LC-MS Oligonucleotide Impurity Analysis: Trace Peak Centric vs Progressive Deconvolution Data Processing

Protein Metrics

Steven Broome, Ignat Shilov, St John Skilton, and Gary Wilson

Protein Metrics Boston, MA

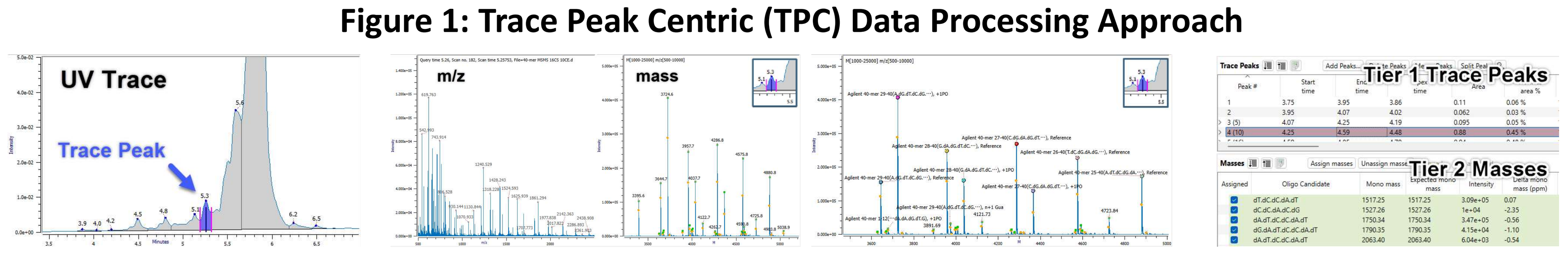
Summary

- Vendor neutral data processing and reporting software for oligonucleotide impurity analysis
- Assessment of novel progressive deconvolution data processing approach
- Different relative quantitation approaches compared including UV, MS, and UV-MS
- Accelerating oligonucleotide LC-MS data analysis and reporting

Introduction

Reliable detection and relative quantitation of synthetic oligonucleotide impurities by LC-MS is essential for process control. The data processing approach used directly impacts impurity detection coverage and relative quantitation consistency. Here we compare two approaches, **Trace Peak Centric (TPC)** and **Progressive Deconvolution (PD)**, using Protein Metrics Byos Oligo software.

Figure 1: Trace Peak Centric (TPC) Data Processing Approach



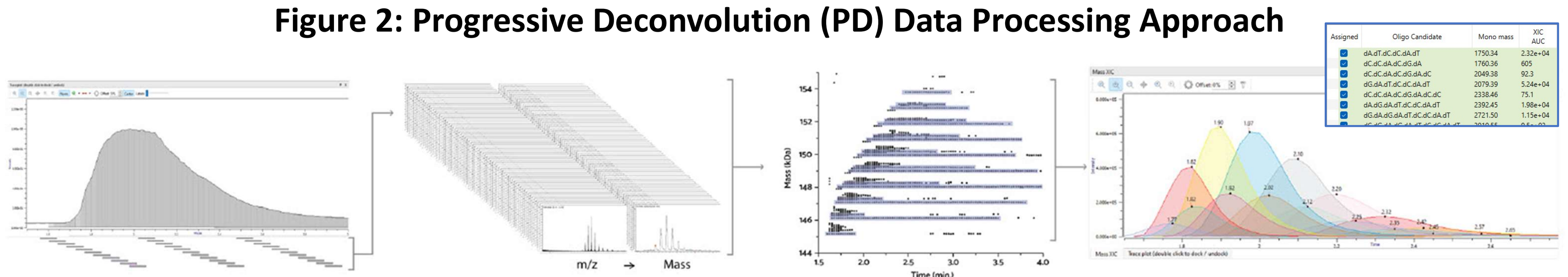
Step 1: Trace peak picking
Peak finding or targeted peak list

Step 2: Sum & deconvolute
m/z → neutral mass per trace peak

Step 3: Mass peak picking
Mass peak picking per trace peak

Output: Trace peaks/masses
Trace peak area and mass intensity quantitation

Figure 2: Progressive Deconvolution (PD) Data Processing Approach



Step 1: Overlapping time slices
Fixed-width windows across the elution range of interest

Step 2: Sum & deconvolute
m/z → neutral mass per slice

Step 3: Group mass features
Signal processing find high-confidence masses

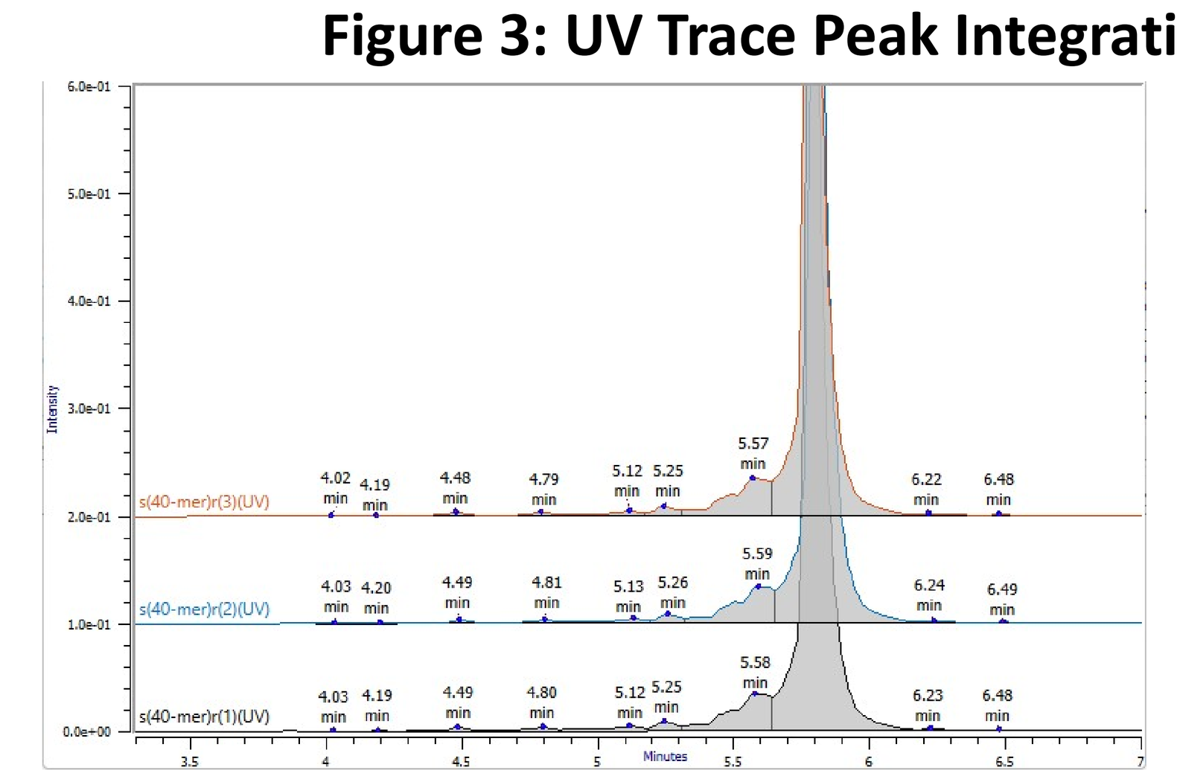
Output: Mass features
Mass XIC AUC quantitation

Methods

Triplicate Agilent DNA 40mer LC-HRMS datafiles were analyzed using **Byos Oligo (v6.0)** with TPC and PD data processing workflows (Figures 1 and 2). Monoisotopic masses were matched to the FLP and defined impurity types (clips, shortmers, longmers, substitutions, and chemical modifications) within 10 ppm. Relative quantitation was estimated using UV-only, UV-MS, and MS-only approaches. Automated report generation was performed using Byos template-driven reporting.

Results – UV relative quantitation (TPC)

Figure 3: UV Trace Peak Integration and % Relative Peak Area



		Sample	40mer				
		Replicate	1	2	3	[Avg]	[RSD]
Sample Peak #	Peak Comment						
1	Impurity		0.03	0.03	0.03	0.03	1.83
2	Impurity		0.05	0.05	0.05	0.05	1.64
3	Impurity		0.44	0.44	0.45	0.44	0.51
4	Impurity		0.48	0.47	0.48	0.48	0.81
5	Impurity		0.88	0.90	0.88	0.89	0.96
6	Impurity		0.86	0.87	0.90	0.88	2.13
7	Impurity		6.91	6.91	7.00	6.94	0.78
8	FLP		89.99	89.96	89.84	89.93	0.09
9	Impurity		0.26	0.27	0.26	0.26	0.66
10	Impurity		0.09	0.10	0.11	0.10	8.30

10x UV trace peaks were found per sample and integrated using Byos AutoCompute trace peak finder.

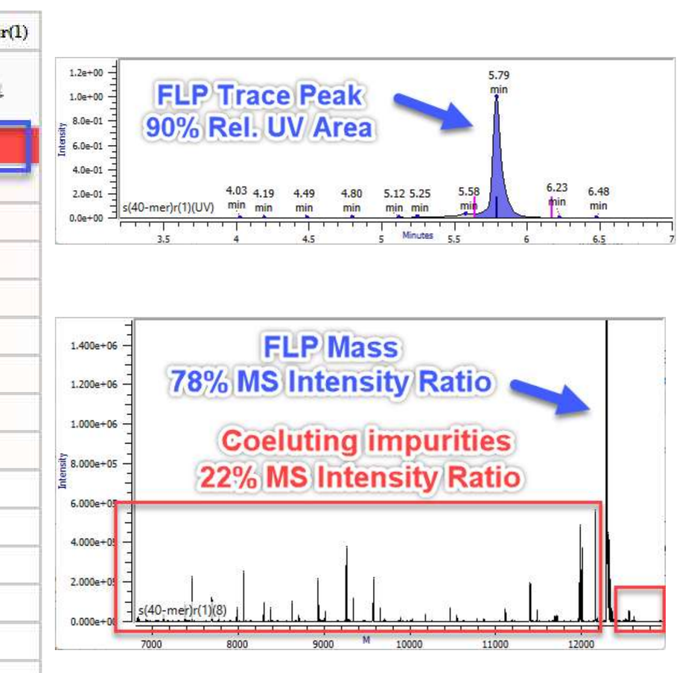
Byos automatically calculates relative UV peak area, estimating 90% FLP purity (RSD < 10%).

Results – UV-MS relative quantitation (TPC)

Figure 4: UV-MS calculation example

			Sample name	s(40-mer)r(1)
			Rel% UV Area	89.99 ↓
Oligo name	Mono mass	*MS Ratio%		
Agilent 40-mer, Reference	12286.12	78.22		70.39
Agilent 40-mer, n-1 Ade	11973.06	3.17		2.85
Agilent 40-mer 11-40(A.dG.dT.dG.dA.···), Reference	9259.60	2.46		2.21
Agilent 40-mer 2-40(C[illegible]	[illegible]	[illegible]		2.20
Agilent 40-mer 15-40(A.dC.dA.dG.dC[illegible]	[illegible]	[illegible]		1.47
Agilent 40-mer 17-40(A.dG.dC.dA.dA.···), [illegible]	[illegible]	1.46		1.31
Agilent 40-mer 10-40(A.dA.dG.dT.dG.···), Reference	9572.65	1.42		1.28
Agilent 40-mer 12-40(G.dT.dG.dA.dC.···), Reference	8930.53	1.40		1.26
Agilent 40-mer 4-40(A.dC.dG.dA.dC.···), Reference	11394.94	1.25		1.12
Agilent 40-mer 11-40(A.dG.dT.dG.dA.···), +1PO	9339.55	0.79		0.71
Agilent 40-mer 14-40(G.dA.dC.dA.dG.···), Reference	8297.44	0.65		0.58
Agilent 40-mer 13-40(T.dG.dA.dC.dA.···), Reference	8626.50	0.63		0.57
Agilent 40-mer 10-40(A.dA.dG.dT.dG.···), +1PO	9652.64	0.46		0.42
Agilent 40-mer 7-40(A.dC.dC.dA.dA.···), Reference	10463.82	0.45		0.40
Agilent 40-mer 14-40(G.dA.dC.dA.dG.···), +1PO	8377.41	0.44		0.40
Agilent 40-mer 15-40(A.dC.dA.dG.dC.···), Reference	7984.39	0.42		0.38
Agilent 40-mer 5-40(C.dG.dA.dC.dC.···), Reference	11105.90	0.41		0.37
Agilent 40-mer 4-40(A.dC.dG.dA.dC.···), +1PO	11474.91	0.39		0.35
Agilent 40-mer 8-40(C.dC.dA.dA.dG.···), Reference	10174.75	0.25		0.23
Agilent 40-mer 16-40(C.dA.dG.dC.dA.···), +1PO	7775.29	0.24		0.21
Agilent 40-mer 7-40(A.dC.dC.dA.dA.···), +1PO	10543.79	0.22		0.20
Agilent 40-mer 3-40(C.dA.dC.dG.dA.···), Reference	11708.04	0.20		0.18
Agilent 40-mer 13-40(T.dG.dA.dC.dA.···), +1PO	8706.48	0.20		0.18
Agilent 40-mer 2-40(C.dC.dA.dC.dG.···), n-1 Ade	11683.99	0.17		0.16
Agilent 40-mer, n+1 Ade	12599.18	0.17		0.15
Agilent 40-mer 9-40(C.dA.dA.dG.dT.···), Reference	9885.72	0.15		0.14
Agilent 40-mer, n-1 Gua	11957.09	0.12		0.11
Agilent 40-mer 7-40(A.dC.dC.dA.dA.···), n+1 Ade	10776.92	0.11		0.10
Agilent 40-mer 5-40(C.dG.dA.dC.dC.···), +1PO	11185.92	0.08		0.07

FLP 70% MS Intensity Ratio adjusted (UV-MS)



To account for coeluting impurities, Byos automatically adjusts relative UV peak area based on MS intensity ratios calculated for each trace peak (UV-MS). Using this approach, we estimate 70% FLP purity (RSD < 10%).

Figure 5: UV-MS FLP and top 5 impurities

	Sample	40mer				
	Replicate ←	1	2	3	[Avg] ↓	[RSD]
Oligo name						
Agilent 40-mer, Reference		70.62	67.46	71.63	69.90	3.11
Agilent 40-mer, n-1 Ade		2.85	3.17	2.62	2.88	9.58
Agilent 40-mer 11-40(A.dG.dT.dG.dA.···), Reference		2.47	2.67	2.46	2.53	4.61
Agilent 40-mer 2-40(C.dC.dA.dC.dG.···), Reference		2.20	2.42	2.00	2.21	9.65
Agilent 40-mer 17-40(A.dG.dC.dA.dA.···), +1PO		1.58	1.83	1.54	1.65	9.67
Agilent 40-mer 15-40(A.dC.dA.dG.dC.···), +1PO		1.55	1.79	1.53	1.62	8.87

Results – MS relative quantitation (TPC and PD)

Relative quantitation using deconvoluted mass Intensity (TPC) and XIC AUC (PD). Intensity yielded superior reproducibility (%RSD < 20%). The lower reproducibility observed with PD XIC AUC is likely due to limited MS scans per chromatographic peak; faster acquisition rates or broader LC peaks would improve reliability.

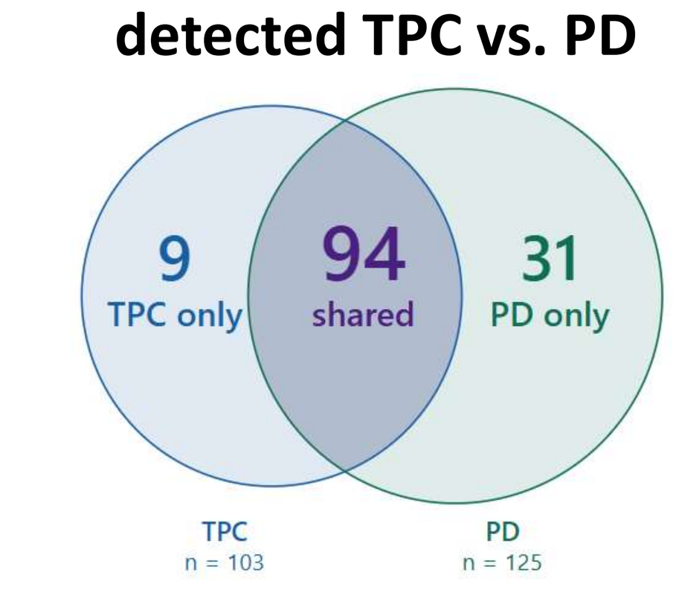
Figure 6: MS only relative quantitation TPC vs. PD (top 10 masses detected by abundance)

		Trace peak centric					Progressive deconvolution				
Oligonucleotide	Mono mass	Rep 1	Rep 2	Rep 3	Avg	%RSD	Rep 1	Rep 2	Rep 3	Avg	%RSD
Agilent 40-mer, FLP	12286.11	39.21%	37.20%	39.79%	38.73%	3.51%	38.38%	36.42%	37.35%	37.38%	2.62%
Clip (34–40)	2079.40	3.31%	3.33%	3.45%	3.36%	2.13%	3.79%	3.89%	5.55%	4.41%	22.40%
Clip (34–40) +3'PO	2159.36	2.54%	2.62%	2.41%	2.52%	4.29%	3.43%	3.44%	3.28%	3.39%	2.69%
Clip (29–40)	3643.65	2.19%	2.39%	2.15%	2.24%	5.75%	2.45%	2.52%	2.34%	2.44%	3.87%
Clip (11–40)	9259.59	2.17%	2.25%	2.17%	2.20%	2.09%	0.71%	0.78%	1.23%	0.90%	31.16%
Clip (17–40)	7382.28	2.00%	2.15%	1.99%	2.04%	4.27%	2.39%	2.55%	2.36%	2.43%	4.02%
Clip (25–40)	4878.85	1.93%	1.95%	1.99%	1.96%	1.57%	1.68%	1.71%	1.06%	1.48%	24.68%
Clip (21–40)	6138.07	1.98%	1.99%	1.85%	1.94%	3.86%	1.45%	1.47%	1.30%	1.41%	6.57%
Clip (25–40) +3'PO	4958.82	1.76%	1.80%	1.80%	1.79%	1.07%	1.99%	1.97%	1.94%	1.97%	1.30%

%RSD > 20% | %RSD ≤ 20% | *Trace peak centric: Intensity | Progressive deconvolution: XIC AUC*

Results – Comparing TPC and PD

Figure 7: Impurities detected TPC vs. PD



TPC and PD: Detect FLP and >100 impurity masses (below 0.01% Rel MS abundance), with rel. abundance estimated for each identified mass.

TPC Benefits: Enables UV, MS, and UV-MS based quantitation. UV-MS provides a hybrid approach with advantages over UV-only and MS-only methods. Simpler, faster processing makes TPC well-suited for high-throughput analysis.

PD Benefits: Signal processing limits noise, enabling detection of very low abundance impurities. A single mass assignment per impurity simplifies interpretation and avoids duplicate assignments seen across multiple trace peaks.

Conclusion

TPC and PD approaches both successfully identified the FLP and >100 impurity masses, including n-1, n+1, clips (6–39mer), and other modifications. UV-only, MS-only, and UV-MS quantitation provide complementary approaches for estimating relative abundance. The analysis and reporting are automated using Byos Oligo. For this dataset PD demonstrates higher sensitivity (+31 impurity mass assignments), while TPC relative quantitation showed superior reproducibility compared to PD, which would benefit from more MS scans per chromatographic peak. TPC and PD offer unique and complementary advantages.