Quantification of Large Oligonucleotides using High Resolution MS/MS on the TripleTOF® 5600 System

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Key Challenges of Oligonucleotide Bioanalytical Assay

1. The bioanalysis of oligonucleotides as therapeutics requires sensitive, specific and robust analysis.
2. Many ELISA-based oligonucleotide measurements do not accurately distinguish large metabolites from the full-length oligonucleotide of interest.
3. ELISA- and UV-based measurements have limited dynamic range which complicates quantitative analysis of oligonucleotides in complex matrices.

Key Benefits of MRM^{HR} Workflow for Oligonucleotide Bioanalytical Assay

1. High sensitivity MS/MS enables the quantitative MRM^{HR} workflow, providing high selectivity in biological matrices.
2. High resolution, accurate mass MS/MS spectra enable qualitative verification of oligonucleotide sequences.
3. The MRM^{HR} workflow offers a dynamic range of two to three orders of magnitude.

Unique Features of MRM^{HR} Workflow on TripleTOF® 5600 System

1. Summing of multiple ion transitions to increase both sensitivity and selectivity of quantitation.
2. Accelerated method development times, since ion transitions can be selected post-acquisition to eliminate background interferences.
3. High multiplexing due to high acquisition speeds (up to 100 spectra per second) for simultaneous quantitation of multiple species, including multiple oligonucleotide sequences and/or their metabolites.

Figure 1: The TripleTOF® 5600 System has the speed and sensitivity to deliver high-throughput targeted quantitation of many species in a single run. This example focuses on using MRM^{HR} workflow to quantify a synthetic oligonucleotide from human plasma, demonstrating post-acquisition fragment ion selection and summing product ions to achieve the highest possible sensitivity and selectivity.

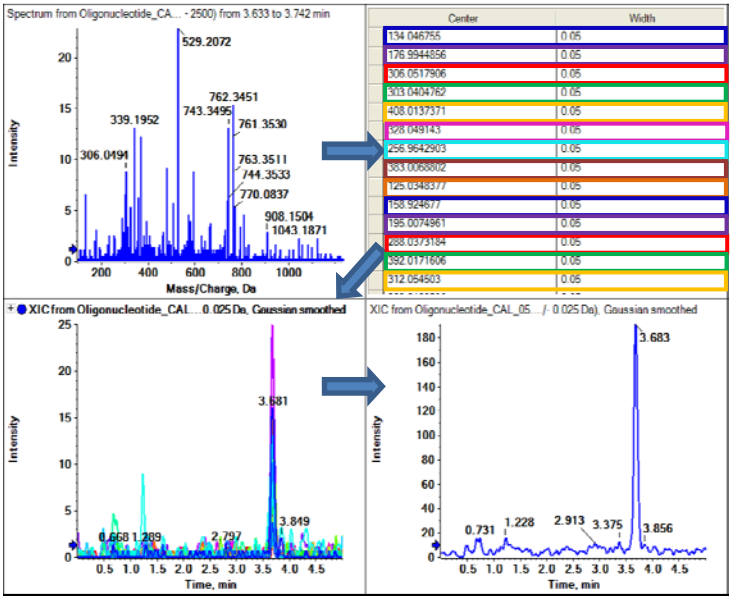

Figure 2: Principle of MRM^{HR} Workflow Quantitation. Looped full scan MS/MS spectra are acquired for each precursor. Selected fragments are then extracted post-acquisition using narrow extraction widths (in this case 50 mDa) to produce high resolution XICs. These XICs can be used individually or summed, depending on which provides the best selectivity and sensitivity for quantitation.

Introduction

Quantitative analysis of synthetic oligonucleotides in biological matrices is an important aspect of pharmacokinetic (PK), toxicokinetic (TK) and metabolic pathway studies in drug development[1]. With an increasing number of oligonucleotide based drugs in research pipelines, the acceleration of the drug development process by reducing the time spent on method development, and by performing simultaneous qualitative structural analysis with quantitative analysis are crucial advantages in any potential quantitation approach[1].

Current LC-MS approaches to oligonucleotide quantitation predominantly use multiple reaction monitoring (MRM), however the complex fragmentation pathways of oligonucleotide species coupled with the variability of matrix effects mean that it can be difficult to predict the sensitivity and selectivity of a given MRM transition without significant optimization[1]. These effects limit the utility of low resolution quantitation methods both in terms of the achievable limits of quantitation and in sample throughput, particularly when quantifying large numbers of potential drug candidates of different sequences, and their metabolites.

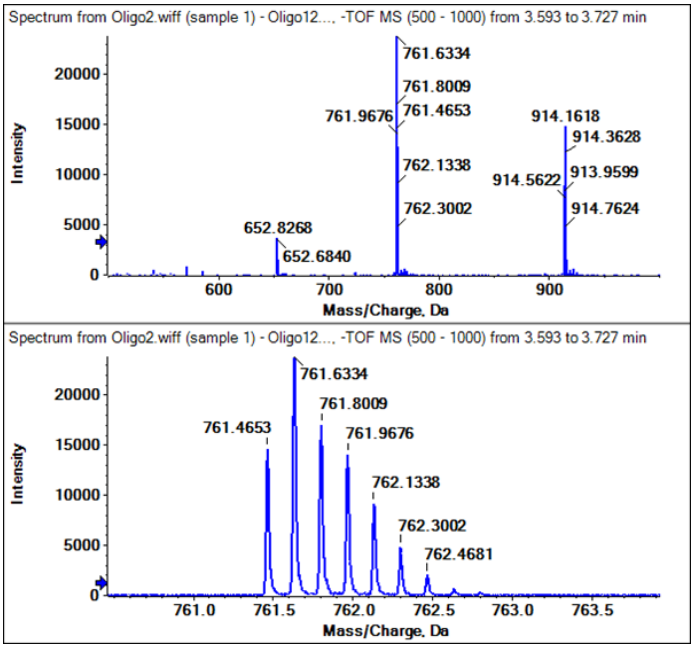

Figure 3: TOFMS Analysis of Intact Oligonucleotide 1 acquired on the TripleTOF® 5600 system (top). Isotope resolution of higher charge states is achieved with a resolution of 30,000-40,000, as illustrated by zooming in on the 6- charge state (bottom). This resolution is achieved at TOF MS scan speeds of 10 MS spectra per second.

Materials and Methods

Sample Preparation

The synthetic DNA Oligonucleotide 1 was spiked into human plasma over a concentration range of 0.025 to 10 nM. Oligonucleotide 2 was used as an internal standard.

LC Conditions

LC System	Shimadzu Prominence XR UFLC
Analytical column	Waters Acquity BEH, 50 x 2.1 mm, 1.7 µm, temp.= 60 ºC
Analytical flow	0.40 ml/min (initial 24 hour flush)
Mobile Phase A	Water (15 mM TEA, 400 mM HFIP)
Mobile Phase B	50:50 Methanol:Water (15 mM TEA, 400 mM HFIP)

Gradient conditions

Time (min)	Mobile phase A%	Mobile phase B%
0.5	*75*	*25*
5	*50*	*50*
5.1	*0*	*100*
5.5	*0*	*100*
5.6	*75*	*25*
6	*75*	*25*

MS Conditions

MS System	TripleTOF® 5600+ system with a DuoSpray™ Source
Ionization Mode	ESI with Negative Mode
TOF MS range	m/z 100-2500 at 250 msec accumulation time
MRM^{HR}	2 product ions each 250 msec
Collision energy spread	-40 ± 4 eV
Source temperature	550°C

Software

Data acquisition	Analyst TF™ 1.5.1 Software
Data review	PeakView™ 1.2 Software
Deconvolution	BioAnalyst™ Software
Quantitation	MultiQuant™ 2.1 Software

Results and Discussion

MS and MS/MS Analysis of Oligonucleotides. TOFMS analysis of Oligonucleotide 1 showed a charge state envelope consisting of $[M-5H]^{5-}$, $[M-6H]^{6-}$ and $[M-7H]^{7-}$ ions (Figure 3) with a resolution of approximately 36,000. Inspection of the TOFMS spectrum showed that the system passivation process had reduced adduct formation to less than 5% relative to the fully protonated form, thus facilitating quantitation from the $[M-6H]^{6-}$ peak (data not shown).

The principle of the MRM^{HR} workflow for quantitation is to acquire full scan TOF MS/MS spectra for each species of interest, and to use high resolution extracted ion chromatograms (XICs) for quantitation, summing multiple transitions where appropriate to achieve optimum sensitivity and selectivity (Figure 2). To develop an MRM^{HR} workflow assay for oligonucleotides 1 and 2, full scan MS/MS spectra were acquired for m/z 761.9 for Oligonucleotide 1, and m/z 745.6 for Oligonucleotide 2. The full scan MS/MS spectra were also used to verify the sequence of Oligonucleotide 1 and 2 (Figure 4). The MS/MS spectra were deconvoluted using BioAnalyst™ Software to enable the singly and multiply-charged fragment ions to be plotted on a mass scale. The sequences were subsequently verified by matching theoretical sequence ions to the fragment ions observed in the deconvoluted spectra.

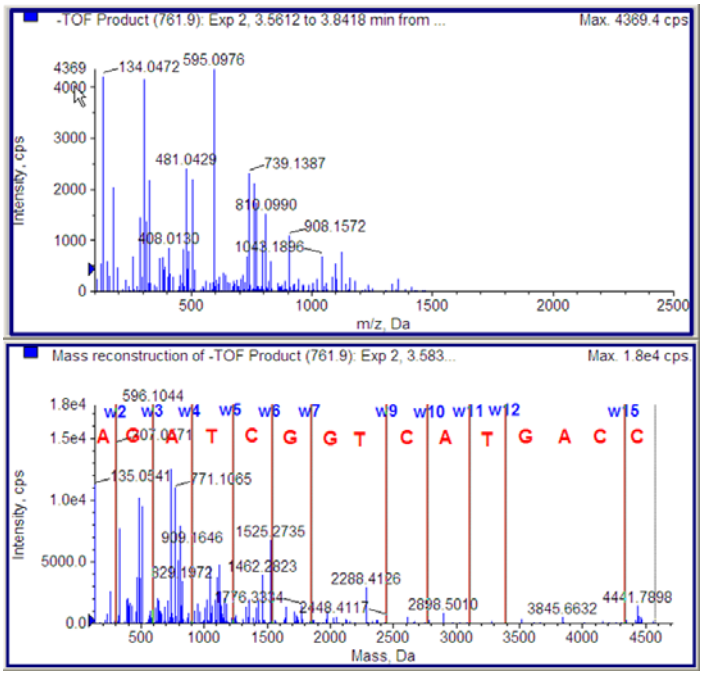

Figure 4: High Quality MS/MS of Oligonucleotide 1 for Characterization and Quantitation Method Development. (top) The full scan MS/MS approach of MRM^{HR} workflow enables full qualitative analysis of any targeted analyte. Full sequencing of Oligonucleotide 1 was achieved on the mass reconstructed MS/MS spectrum (bottom) for fragment ion selection.

MRMHR Workflow Assay Development. Having acquired full scan TOF MS/MS spectra, fragment ions can be selected and extracted post-acquisition for use in quantitation. Figure 2 shows the stepwise process of selecting fragment ions from a full scan MS/MS spectrum, generating multiple high resolution XICs from the selected fragment ions, and summing the XICs to optimize the signal-to-noise. For transitions arising from Oligonucleotides 1 and 2, an extraction width of 50 mDa was used to generate XICs; however it is possible to collect the MS/MS spectra in high resolution mode (>30,000 resolution) and extract with narrower windows to improve selectivity in any given assay if required.

The post-acquisition selection of fragments is a significant advantage of the MRM^{HR} workflow, since the selectivity of specific fragment ions in matrix cannot necessarily be predicted prior to data acquisition. In the case of Oligonucleotide 1, the three most intense fragment ions in the MS/MS spectrum gave poor selectivity when extracted (Figure 5), and therefore could not be used for quantitation. In contrast, other less intense transitions showed excellent selectivity, and were subsequently included in the assay. The final XIC trace was achieved by summing 25 different fragment ion XICs (Figure 5). The final data processing using the summed XICs was performed using MultiQuant™ Software.

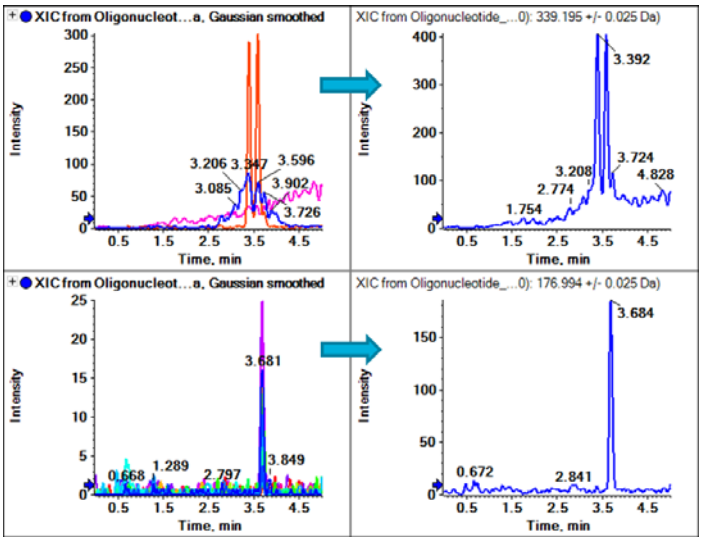

Figure 5: Post-Acquisition Extraction of Structurally Specific Ions. In the case of Oligonucleotide 1, the three most intense fragment ions (top left) are non-selective in plasma at low concentrations and therefore summing of these XICs does not provide a good assay (top right). Because the full scan MS/MS spectrum is acquired in the MRM^{HR} workflow, this allows different fragment ions to be selected and extracted for quantitation post-acquisition (bottom left, summed bottom right), and therefore requires significantly less method development than traditional MRM approaches.

TOF MS vs. MS/MS Quantification Strategies. The MRM^{HR} workflow for quantitation from complex matrices offers significant advantages over the full scan TOF MS approach to quantitation, due to both the high selectivity of the MS/MS based XICs and the ability to remove fragment ions with background interferences, which can impact significantly on the achievable limits of detection and quantification.

Figure 6 shows a comparison of the TOF MS and MRM^{HR} workflows applied to the analysis of Oligonucleotide 1. In the case of the TOF MS quantitation, each peak in the isotopic envelope of the $[M-6H]^{6-}$ charge state was extracted using a 10 mDa extraction window (Figure 6, left). The extracted ion chromatograms from the TOF MS approach show significant matrix interferences that seriously impact the limit of quantification, which is approximately 0.5 nM using this approach.

In contrast, the specificity of the MRM^{HR} workflow produced significantly lower limits of detection and quantification (Figure 6, right). This improvement is due to the significant reduction in noise from the complex matrix.

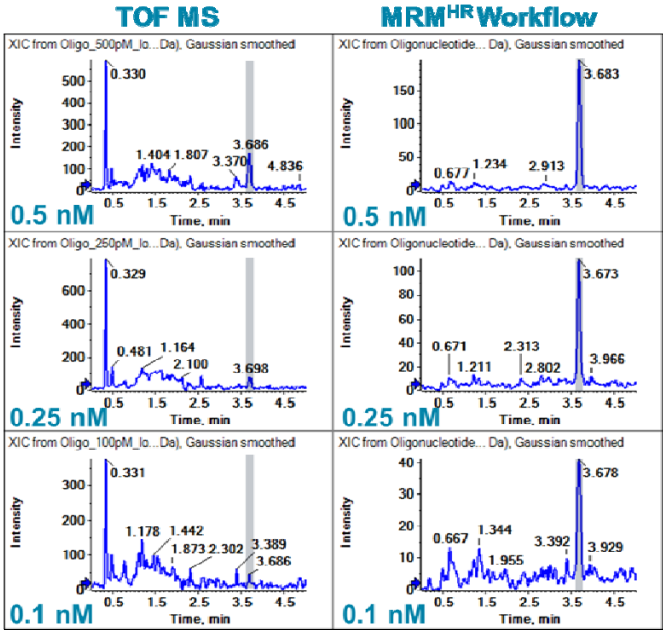

Figure 6: Selectivity of MRM^{HR} Workflow in Complex Matrices Allows Better LLOQs to be Obtained. In the case of Oligonucleotide 1, background interferences in full scan TOF MS result in higher limits of detection and quantitation (0.5 nM), while the selectivity of the MRM^{HR} workflow allows quantitation of concentrations less than 0.1 nM.

Figure 7 shows the calibration plot of Oligonucleotide 1 using the MRM^{HR} workflow with Oligonucleotide 2 used as an internal standard. The %CV values for each concentration analyzed are shown in the embedded table. The correlation coefficient of the response is in excess of 0.99 over ~2 orders of magnitude; the linearity of response at concentrations in excess of 10 nM was not investigated. The lower limit of quantification (LLOQ) using the MRM^{HR} workflow was 0.05 nM (Figure 7), while the LLOQ was 10 fold higher when the same oligonucleotide was analyzed by full scan TOF MS workflow (Figure 6).

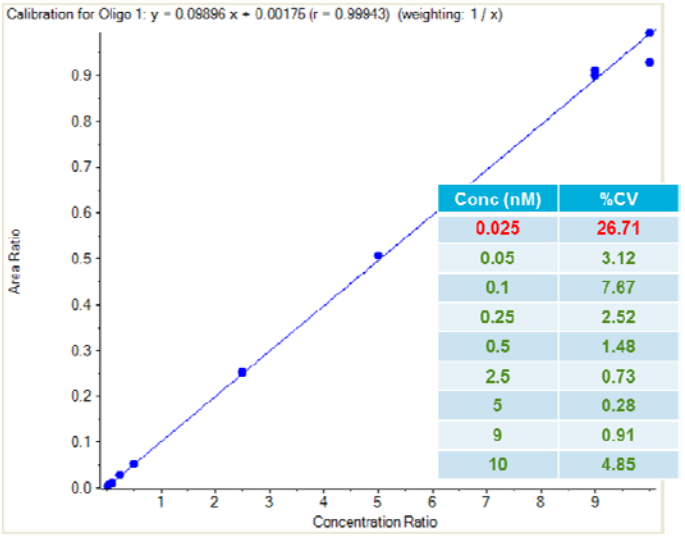

Figure 7: Standard Concentration Curve for Oligonucleotide 1 in Matrix using MRM^{HR} Workflow. Concentration curve for Oligonucleotide 1 in plasma, using Oligonucleotide 2 as an internal standard. Using MRM^{HR}, excellent linearity was observed, with a lower limit of quantitation of 0.05 nM.

Conclusions

1. The TripleTOF® 5600 system offers sensitive, high-resolution analysis of large oligonucleotides, with the opportunity to perform both qualitative and quantitative analysis in a single run.
2. Using a targeted MRM^{HR} workflow, looped full scan TOF MS/MS spectra of a ~4.5 kDa synthetic oligonucleotide were acquired with a resolution of ~16,000 and XICs were generated from specific fragment ions to achieve a highly sensitive and selective quantitative assay.
3. The ability to quantify oligonucleotides from complex matrices with minimal assay optimization offers the opportunity for high-throughput analysis of potential oligonucleotide-based therapeutics. Upfront method development is highly simplified and consists of specifying a theoretical oligonucleotide m/z and a collision energy, the remainder of the analysis being done post-acquisition.

References

1. Z. J. Lin, W. Li and G. Dai, J. Pharm. Biomed. Anal. 44

Considerations for Handling Therapeutic Oligonucleotide Reference Standard and Sample Extraction Tips

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Handling Reference Standard Material

The Oligonucleotide API (active pharmaceutical ingredient in GMP Studies) or test article (in GLP Studies) is highly hygroscopic and the water content of an oligonucleotide is related to its environment. Additionally, concentrated solution such as those observed in a clinical setting can be very viscous; both of these traits can lead to inaccuracies in the Assay. To prevent inaccuracies due to the hygroscopic nature of the API, one of two options are presented. The drug substance can be handled only in a room that enables precise temperature and humidity control. The glaring limitation of this approach is that not every site has this available. The alternate option is to equilibrate the API to ambient temperature and humidity prior to handling and using the UV absorbance (typically at 260 nm) and purity to determine the actual concentration. The latter approach assumes knowledge of the oligonucleotide purity. The viscosity issue can be overcome by relying on a gravimetric technique as opposed to a volumetric one.

Dealing with Non-Specific Binding

Oligonucleotides are prone to non-specific binding with the container/closure system, components in biological matrices and components of the LCMS system used for analysis. Non-specific binding to sample containers are more pronounced at lower concentrations and can be helped by either storing the solutions in Type I glass containers or using EDTA or other preservative with plastic container. Regardless of oligonucleotide concentration EDTA is a useful preservative as it will chelate divalent cations which are required for nuclease activity. To prevent non-specific binding to components of the LCMS system, one option is to replace as much stainless steel as possible and replace with PEEK or similar tubing; of course this is only applicable to relatively low pressure systems as the PEEK tubing will not tolerate as high a pressure. Another little trick is to add a minute amount (µM quantities) of EDTA to the mobile phase again to chelate any divalent cations present. Dealing with the non-specific binding of oligonucleotides to components in the biological matrices is more involved and intricate given that oligonucleotides carry lots of negative charge on their phosphodiester-based backbone.

The negative charge imparts a strong affinity for ubiquitous cations such as Na^+ and K^+ as well as other matrix components.

Sample Extraction Tips

There are many ways to separate these matrix components from the therapeutic oligonucleotide including protein precipitation, liquid-liquid extraction, solid phase extraction and various combinations of these procedures. Simple protein precipitations with organic solvents such as acetonitrile are met with limited success as they are prone to low recoveries and the ubiquitous cations are not necessarily removed.

Several liquid-liquid extraction techniques have met with some success, particularly using a phenol/chloroform solution for the LLE. Often, the LLE will include a step that adds a detergent or other modifier to break up any complexes between the oligonucleotide and matrix components. The proteins will partition into the organic layer while the oligonucleotide is left in the aqueous portion along with other polar matrix components. These remaining matrix components can and do interfere with LCMS. For this reason, the liquid-liquid extract can be further treated by solid phase extraction. Adsorption of the oligonucleotide to a reverse phase solid packing material can be enhanced by the addition of a modifier to both the loading and elution solvent; typically an ion pairing agent and/or ammonium hydroxide are used. Once the oligonucleotide is adsorbed onto the solid phase, washing and elution. This combination of LLE followed by SPE is laborious, time consuming and the ability to automate for a larger number of samples is limited. A few years ago Phenomenex came out with their Clarity OTX system which is a mixed-mode (weak anion exchange and reverse phase) SPE cartridge along with buffers designed to work with the cartridge to clean up oligonucleotide samples. The Clarity OTX offers a quicker method for sample preparation from biological matrices with adequate recoveries.