Application Note

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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 LC/MS 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, then characterized for performance on a range of synthetic DNA and RNA, 18 to 100mer in length.

Experimental

For the Fast LC method, an Agilent 1290 Infinity II multisampler 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 (Figure 1). 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, versus ~5 seconds for the RapidFire method). For the RapidFire method (Figure 2), the system performed a 6-second desalting (Pump 1, State 2) followed by a 6-second elute (Pump 3, State 4) on each sample. All resulting data were analyzed using MassHunter Bioconfirm B07.

Figure 1. Fast LC method using an Agilent 1290 Infinity II multisampler equipped with dual injection needles.

Figure 2. Agilent RapidFire 400 high-throughput mass spectrometry system coupled to an Agilent Ultivo triple quadrupole LC/MS.

Fast LC conditions

Agilent 1290 Infinity II Binary Pump, Multisampler with Dual Needles	
Column	Agilent AdvanceBio Oligo UHPLC Guard column, 2.1 × 5 mm, 1.7 µm (p/n 821725-921)
Column Temperature	Room temperature
Injection Volume	10 µL
Smart Overlap	Enabled, alternating needle
Autosampler Temperature	5 °C
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	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

Agilent 6545 LC/Q-TOF	
Ion Polarity	Dual AJS Negative
Data Storage	Both (centroid and profile)
Gas Temperature	350 °C
Drying Gas Flow	13 L/min
Nebulizer Gas	60 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	2,000 V
Fragmentor	200 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	400 to 3,200 *m/z*
Acquisition Rate	10 spectra/sec

RapidFire conditions

Agilent RapidFire 400		
Cartridge	Agilent PLRP-S, 30 µm, 1,000 Å, 4 µL bed volume	
Cartridge Temperature	Room temperature	
Injection Volume	10 µL	
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	Re-equilibrate	500 msec

Agilent 6545 LC/Q-TOF	
Ion Polarity	Dual AJS Negative
Data Storage	Both (centroid and profile)
Gas Temperature	275 °C
Drying Gas Flow	11 L/min
Nebulizer Gas	35 psi
Sheath Gas Temperature	325 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	2,000 V
Fragmentor	200 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Mass Range	400 to 3,200 *m/z*
Acquisition Rate	4 spectra/sec

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 postacquisition. Figure 3 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.

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. Figure 4 shows the overlaid pump pressure traces from 24 injections. The traces are superimposed, revealing good gradient reproducibility.

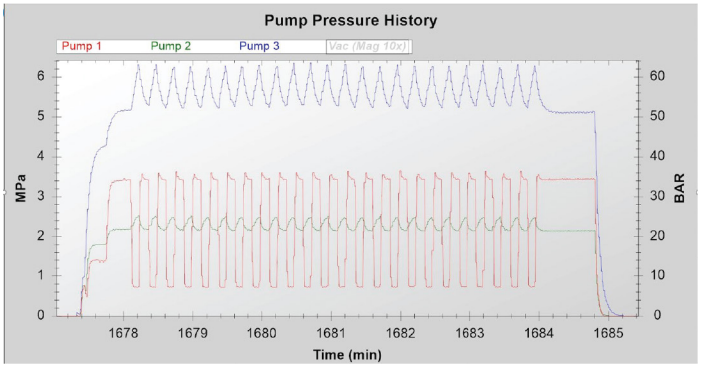


Figure 3. Overlay of three RapidFire pumps for 24 replicate injections.

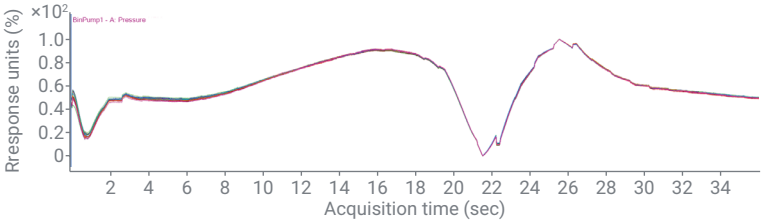
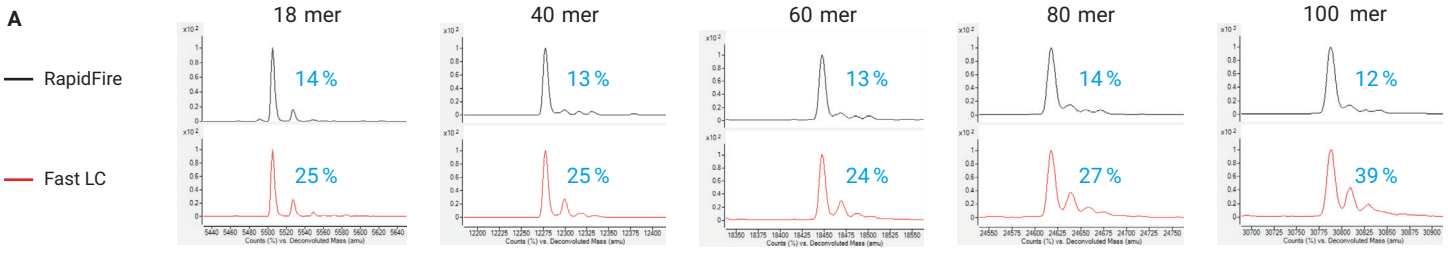


Figure 4. Pump pressure traces for 24 injections, revealing good reproducibilty.

Desalting and signal intensity

Figure 5 shows the deconvoluted spectra from unpurified 18, 40, 60, 80, and 100mer oligos acquired using the RapidFire method (black) and the Fast LC method (red). Figure 5A 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 µL bed volume cartridge, which results in 15 cartridge volumes of wash. Figure 5B 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 Figure 7), 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 vsersus 0.6 mL/min for RapidFire), faster acquisition rate (10 vsersus 4 spectra/sec for RapidFire), and less efficient desalting.

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.

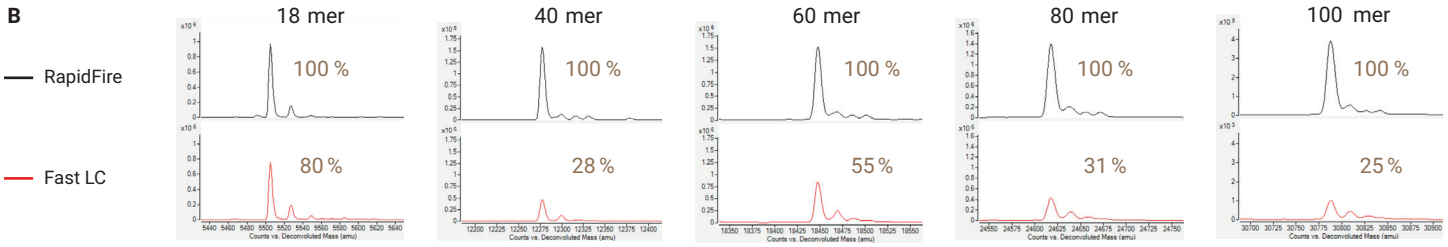


Figure 5. Deconvoluted spectra from unpurified oligos, acquired using the RapidFire and Fast LC methods.

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) using cartridges with a small resin volume (4 µL), and eluting in the reverse direction to minimize analyte/cartridge interactions. Figure 6 shows the overlaid total ion chromatograms (TIC) for all 19 samples.

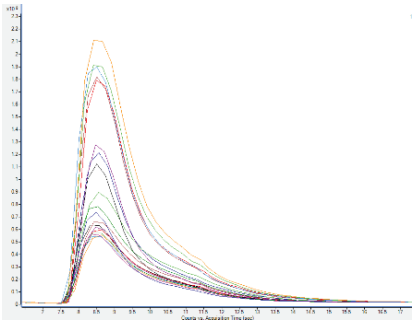


Figure 6. TIC for 19 samples. In the RapidFire method, all oligos had equivalent retention time.

Oligo retention – Fast LC

In contrast to the RapidFire method, variable retention times were observed with the Fast LC method. Figure 7A shows the overlaid TIC for 19 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 7B 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.

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 18 and 20mer was run. Figure 7C shows the TIC, revealing the oligos produced peaks which the software integrated separately. Figure 7D 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).

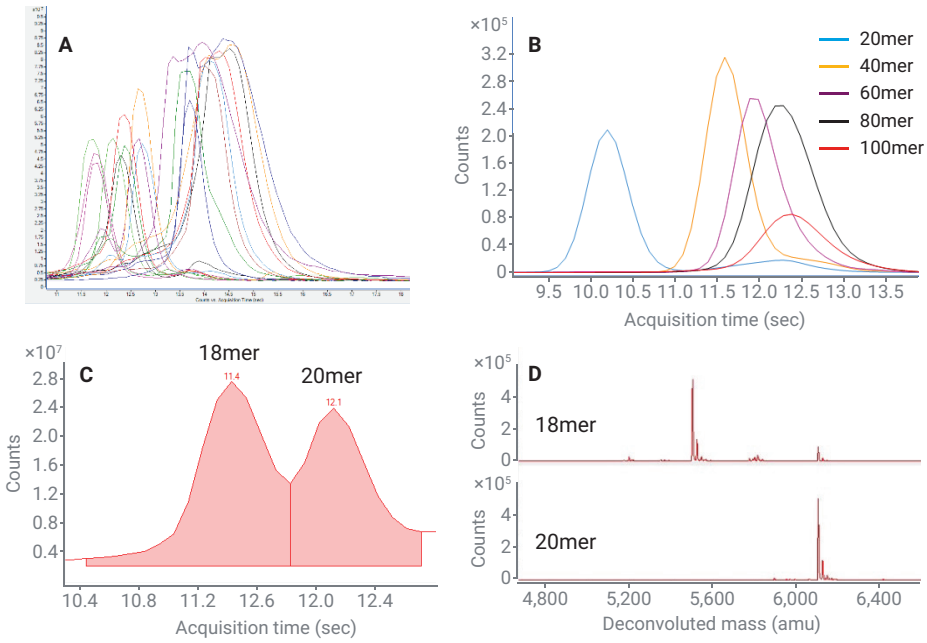


Figure 7. (A) Differential RT from the Fast LC method. (B) Overlaid EIC from an oligo mixture. (C) Separation of 18 and 20mer by Fast LC. (D) Deconvoluted spectra showing how oligo separation can simplify data interpretation.

Low-abundance impurity analysis

High-throughput purity assessment of oligos can be done by mass-resolving the products from a single chromatographic peak. Often, 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. Figure 8 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).

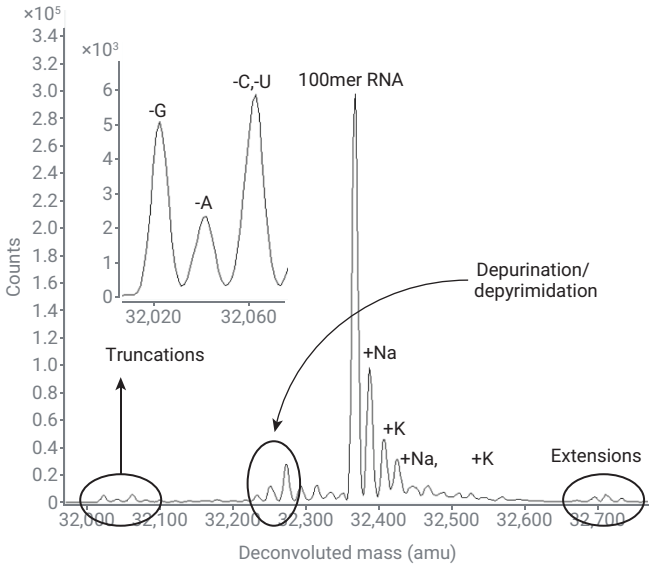


Figure 8. Deconvolution revealing low-abundance imprities.

Conclusion

- 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, 5,760 a day) while the Fast LC method sustained a throughput of 40 seconds per sample (90 samples an hour, 2,160 a day).
- The RapidFire method desalted oligos more efficiently than Fast LC, approximately 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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