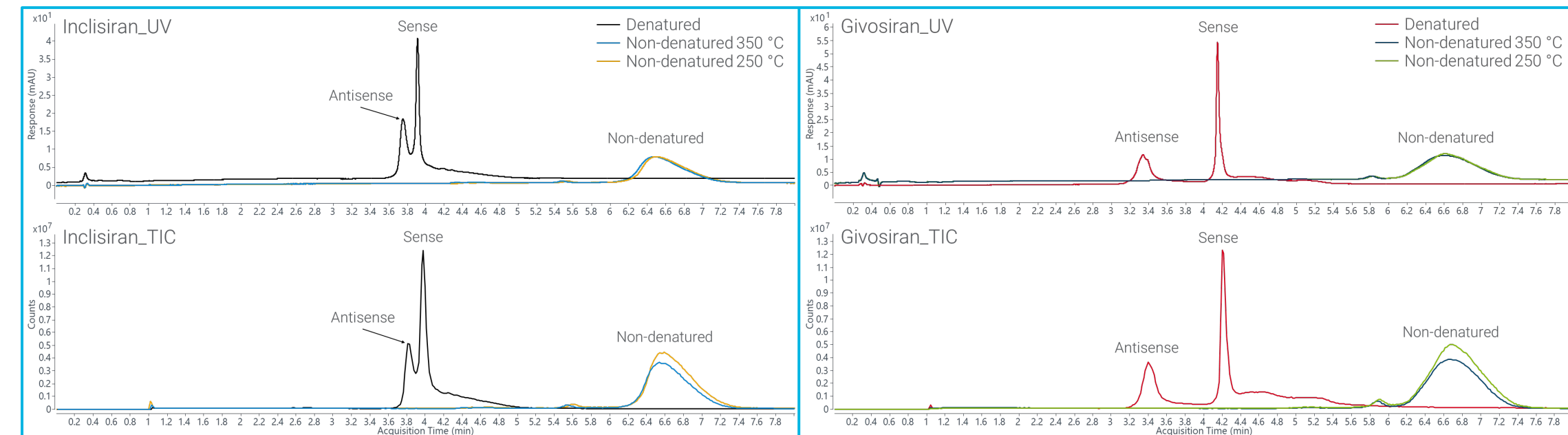


Figure 2. UV and total ion chromatograms of denatured and non-denatured inclisiran (left) and givosiran (right).

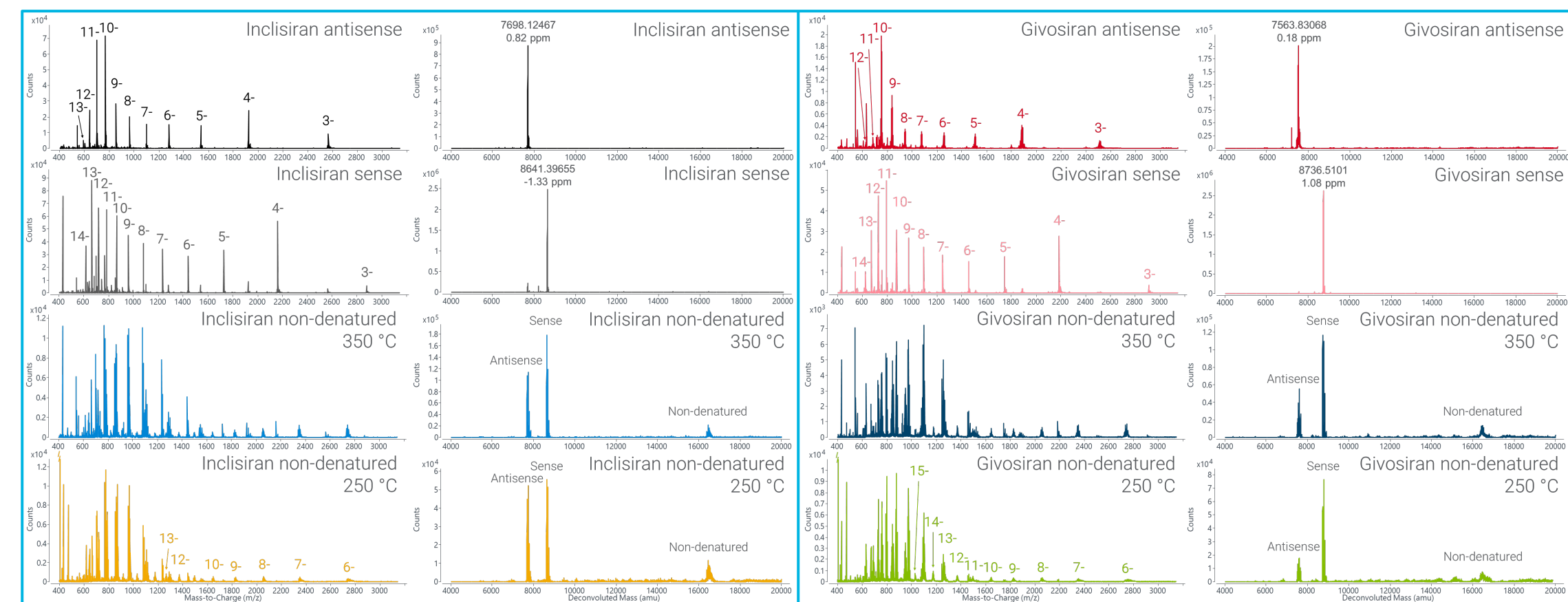


Figure 3. Raw and deconvoluted MS spectra of inclisiran (left) and givosiran (right)

Sequence confirmation of siRNA

As one example, inclisiran was analyzed using targeted MS/MS. 100% sequence coverage was achieved for both the antisense and sense strands of inclisiran, as shown in Figure 4.

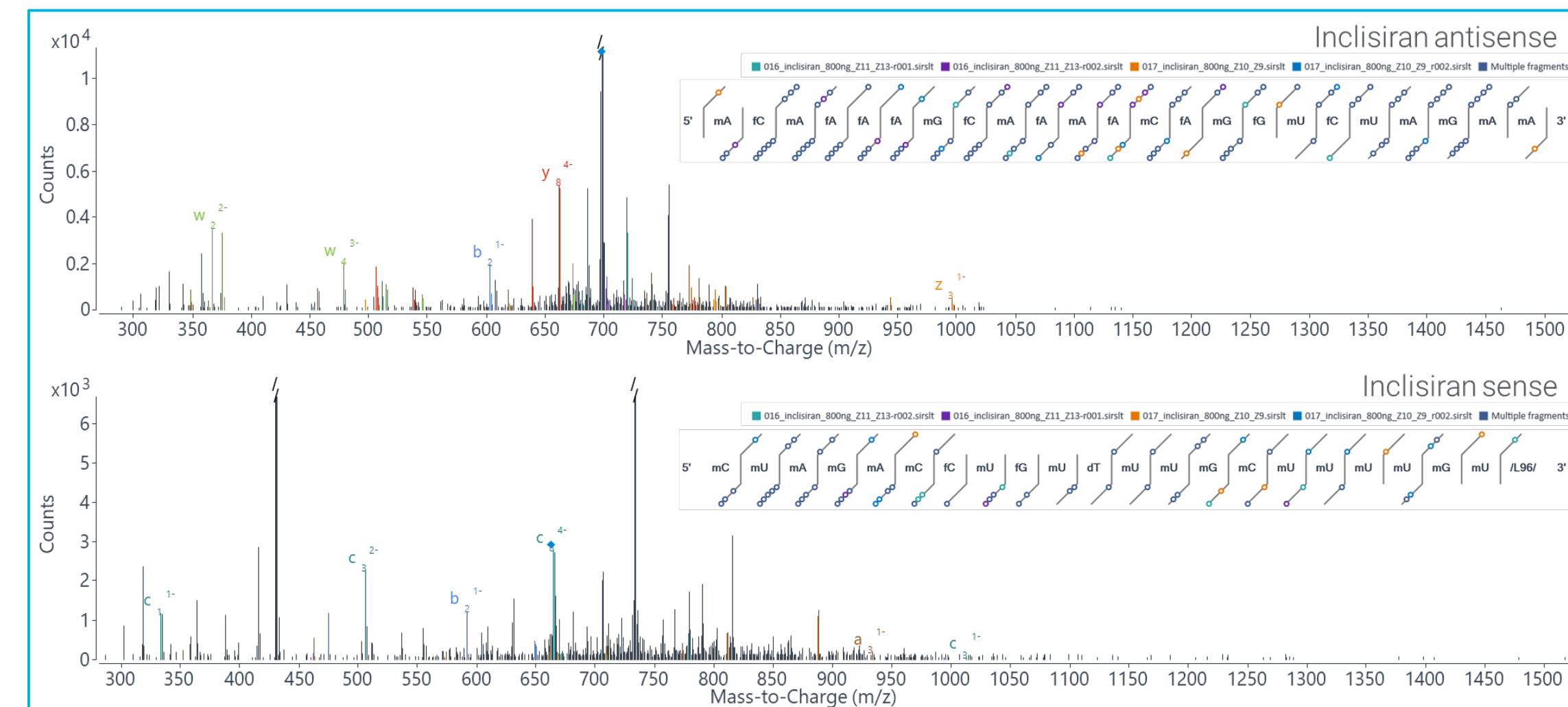


Figure 4. MS/MS analysis of inclisiran, measured by 6545XT. Sequence coverage was achieved by targeting the -10 and -11 charge states of the antisense strand, and the -9 and -13 charge states of the sense strand, in duplicate.

Reporting

Ready-to-use report templates are available for the analysis of oligonucleotides, including applications such as target and impurity analysis as well as sequence confirmation. A built-in report builder supports customized report design, enabling reports to be generated automatically and efficiently. An example of a sequence confirmation report for the antisense strand of inclisiran is shown in Figure 5 (right).

Table 4. Consumables for the solution.

Description	Part Number
Agilent Altura Oligo HPH-C18, 2.1 × 50 mm, 2.7 µm	227205-702
Agilent InfinityLab bio solvent bottle, PP, 1 L, with cap	9301-6028
Agilent InfinityLab bio solvent inlet filter, 10 µm, PEEK	5320-0070
Agilent InfinityLab Quick Connect LC fitting	5067-5965
Agilent InfinityLab Methanol for LC/MS	5191-5111
Agilent InfinityLab Water for LC/MS	5191-5121
Agilent Polypropylene Vials	5188-2788
Agilent Snap Top Caps	5182-3458

BioConfirm Sequence Confirmation Report

[illegible]

Figure 5. Report generated using the existing report templates in BioConfirm..

Conclusions

- An integrated software platform, together with new hardware and consumables, enables end-to-end data acquisition, analysis, and reporting for siRNA.
- Intelligent sample sequence management is supported through task actions, wait actions, sample custom parameters, and additional advanced features.

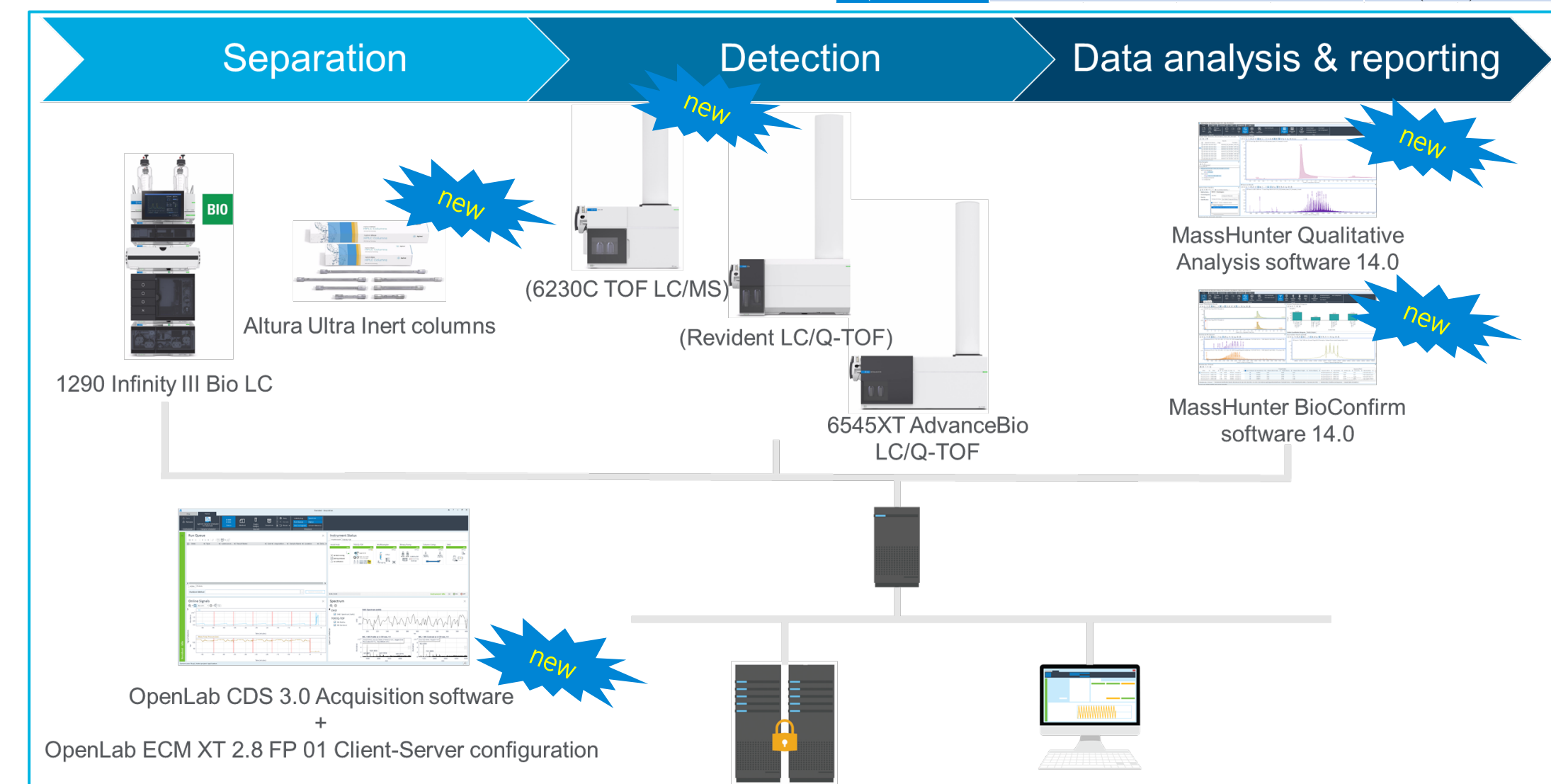


Figure 1. Solution overview.