Poster Reprint

CASSS MS 2021

Oligonucleotide Analysis by Mass Spectrometry: Sequence Display and Automation is no longer a Sisyphean Task

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1

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Introduction

As the world has learned over the past year, mRNA vaccines are safe, effective, and quick to develop. Along with mRNA's, there are a variety of oligonucleotide therapeutics in clinical trials or already on the market, including antisense oligonucleotides, siRNA's, miRNAs, and aptamers.

Mass spectrometry (MS) analysis of oligonucleotides, however, is far from routine, and especially MS data analysis may be viewed as expensive, difficult, specialized, and tedious – a task for Sisyphus! Many oligonucleotide drug makers do not yet know what MS has to offer, and rely on sequencing, chromatography, and other assays. Here we demonstrate that MS – with appropriate software! – can assay a mixture of oligonucleotides quickly and accurately, down to trace impurities below 1% of intended product.

Methods – Experimental

We ordered two DNA oligonucleotides, a 21-mer and a 40-mer from Integrated DNA Technologies (IDT).

Instrumentation

An Agilent 1290 Infinity II UHPLC was attached to an Agilent 6545 Q-TOF. The 1290 LC consisted of a binary gradient pump, multisampler, column compartment, and Diode Array Detector. The 6545 MSD utilized an Agilent Jet Stream ESI Source.

Sample Preparation

The two DNA oligonucleotide strands were prepared at a concentration of 0.5 mg/mL in ultra-pure water. These samples were then desalted using Amicon® Ultra 0.5 mL Centrifugal

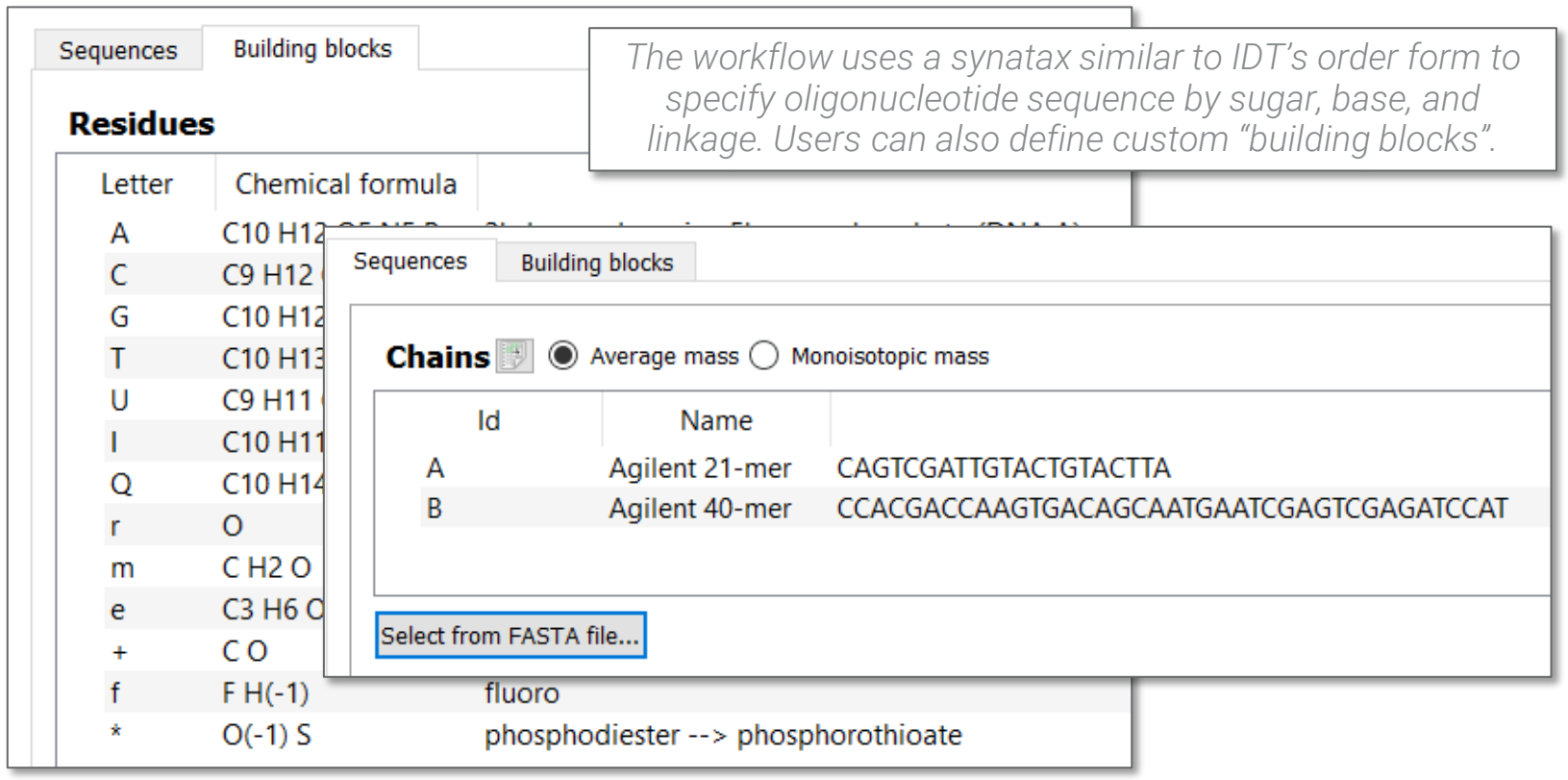
LC Parameters		
Mobile Phase A	200 mM HFIP + 8 mM TEA + 5% (v:v) MeOH	
Mobile Phase B	MeOH	
Flow Rate (mL/min)	0.2	
Injection Volume (µL)	3	
Gradient	Time (min)	%B
	0	0
	1.5	0
	6	30
	6.5	0
	13	0
Column Temperature (°C)	50	

6545 Q-TOF MS Parameters	
Drying Gas Temperature (°C)	325
Drying Gas Flow (L/min)	10
Nebulizer Pressure (psig)	28
Sheath Gas Temperature (°C)	325
Sheath Gas Flow (L/min)	10
Capillary Voltage (V)	4000
Nozzle Voltage (V)	1000
Fragmentor Voltage (V)	110
Acquisition Mode	Targeted MS/MS
MS Acquisition Rate (spectra/s)	1
MS Acquisition Time (ms/spectrum)	1000
MS/MS Acquisition Rate (spectra/s)	1
MS/MS Acquisition Time (ms/spectrum)	1000

We acquired MS2 spectra of various precursor charge states, and used various collision energies..

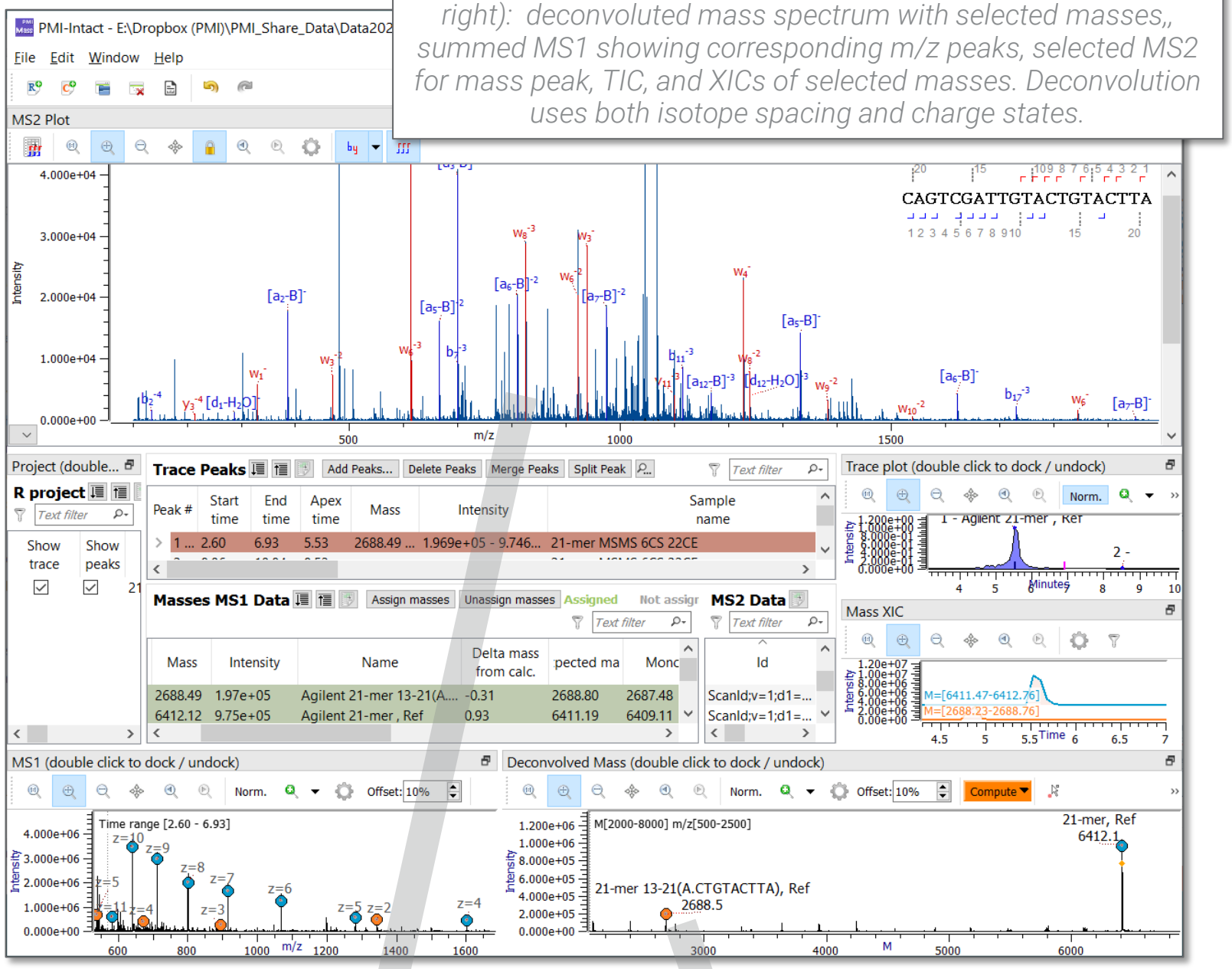
Data Analysis for the 21-mer

We used the new oligonucleotide workflow in Protein Metrics Byos™ to analyze and visualize the MS data for the 21-mer with z = 6- and collision energy 22.

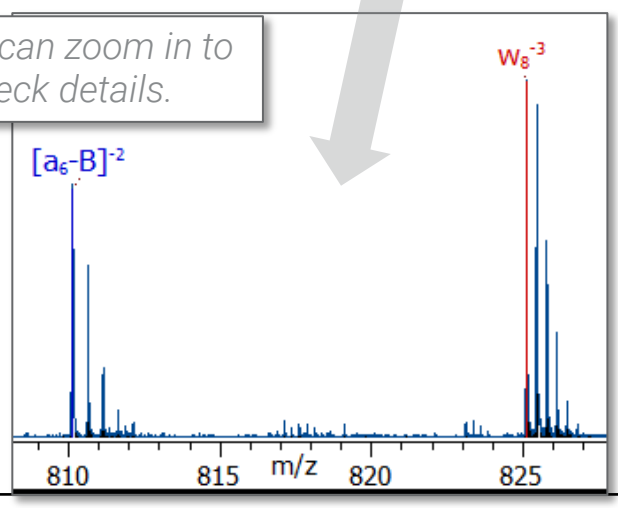


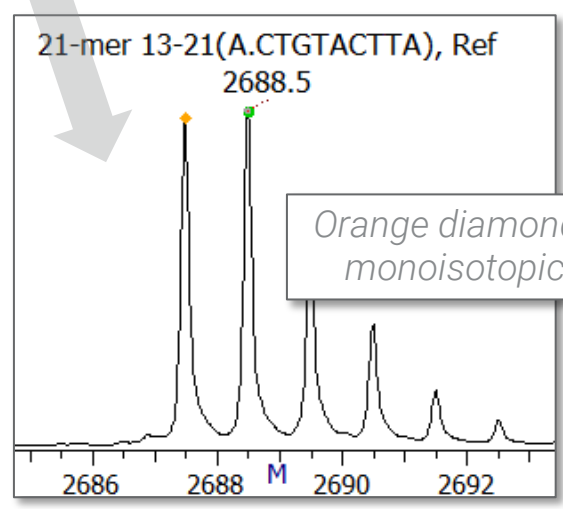
The workflow uses a synatax similar to IDT's order form to specify oligonucleotide sequence by sugar, base, and linkage. Users can also define custom "building blocks".

The interactive application shows plots (clockwise from lower right): deconvoluted mass spectrum with selected masses,, summed MS1 showing corresponding m/z peaks, selected MS2 for mass peak, TIC, and XICs of selected masses. Deconvolution uses both isotope spacing and charge states.



Users can zoom in to check details.

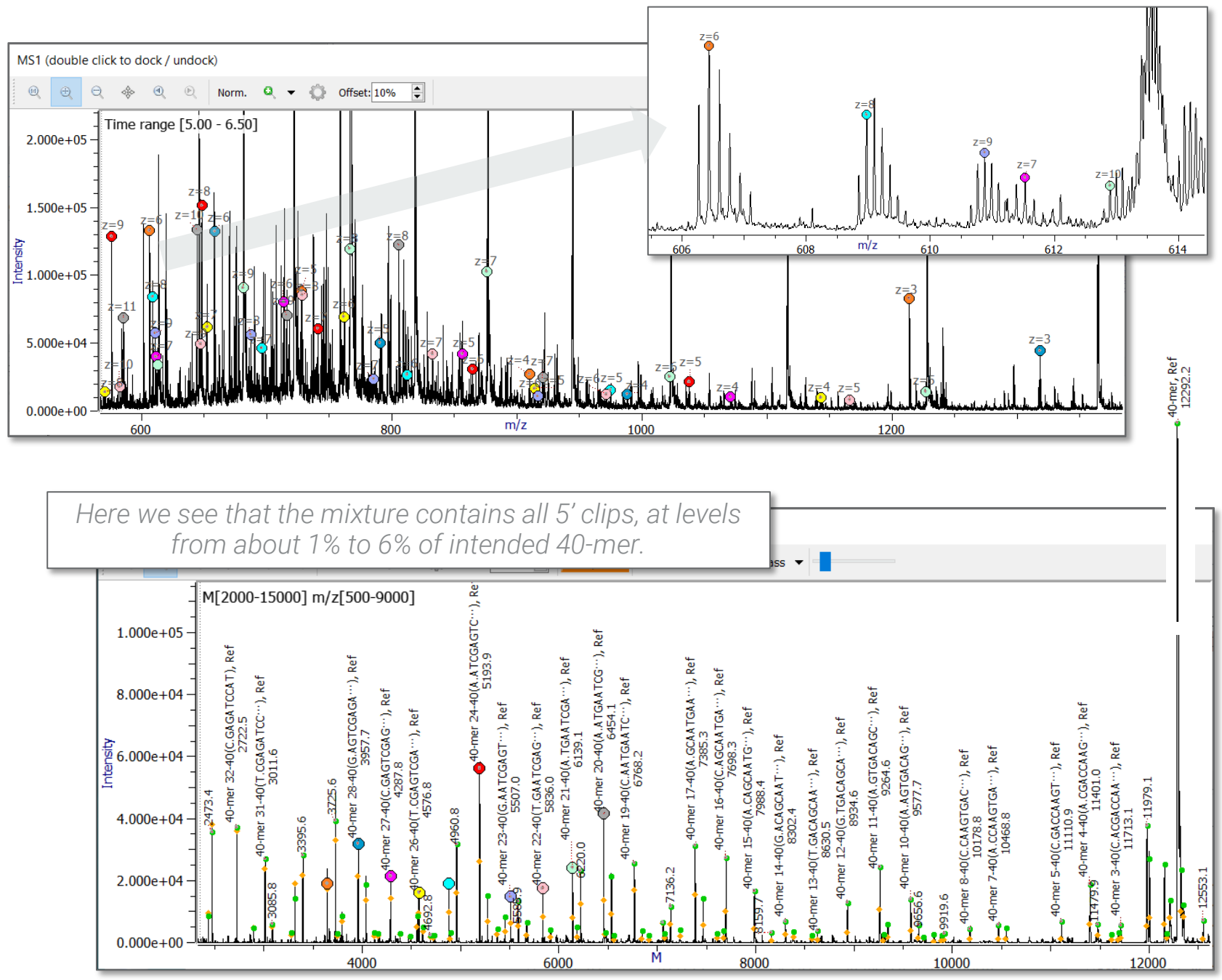




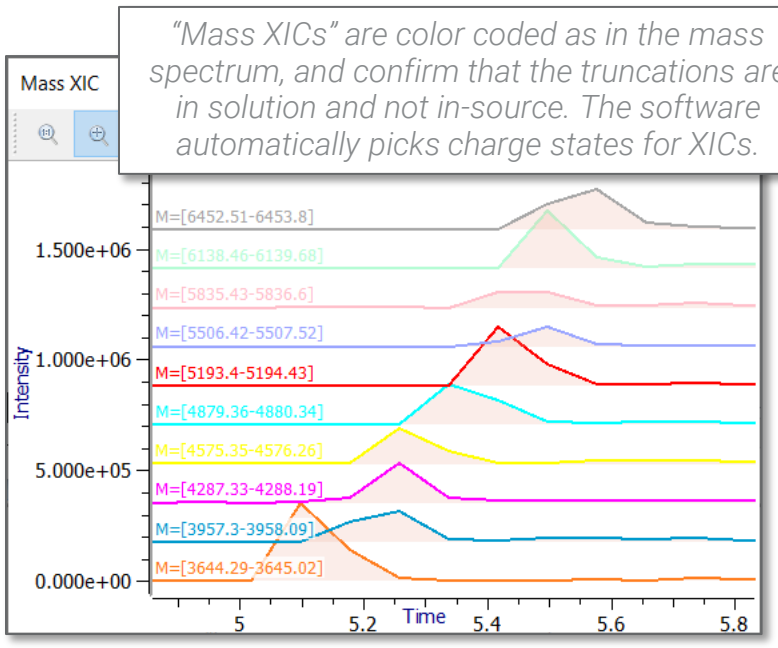
Orange diamond shows monoisotopic mass

Data Analysis for the 40-mer

This panel shows the 40-mer. Byos™ allows the user to analyze arbitrary time slices, but here we analyzed all the MS1 data at once to show the high dynamic range of the deconvolution algorithm.

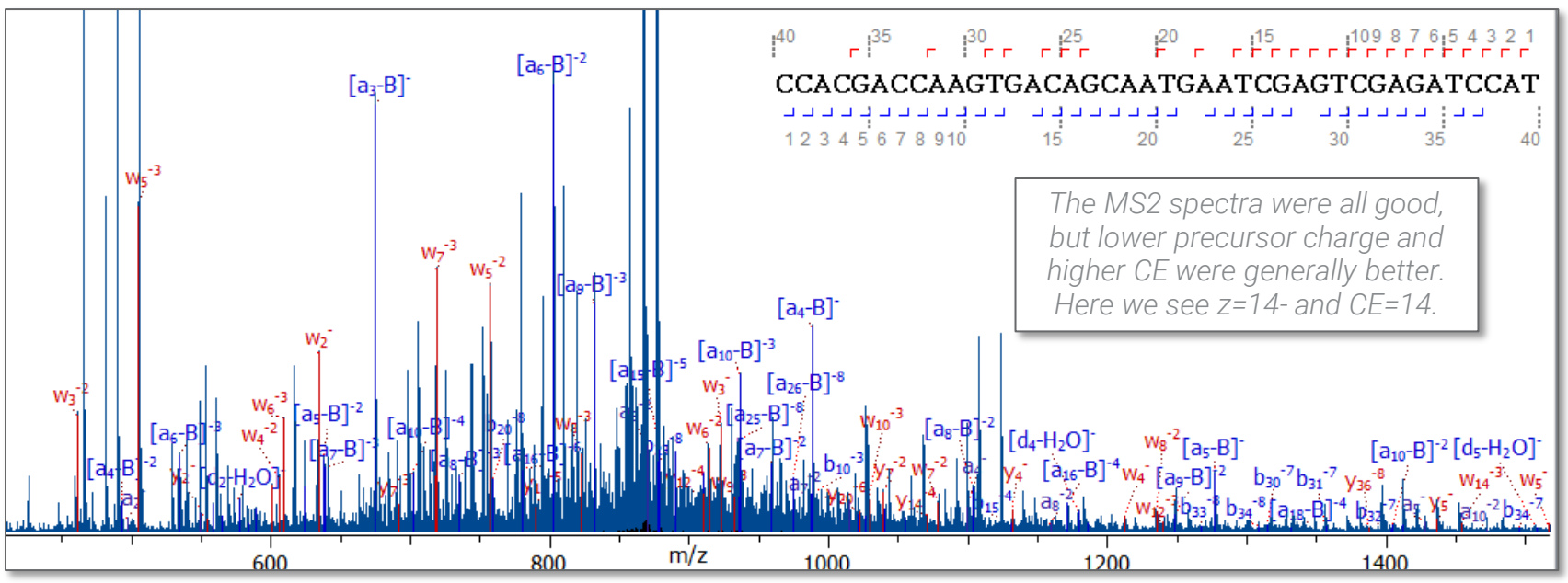


Here we see that the mixture contains all 5' clips, at levels from about 1% to 6% of intended 40-mer.



"Mass XICs" are color coded as in the mass spectrum, and confirm that the truncations are in solution and not in-source. The software automatically picks charge states for XICs.

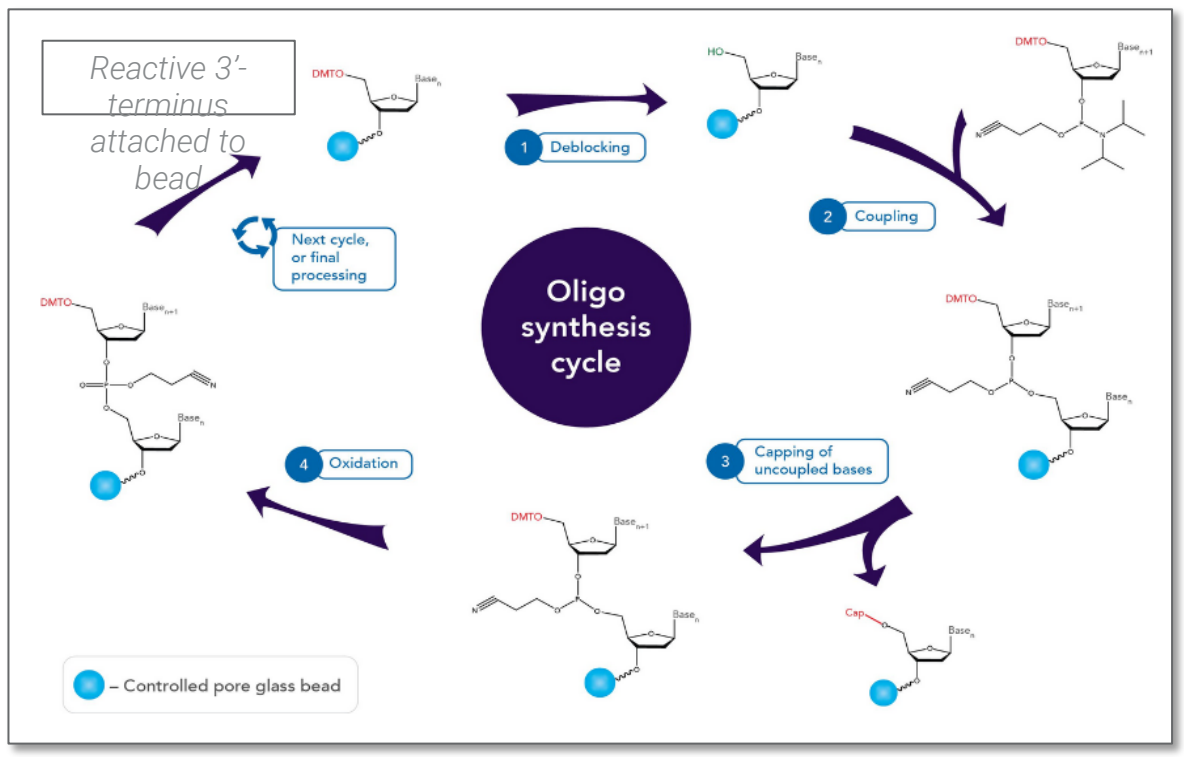
Reports include bar charts, heat maps, etc. Here we see that the 40-mer is 62% of total mass intensity. Truncations are color coded by the next 5' base.



The MS2 spectra were all good, but lower precursor charge and higher CE were generally better. Here we see z=14- and CE=14.

Synthesis Byproducts

Why do the synthesized DNA samples contain 5’ truncations? The answer lies in IDT’s phosphoramidite 3’ to 5’ synthesis. IDT claims coupling efficiency of 99.4%, which allows synthesis of DNA sequences up to about 200 nucleotides. The intensity of the 40-mer peak in the bar chart at the left lets us estimate efficiency. E^{40} = 62% implies E = 98.8%. (This is an underestimate because the 40-mer peak has more isotopes than the lower-mass peaks.)



From https://www.idtdna.com/pages/education/decoded/article/oligo-synthesis-why-idt-leads-the-oligo-industry

Summary

We believe that MS analysis is under-utilized in oligonucleotide analysis. Oligonucleotides are actually more amenable to MS analysis than peptides and proteins due to: propensity to hold (negative) charge, less heterogeneity, fewer modifications, elution order determined by length, and good collisional fragmentation. The only weak point is that oligonucleotides tend to have higher mass, about 3x the mass of the peptides that they encode.

MS analysis, however, has been held back by a lack of software. Here we have shown an upcoming Byos workflow for oligo analysis. This workflow has a number of features that are especially useful for oligos:

- Charge deconvolution using isotope spacing and / or multiple charge states. Masses < 5 kDa often have only two or three distinct charge states; masses > 15 kDa may not be isotope-resolved; and mass in between may have weak signal in both “channels”.
- Fully integrated MS1 and MS2 analysis
- Automatic peak assignment for both MS1 and MS2, including truncations, which are common in oligos
- “Mass XICs” for tracking the elution profiles of co-eluting masses in complex matrixes
- Integrated, customized reporting

Sisyphus by Jeffrey Hummel

DE44498.5813310185

Published in USA, September 14, 2021

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