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High-throughput Mass Spectrometry Analysis of Synthetic Oligonucleotides: A Comparison of Data from Fast LC and RapidFire Methods

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Introduction

Liquid chromatography (LC) and mass spectrometry (MS) play a vital role in the characterization of synthetic oligonucleotides (oligos), and the appetite for higher throughput analytical methods has increased in the past years alongside the acceleration of oligo production and use. Traditional LCMS of oligos, where separation is desired, can necessitate run times of many minutes. However, not all applications require chromatographic separation and desalting prior to MS measurement can be sufficient. This work describes and compares two methods, Fast LC and RapidFire, for the high-throughput sampling and desalting of oligos. Each method was optimized for speed on 18mers, and then characterized for performance on a range of synthetic DNA and RNA, 18 to 100mer in length.

Experimental

Fast LC Method

LC Conditions, Agilent 1290 Infinity II Binary pump, Multisampler with Dual Needles			
Column	AdvanceBio Oligo UHPLC Guard column, 1.7 um, 2.1 x 5mm pn: 821725-921		
Column temperature	room temperature		
Injection volume	10 µL		
Smart Overlap	Enabled, alternating needle		
Autosampler temp	5 ºC		
Needle wash	Methanol:Water 50:50		
Mobile phase	A = Water + 15 mM TEA + 400 mM HFIP B = Methanol		
Flow rate	1.75 mL/min		
Gradient program	Time (min)	Time (sec)	B (%)
	0.00	0.00	20
	0.03	1.80	20
	0.24	14.4	50
	0.25	15.0	100
	0.30	18.0	100
	0.31	18.6	20
	0.59	35.0	20
Stop time	0.60 min		
Post time	0.00 min		

6545 LC/Q-TOF Conditions	
Ion Polarity	Dual AJS Negative
Data Storage	Both (Centroid and Profile)
Gas temperature	350 ºC
Drying gas flow	13 L/min
Nebulizer gas	60 psi
Sheath gas temperature	350 ºC
Sheath gas flow	12 L/min
Capillary voltage	3500V
Nozzle voltage	2000V
Fragmentor	200 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	400 – 3200 *m/z*
Acquisition Rate	10 spectra/sec

RapidFire Method

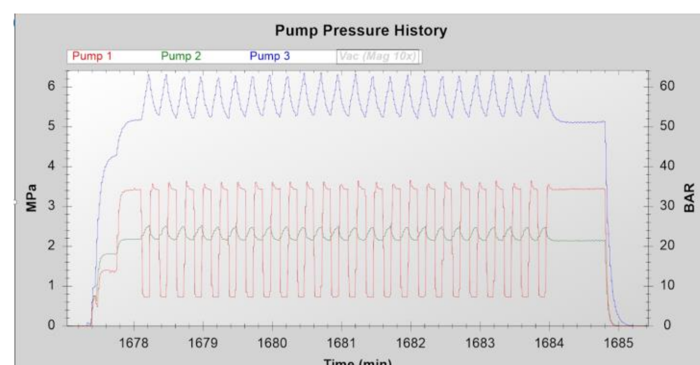
RapidFire Conditions		
Cartridge	PLRP-S, 30 um 1000A, 4 ul bed volume	
Cartridge Temperature	room temperature	
Injection volume	10 uL	
Pump 1	Water + 7.5 mM TEA + 200 mM HFIP	1.2 ml/min
Pump 2	50% Methanol + 7.5 mM TEA + 200 mM HFIP	0.6 ml/min
Pump 3	50% Methanol + 7.5 mM TEA + 200 mM HFIP	0.6 ml/min
State 1	Aspirate sample (sip sensor on)	600 msec
State 2	Load/wash (desalt)	6,000 msec
State 3	Extra wash	0 msec
State 4	Elute (inject)	6,000 msec
State 5	Reequilibrate	500 msec

6545 LC/Q-TOF Conditions	
Ion Polarity	Dual AJS Negative
Data Storage	Both (Centroid and Profile)
Gas temperature	275 ºC
Drying gas flow	11 L/min
Nebulizer gas	35 psi
Sheath gas temperature	325 ºC
Sheath gas flow	11 L/min
Capillary voltage	3500V
Nozzle voltage	2000V
Fragmentor	200 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	400 – 3200 *m/z*
Acquisition Rate	4 spectra/sec

For the Fast LC method, an Agilent 1290 Infinity II multi-sampler was equipped with dual injection needles that alternated between samples with smart overlap, providing analysis from one needle at the same time as sample draw from the other. The run time was further optimized by a fast gradient at high flow running through a guard column attached directly to the analytical nebulizer of the MS. The high flow rate for the Fast LC method was required to desalt the oligos quickly. In turn, the Fast LC acquisition rate was set to 10 spectra/sec to ensure at least 15 points across all chromatographic peaks (which were ~2 seconds wide, vs ~5 seconds for the RapidFire method). For the RapidFire method, the system performed a six second desalting (Pump 1, State 2) followed by a six second elute (Pump 3, State 4) on each sample. All resulting data were analyzed using MassHunter Bioconfirm B07.

Results and Discussion

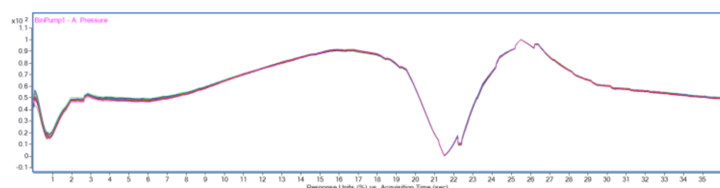
Throughput and Reproducibility – RapidFire



The throughput of the RapidFire method is determined by the sum of the five states (~13 seconds, see experimental) plus ~1.5 seconds for plate stage motion, and was just under 15 seconds per sample. For RapidFire MS, to circumvent the delay times associated with MS acquisition start/stop, a single data file is acquired per sample set and parsed post-acquisition. This figure shows the pressure for all three RapidFire pumps as one continuous file for a set of 24 replicate injections. For each pump, the pressure peaks and valleys were steady, and in the range between 0.5 and 10 MPa, consistent with a stable method.

Results and Discussion

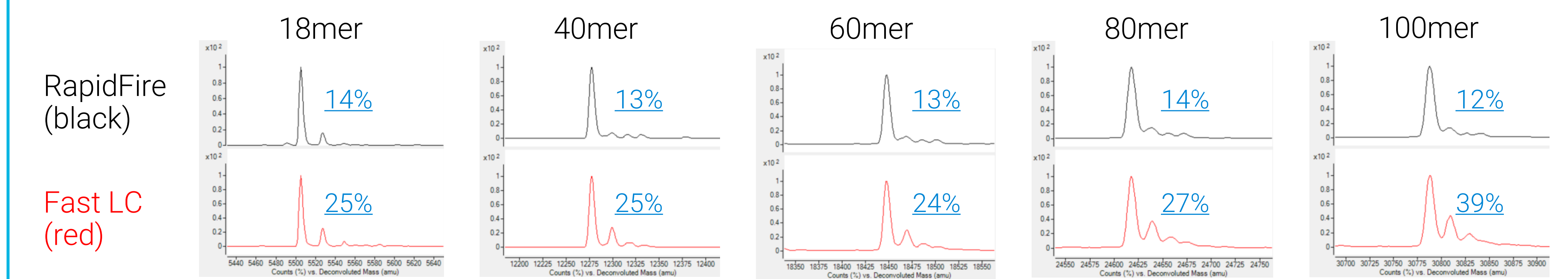
Throughput and Reproducibility – Fast LC



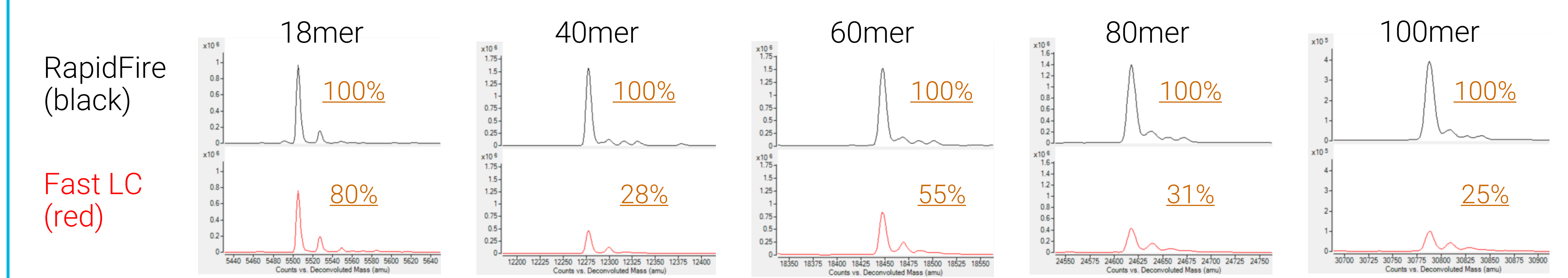
The throughput of the Fast LC method is determined by the gradient program (~35 seconds, optimized within the time of next sample draw) plus MS acquisition stop/start (~5 seconds), and was 40 seconds per sample. This figure shows the overlaid pump pressure traces from 24 injections. The traces are superimposed, revealing good gradient reproducibility.

Desalting and Signal Intensity

Scaled to largest peak in each spectrum. The percent salt adducts, relative to target peak, are in blue.



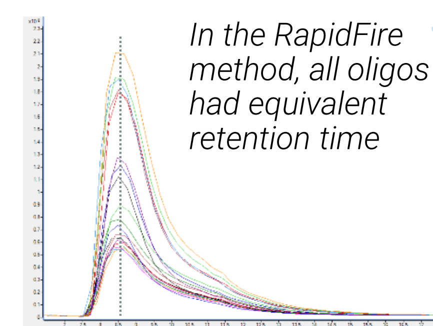
Linked Y-axis. The intensity of the target peaks for each oligo size are indicated in brown.



The panels above show the deconvoluted spectra from unpurified 18, 40, 60, 80, and 100mer oligos acquired using the RapidFire method (black) and the Fast LC method (red). The top figure represents the data scaled to the largest peak in each spectrum, and shows that the RapidFire method was more efficient than Fast LC at decreasing salt adducts, which appear as peaks +22 (Na) and +38 (K) Da. The relative percent of adducts, to the target peak, for each spectrum are indicated in blue. Very efficient desalting by the RapidFire method derives from the 6 second State 2 (see experimental) on the 4 ul bed volume cartridge, which results in 15 cartridge volumes of wash. The bottom figure shows the same data as on top but with the Y-axis for each oligo size linked. Comparison of the absolute peak heights shows the Fast LC method provides less abundant target MS signals, which are indicated for each oligo in brown. Despite the separative characteristics of Fast LC (see below) which can decrease ion suppression and thereby increase signal, the lower signals from Fast LC are the combined result from higher pump flow rate (1.75 vs 0.6 ml/min for RapidFire), faster acquisition rate (10 vs 4 spectra/sec for RapidFire), and less efficient desalting.

Oligo Retention - RapidFire

To evaluate oligo separation by the two methods, nineteen unique DNA and RNA samples ranging from 18 to 100mer in length were measured. In the RapidFire method, all of the oligos eluted from the cartridge at the same retention time. This result was expected as the RapidFire is specifically designed to prevent separation by switching from low to high organic conditions instantly (by valving), utilizing cartridges with a small resin volume (4 ul), and eluting in the reverse direction to minimize analyte/cartridge interactions. This figure shows the overlaid total ion chromatograms (TIC) for all nineteen samples.



Results and Discussion

Oligo Retention – Fast LC

In contrast to the RapidFire method, variable retention times were observed with the Fast LC method. Figure A shows the overlaid TIC for nineteen unique DNA and RNA samples ranging from 18 to 100mer in length. For these samples, the retention times varied within a 7 second window. Figure B shows overlaid extracted ion chromatograms for a 20, 40, 60, 80, and 100mer that were injected as a single mixture, illustrating resolution of these products by a combination of chromatography and mass.

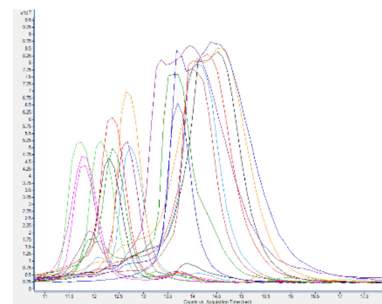


Figure A. Differential RT from the Fast LC method

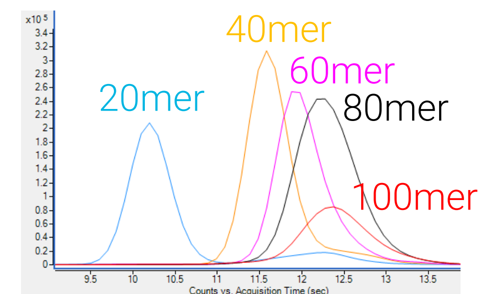


Figure B. Overlaid EIC from an oligo mixture

To evaluate the ability of the Fast LC method to separate and produce distinct deconvolution results for two oligos that were close in size, a 1:1 mixture of 18mer and 20mer was run. Figure C shows the TIC, revealing the oligos produced peaks which the software integrated separately. Figure D shows the resulting deconvoluted spectra, revealing the two species, and their respective impurities. This separation could be easily improved by small changes to the gradient program (not shown).

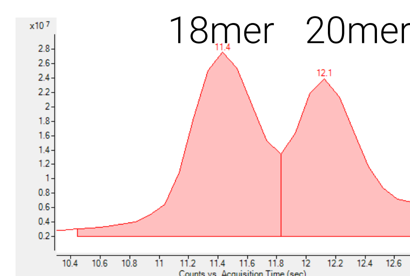


Figure C. Separation of 18 and 20mer by Fast LC

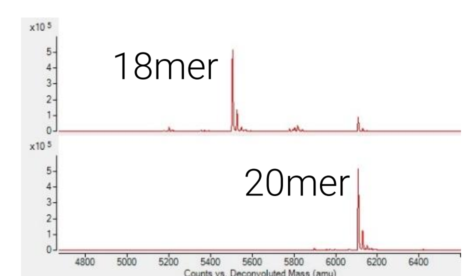
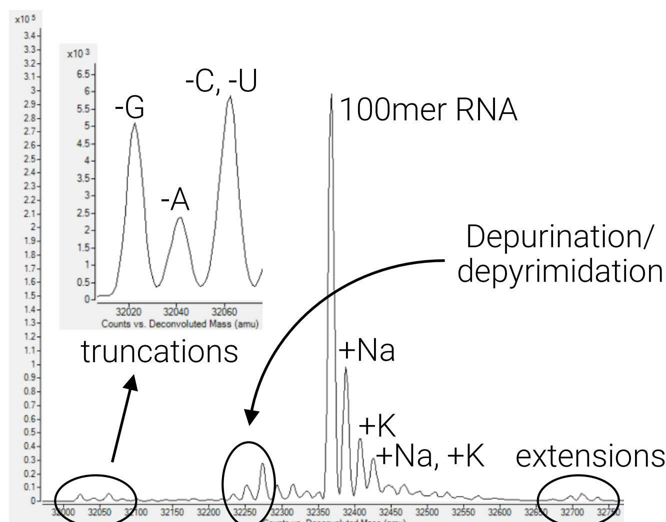


Figure D. Deconvoluted spectra showing how oligo separation can simplify data interpretation

Low Abundance Impurity Analysis



High-throughput purity assessment of oligos can be done by mass resolving the products from a single chromatographic peak. Oftentimes, there are many low abundance impurities coeluting with the highly abundant target, making MS measurement with a wide dynamic range, as well as software that can deconvolute complicated spectra, critical. To evaluate the detection of low abundance impurities in the same chromatographic peak as the main product, the RapidFire method was used to analyze a 100mer guide RNA. This figure shows that despite zero chromatographic separation, the deconvolution results reveal 100mer RNA as well as numerous impurities, many with a relative area as low as ~0.5%. As expected, this dynamic range was even better with separative/lower throughput methods (data not shown).

Conclusions

- Both the RapidFire TOF and Fast LC TOF methods produced reproducible and high quality data for synthetic oligos.
- The RapidFire method sustained a throughput of 15 seconds per sample (240 samples an hour, 5760 a day) while the Fast LC method sustained a throughput of 40 seconds per sample (90 samples an hour, 2160 a day).
- The RapidFire method desalted oligos more efficiently than Fast LC, about 2- to 3-fold as oligo size increased.
- The Fast LC method produced less intense target signal than RapidFire, from 80 to 25% as oligo size increased.
- Small changes to the Fast LC method, with some compromise to throughput, further improved its performance.
- The Fast LC method afforded some separation of oligo species, a characteristic that could simplify the interpretation of data from mixtures and could also be adjusted to balance the throughput and separation needs of the application.
- In spite their speed over separation approach, both high-throughput systems provided excellent oligo data by mass resolving large numbers of low abundance impurities.

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