

Poster Reprint

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# Oligo Purity Analysis and Sequence Confirmation by LC/MS without Ion Pairing Reagents — Sample to reports in about 5 minutes

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## Introduction

Oligonucleotides are commonly analyzed by LC/MS in negative ion polarity mode using ion-pair reverse-phase (IPRP) methods. However, because many ion-pairing reagents persist in the analytical system long after their use and present a very strong MS response in positive ion polarity, using the same analytical system for multiple applications can be challenging. LC/MS of oligos using HILIC chromatography can be an alternative to IPRP conditions for a wide range of oligo targets. HILIC separations typically have long run times. In this work, we optimized and applied a 1-minute Fast LC HILIC method in conjunction with MS1 acquisition for purity/impurities characterization and auto MS/MS acquisition for sequence confirmation on multiple oligos approximately 20mer in length.

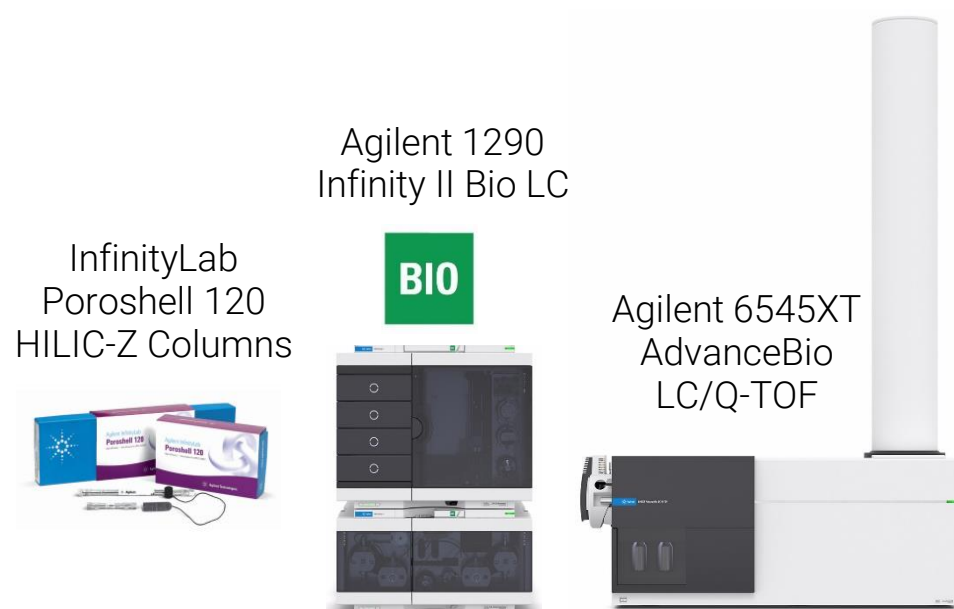


Figure 1. LC/MS Configuration

Table 1. LC/MS method used in the study

Agilent 1290 Infinity II Bio LC Conditions			
Column	InfinityLab Poroshell 120 HILIC-Z UHPLC guard, 2.7 $\mu$ m, 2.1 x 5mm (pn: 821725-947)		
Injection volume	0.25 mL		
Mobile phase	A = 90% Acetonitrile : 10% Water + 15 mM Ammonium Acetate B = 10% Acetonitrile : 90% Water + 15 mM Ammonium Acetate		
Gradient program	Time (min)	B (%)	Flow (mL/min)
	0.00	45	0.6
	0.40	75	0.6
	0.41	75	1.75
	0.60	75	1.75
	0.61	45	1.75
Stop time	1.00 min		

## Experimental

### Oligonucleotide Samples Analysis

LC/MS analyses were conducted on a 1290 Infinity II Bio LC system coupled with a 6545XT AdvanceBio LC/Q-TOF equipped with an Agilent Dual Jet Stream ESI source (Figure 1) [1]. LC separation was obtained with an InfinityLab Poroshell 120 HILIC-Z UHPLC guard column at room temperature. For purity analysis, MS1 data were acquired from 400 to 3200  $m/z$ . For sequence confirmation, auto MS/MS data were acquired with up to 3 precursors per cycle (with >3 charges, prioritized by charge state then abundance) (Table 1). The resulting data were processed with Agilent MassHunter BioConfirm software 12.0 automatically after acquisition. [2,3]

Table 1. LC/MS method used in the study (continued)

6545XT AdvanceBio LC/Q-TOF Source Conditions	
Ion Polarity	Dual AJS Negative
Gas temperature	350 $^{\circ}$ C
Drying gas flow	13 L/min
Nebulizer gas	35 psi
Sheath gas temperature	400 $^{\circ}$ C
Sheath gas flow	12 L/min
Capillary voltage	3500
Nozzle voltage	2000V
Fragmentor	180 V
6545XT AdvanceBio LC/Q-TOF Acquisition Conditions	
Purity Analysis	
Mass Range	400 – 3200 $m/z$
Acquisition Rate	4 spectra/sec
Sequence Confirmation	
MS Mass Range	400 – 2500 $m/z$
MS Acquisition Rate	10 spectra/sec
MS/MS Mass Range	100 – 2500 $m/z$
MS/MS Acquisition Rate	1 spectra/sec
Isotope Width	Medium ( $\sim$ 4 $m/z$ )
Collision Energies	15 V, 20 V, 25 V or 30 V
Precursor Threshold	Abs. Threshold: 500 counts Rel. Threshold: 15 %
Static Exclusion Range	400 – 600 $m/z$
Table	1700 – 2500 $m/z$

# Results and Discussion

Table 2. List of oligonucleotides analyzed. Calculated masses are monoisotopic masses (matched using the find by formula algorithm).

Oligonucleotide Name	Length	Sequence	Calculated Mass (Da)	Measured Mass (Da)	Mass Accuracy (ppm)
21mer DNA	21	CAGTCGATTGTACTGTACTTA	6408.0961	6408.0941	-0.31
21mer BS	21	CAGTAGATTGTACTGTCCTTA	6408.0961	6408.0960	-0.02
21mer Complement	21	TAAGTACAGTACAATCGACTG	6435.1308	6435.1264	-0.69
PR2	20	GGCCACGCGTCGACTAGTAC	6100.0525	6100.0459	-1.07
PR8	18	CTAGTTATTGCTCAGCGG	5502.9414	5502.9365	-0.88

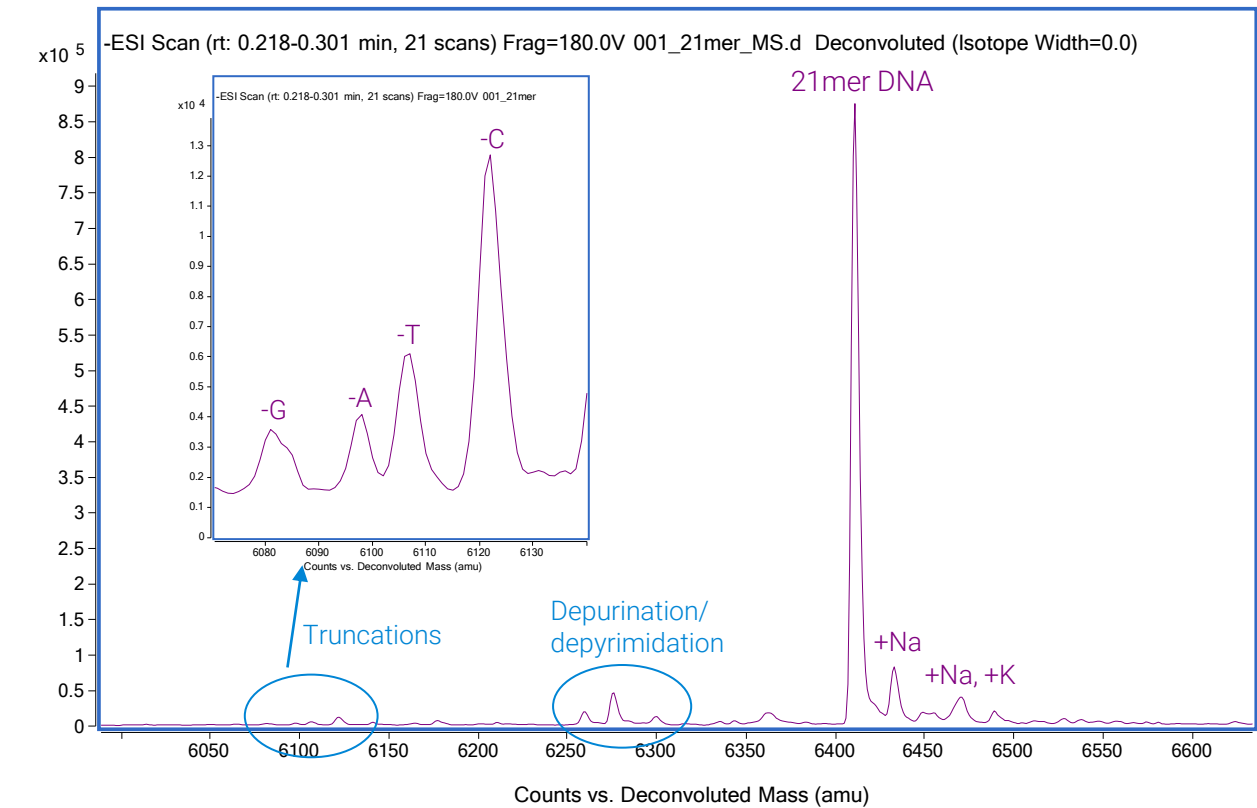


Figure 2. The deconvoluted spectrum for 21mer DNA.

## Purity Analysis

For purity/impurities analysis, oligo mass accuracies were about 1 ppm (Table 2). Multiple low-abundance impurities (many below 0.2 % of target), which are commonly found using extended ion-pairing gradients, were observed and identified. Figure 2 shows the deconvolution result for 21mer DNA.

## Sequence Confirmation Analysis

For sequence confirmation analysis, auto MS/MS acquisition resulted in 1-2 MS/MS spectra per precursor. For all the oligos in the study, the data for 4 injections, each using one collision energy, were aggregated by the software and resulted in complete sequence coverage. Figure 3 shows the data for 21mer DNA.

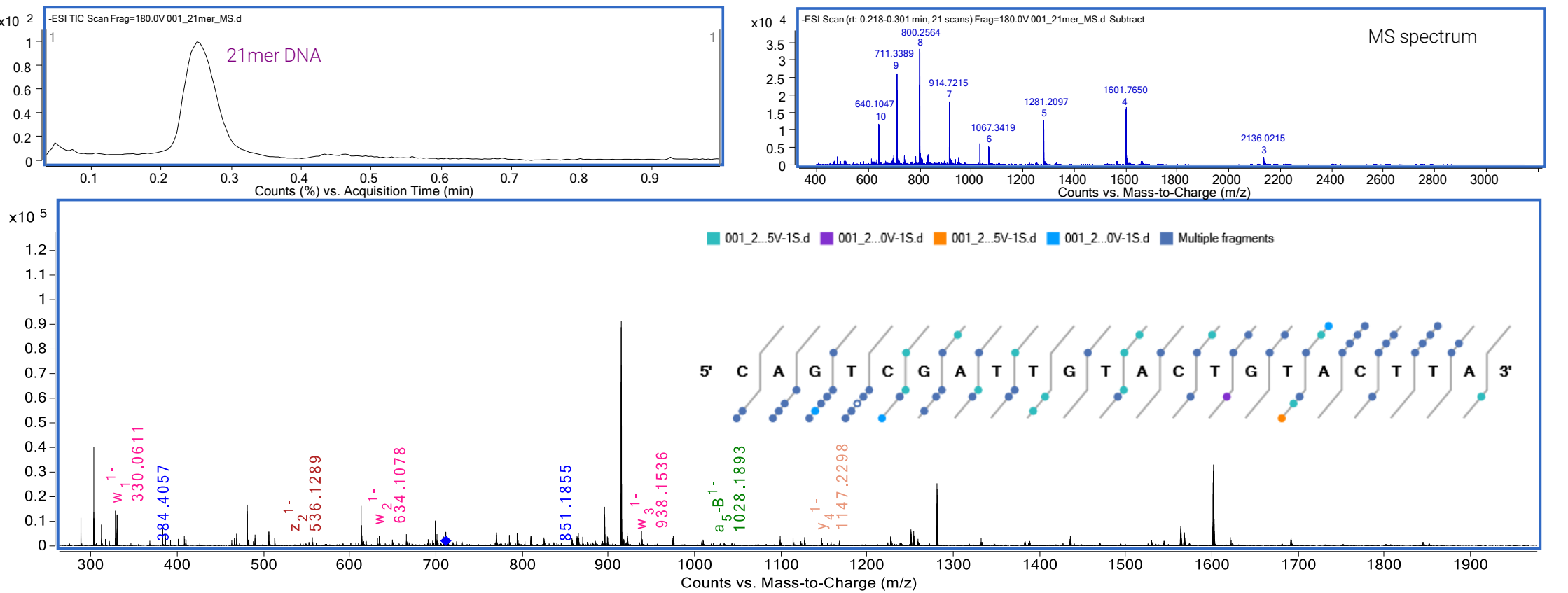


Figure 3. 21mer DNA data. 100% sequence coverage achieved.

Reproducibility and Oligo Retention

Despite the speed of the method, good chromatographic peak shape (6 seconds wide) and retention was achieved. Pump backpressure traces for 50 injections were superimposable, indicating excellent gradient reproducibility (Figure 4). Variable retention were observed with Fast LC HILIC method. Figure 5 shows the overlaid TIC for five oligo samples ranging from 18 to 21-mer in length.

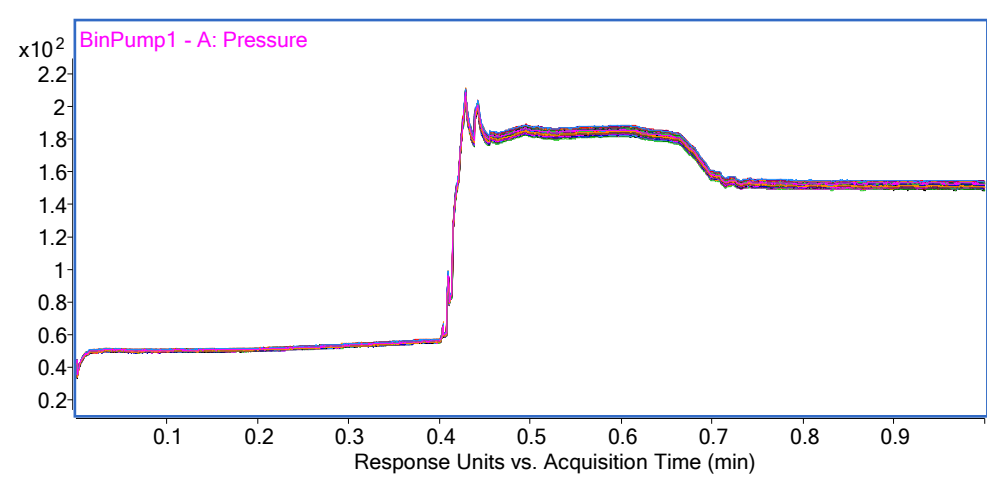


Figure 4. Overlaid pump pressure traces for 50 injections.

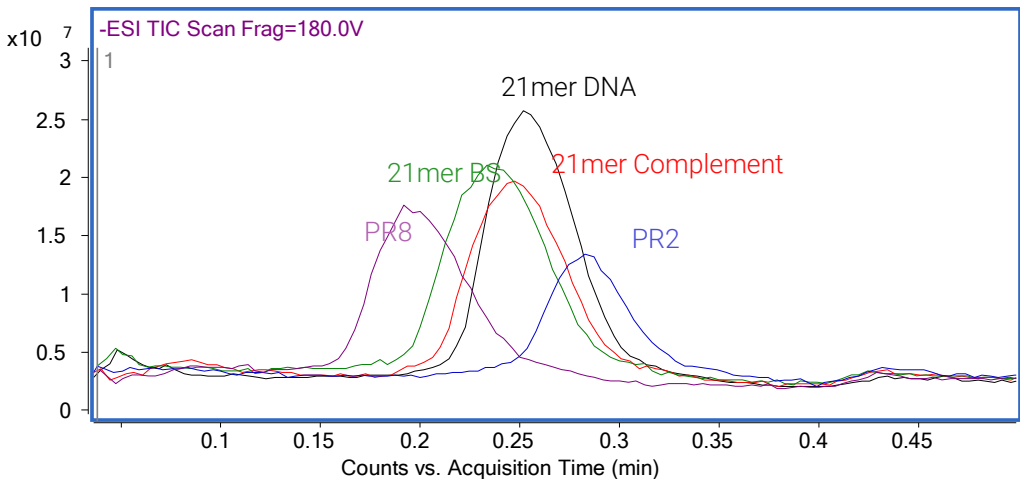
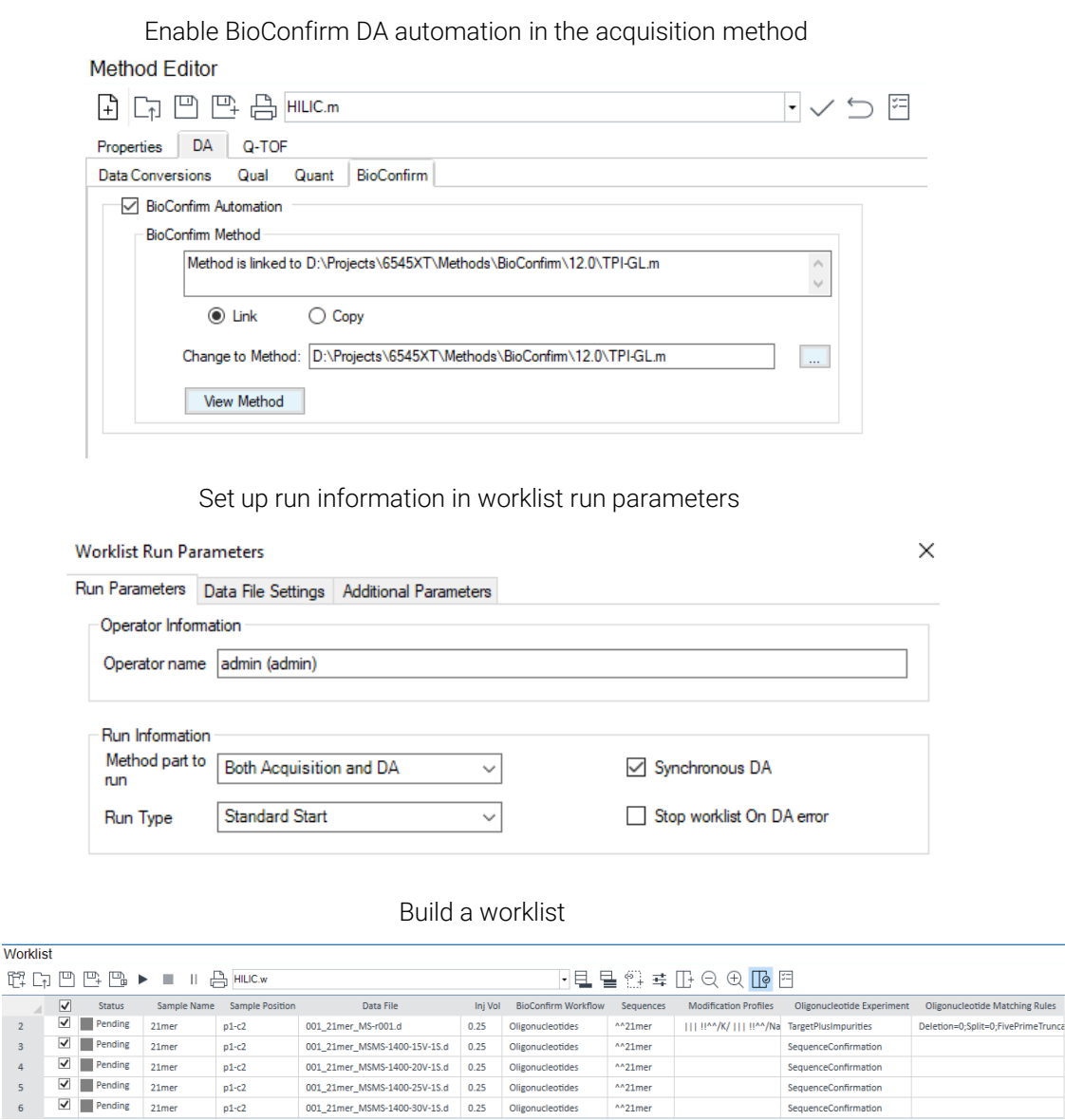


Figure 5. Overlaid TIC for five oligo samples.

Sample to Reports in About 5 Minutes

Data analysis methods for oligo workflows were developed in BioConfirm software [4,5]. Data acquisition and data analysis were synchronized by setting up automation in MassHunter Acquisition (Figure 6).



Conclusions

- This work describes two workflows enabling Fast LC/MS characterization of oligos without ion pairing reagents.
- The analytical results demonstrate that excellent mass accuracy for expected oligonucleotides was achieved.
- 100% sequence coverages were achieved on the five oligonucleotides analyzed.
- Sample to reports in about 5 minutes was achieved with synchronous data analysis.

References

<sup>1</sup>Rye, P.; Yang, Y., Agilent Technologies application note, publication number 5994-3753EN, 2022.

<sup>2</sup>Rye, P.; Schwarzer, C., Agilent Technologies application note, publication number 5994-5631EN, 2023.

<sup>3</sup>Li, G.; Rye, P., Agilent Technologies application note, publication number 5994-5632EN, 2023.

<sup>4</sup>Wong, D.; Rye, P., Agilent Technologies application note, publication number 5994-4817EN, 2022.

<sup>5</sup>Wong, D.; Rye, P., Agilent Technologies application note, publication number 5994-5071EN, 2022.

Figure 6. Automation setup in MassHunter Acquisition.

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