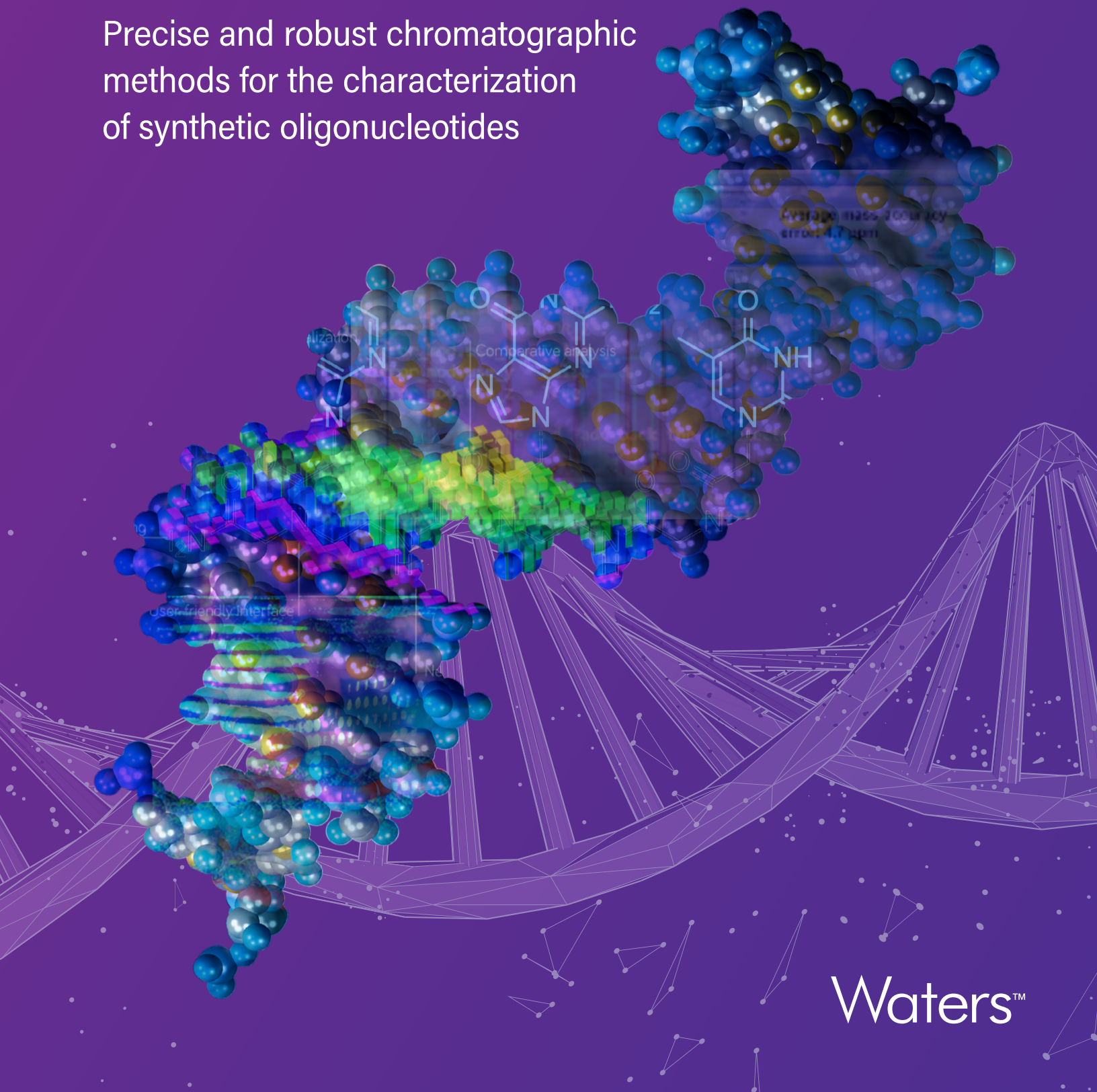
CONSUMABLES GUIDE BOOK

Characterizing Oligonucleotides

Precise and robust chromatographic methods for the characterization of synthetic oligonucleotides



Waters™

A Changing Industry

There has been a resurgence in synthetic oligonucleotides in the past decade, as seen with burgeoning pipelines across the industry. These new drug candidates are providing opportunities to address not only inherited disorders but also historically difficult to treat ailments, such as cardiovascular disease. Synthetic oligos like ASOs, siRNA, and CRISPR sgRNA are typically made using solid-phase chemical synthesis, a process that involves the addition of nucleotides one by one to form a growing polymer. The resulting molecules can therefore exhibit n-1 and other types of impurities related to further conjugation and modification. As therapeutic oligos come to be modified with new chemical moieties, the complexity of these impurities also increases. This presents new analytical challenges. Ever improving characterization techniques based on ion pairing reversed phase LC, anion exchange, HILIC and SEC can help.

CRISPR sgRNA, GalNAc Conjugated Small Interfering RNA (siRNA) and Lipid Conjugated Antisense Oligonucleotide (ASO)

Analytical Chromatography Options

Ion Pairing RPLC

HILIC

Anion Exchange

Size Exclusion

Waters Application Notes and Peer Reviewed Articles

Waters scientists and collaborators are publishing on this subject. Make sure to frequent the Resource Tab on our **waters.com/GTx** website to keep up to date on the literature.

Attribute Testing

A synthetic oligonucleotide drug can contain as many as 5,000 atoms arranged in important 5′ to 3′ sequences along with special modifications precisely located at important residue positions. These compositional details must be confirmed along with the purity of the drug substance material. As this field of pharmaceutical science advances so to does the understanding of critical quality attributes.

IDENTITY

The identity of an oligonucleotide drug substance must be confirmed. Chromatographic retention behavior is often confirmatory. Accurate mass analysis confirms elemental composition.

SEQUENCE CONFIRMATION

Fragmentation analysis by mass spectrometry can be applied to confirm modifications and their position within an oligonucleotide sequence. Enzymatic digestion and oligo mapping can provide comprehensive characterization data as well.

MODIFICATIONS

Modifications can range from 2' position fluoro and methoxyethyl groups to methylated nucleobases. Precise chromatography and accurate mass information can help pinpoint these molecular details.

PURITY

Synthetic oligonucleotides are often purified by anion exchange and reversed phase chromatography. Nevertheless, they can contain a number of different process and product related impurities.

N-1 SHORTMERS

Early termination and incomplete solid state synthesis of oligonucleotides is common. Chromatography can be applied to measure these so-called n-1 shortmer impurities.

OXIDATION

Many oligonucleotides are modified to contain a phosphorothioate backbone. A PS (phosphorothioate bond) can be oxidized and the sulfur atom can swap back to oxygen.

DEAMINATION

The exocyclic amines of the nucleobases contained within an oligonucleotide can be deaminated into carbonyl residues.

DEPURINATION

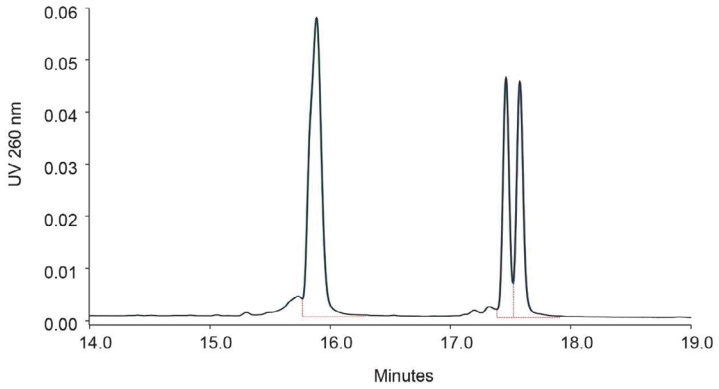
Adenine and guanine can be hydrolyzed from their ribose sugar through a break at the N-glycosidic bond. This is detrimental to the base pairing ability of the oligonucleotide therapy.

DUPLEX VS. SINGLE STRANDS

Small interfering RNA must be formulated to be intact duplex molecules in order to be functionally active and loaded into a RISC (RNA-induced silencing complex) subunit.

IP-RPLC to Confirm Identity and Purity

Ion pairing (IP) reversed phase (RP) liquid chromatography is the *de facto* approach for confirming the identity and purity of an oligo sample. Standard pore size stationary phases are used to separate up to 30-mer oligos, while widepore (300Å) stationary phases are available for larger species; both are available as oligo QC-tested and batch selected materials. IP-RP methods have historically leveraged triethylamine, though hexylamine and diisopropylethylamine have more recently become preferred options. With these methods, MaxPeak™ High Performance Surfaces have provided a step change in separation performance and robustness.



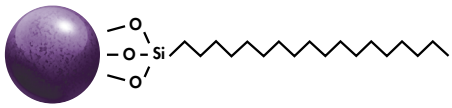
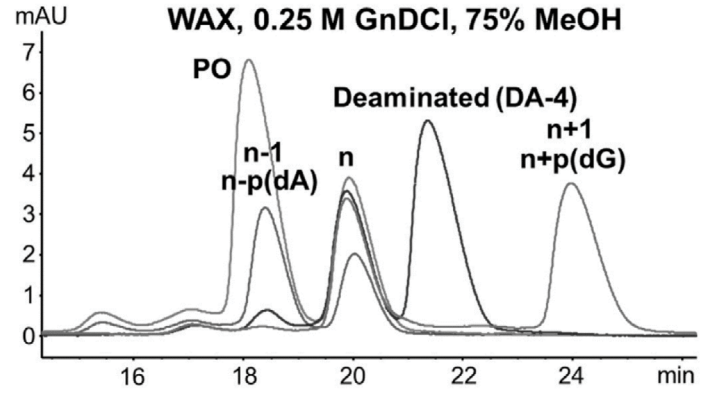


Figure 2. Denaturing IP-RPLC separation of a phosphorothioated siRNA as obtained with an ACQUITY™ Premier Oligonucleotide BEH C_{18} 300Å Column as reported in Waters Application Note 720007362.

AEX for Charge Based LC and New Selectivity

Anion exchange is an inherently good fit for the analysis of oligos and is compatible with a myriad of elution mechanisms. Research has uncovered both non-denaturing uses as well as new types of denaturing methods. Weak anion exchangers show promise for unique selectivity through pH adjustments and gradient tuning. Moreover, ion pairing agents can be added to the mobile phase to attenuate adsorption and partitioning. Ultimately, options for retentivity matter when it comes to developing new AEX methods. The Gen-Pak FAX Column is a weak anion exchanger that has a uniquely low retentivity, while the Protein-Pak HiRes Q Column and its SAX sorbent has comparatively strong retentivity. Both are useful tools for your analytical toolbox.



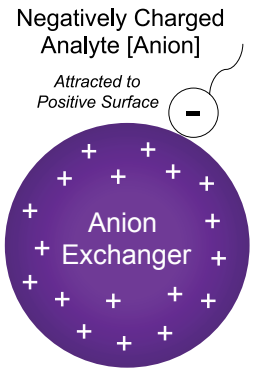
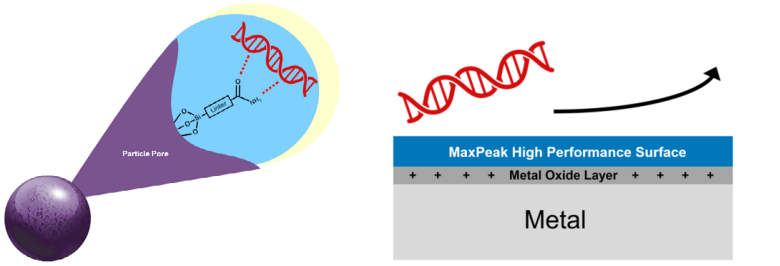


Figure 3. UV Chromatograms obtained for model oligonucleotide mixtures as obtained with a Gen-Pak FAX column. Used with permission from Anal. Biochem. 659 (2022) 114956.

HILIC as an Alternative

Hydrophilic interaction chromatography (HILIC) has garnered significant attention in recent years, due to the strong adsorptive interactions that oligonucleotides have with the associated stationary phases. LC-MS methods with ammonium acetate mobile phases have come to be preferred for ion-pairing free techniques with performance improvements coming from MaxPeak HPS hardware. An amide bonded BEH™ particle provides high efficiency separations that are based on direct H-bonding effects and the partitioning of an oligonucleotide into and out of an adsorbed water layer.



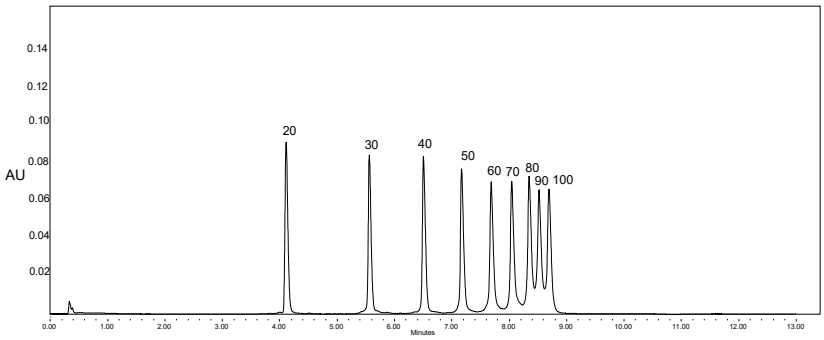


Figure 4. Top: Schematic representation of the GTxResolve Premier BEH Amide hydrogen bonding mechanism and MaxPeak High Performance Surfaces (HPS) as used on column hardware to mitigate adsorptive interactions with oligonucleotides.

Bottom: Separation of the ssDNA 20 to 100 Ladder (186009448) with a GTxResolve™ Premier BEH Amide 300Å column as reported in Application Note 720008456.

siRNA Duplex Formation in Native Conditions

Temperature is a key parameter in HILIC separations as it has a significant impact on the oligonucleotides secondary structure and elution profile. For example, siRNA is comprised of two complementary strands that form a duplex. Evaluation of the duplex formation may be performed to ascertain the presence of excess singled stranded sequences present in the formulation. These unwanted single stranded components can impact the efficacy of the therapeutic or even lead to off-target effects. Analysis of the siRNA in native (non-denaturing) conditions at lower column temperatures stabilizes the duplex structure for qualitative and quantitative characterization of secondary species.

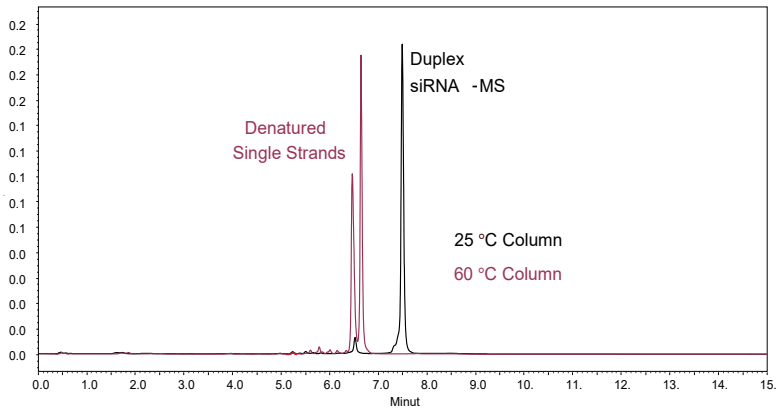


Figure 5: Denaturing and non-denaturing analysis of siRNA LC-MS Standard demonstrating how column temperature can be utilized to separate the duplex or analyze it in its native form.

SEC for Native LC-UV-MS

Size exclusion chromatography (SEC) has not yet been put to regular use for the analysis of synthetic oligonucleotides. However, there is potential for it to become a powerful tool. It can be applied to achieve fast desalting native SEC-MS and be used to assay duplex siRNA for product-related, single stranded impurities. Columns constructed with 100 to 300Å pore diameter packing materials have a fractionation range suitable for separating 5 to 150-mer oligonucleotides.

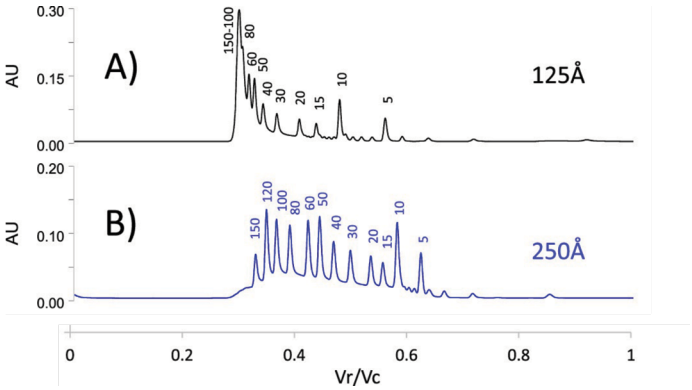


Figure 6: SEC separation of dA 5 to 150-mer species as published in Anal. Chem. 2023, 95, 38, 14308–14316.

Notes

Top-Down and Bottom-Up sgRNA Analysis

Comprehensive characterization of CRISPR sgRNA calls for a dual-pronged approach to confirm identity, assess purity, and mapping terminal modifications. Detailed analysis is essential to evaluate and mitigate the risk of off-target effects, which could lead to unintended genetic mutations if not carefully monitored. Advanced structural characterization of the guide RNA can be performed using both intact (top-down) and post-digestion (bottom-up) methodologies. The top-down approach examines the intact molecule without prior digestion providing a holistic view of the sgRNA, enabling precise measurement of its molecular weight, detection of any unexpected modifications, and verification of sequence integrity. In the bottom-up approach, the sgRNA is treated with a RNase, cleaving the strand into smaller fragments that are more suitable for precise LC-MS analysis. RapiZyme™ MC1 and RapiZyme Cusativin are recombinant endonucleases with complementary specificities that generate longer marker oligonucleotides with unique masses. The theoretical cleavage properties of each enzyme are integrated into the waters_connect MAP Sequence app, facilitating streamlined sequencing and data interpretation.

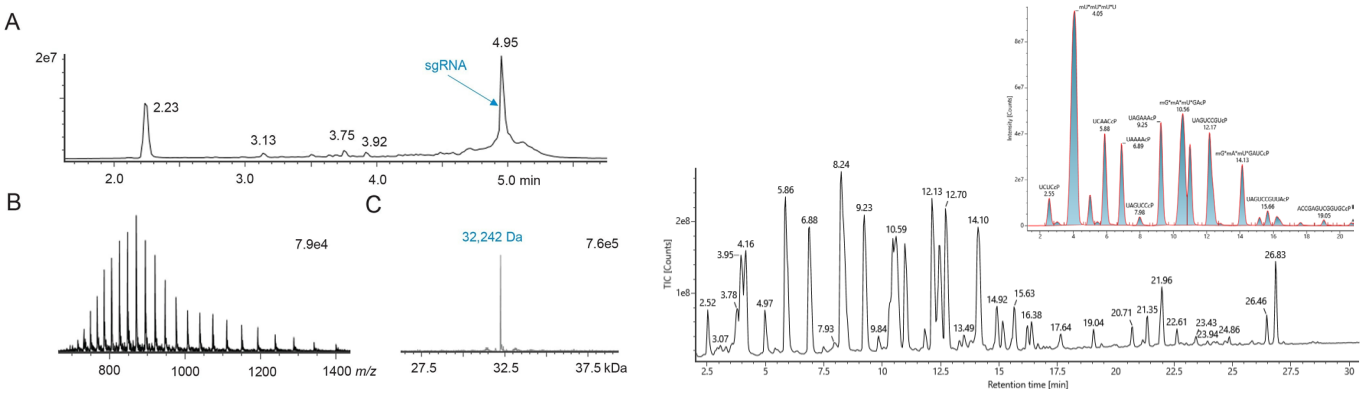
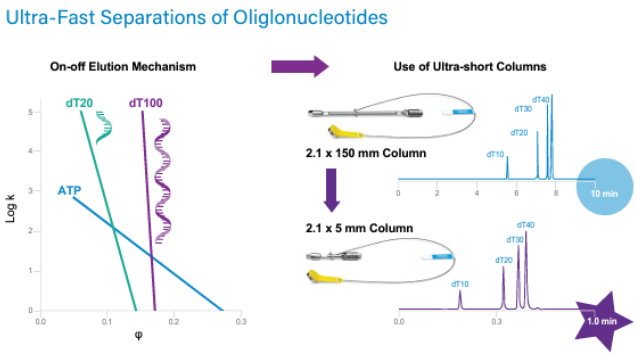


Figure 7. Top-down and bottom-up analysis of sgRNA. The left side of the figure depicts a IPRP LC-MS analysis of intact sgRNA and the right an annotated TIC of the sgRNA sample digested with RapiZyme MC1 10,000 Units (186011190).

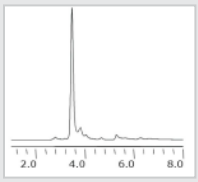
Rapid LC Separations for Fast LC-MS Quantitation

Bioanalytical labs need analytical solutions that are robust, scalable and fast to maximize performance and productivity. As demonstrated in a 2023 publication by Honorine Lardeux et. al.*, oligonucleotides exhibit a strong on/off elution mechanism, making them amenable to fast separations using ultrashort columns. Reversed-phase and HILIC columns with a 50 mm length are commonly used today, but to further accelerate separations, increase throughput and reduce costs, new ACQUITY™ Premier Oligonucleotide BEH™ C_{18} and GTxResolve™ Premier BEH Amide 300 Å Ultrashort Columns with a 20 mm length are now available from Waters.

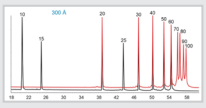


**High-Throughput Chromatographic Separation of Oligonucleotides: A Proof of Concept Using Ultra-Short Columns by Honorine Lardeux et.al. (Analytical Chemistry 2023, 95, 27, 10448-10456).*

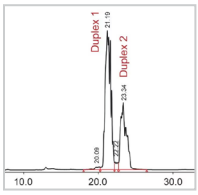
Application Notes



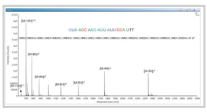
[Properties of the Gen-Pak™ FAX Column and Its Utility for Anion Exchange Analysis of Large Molecule Biologics]



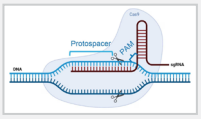
[Enhanced Resolution for Longer Oligonucleotide Analytes With a MaxPeak™ Premier Oligonucleotide BEH C_{18} 300 Å Column]



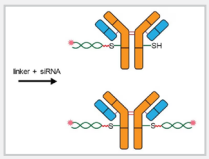
[Analysis of siRNA Duplexes at Non-Denaturing UPLC Conditions Using MaxPeak Premier Column Technology]



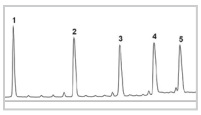
[Analysis of Oligonucleotide Impurities on the BioAccord System with ACQUITY Premier]



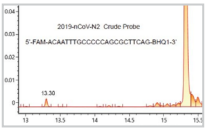
[CRISPR Single Guide RNA Characterization by IP- RP-LC-MS with a Premier Oligonucleotide BEH 300 Å C_{18} Column]



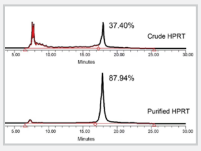
[Analysis of Antibody siRNA Conjugate Using BioAccord System]



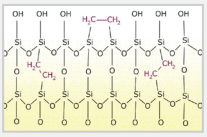
[Improved Peak Recovery and Peak Shape of Oligonucleotides Using Waters Premier Columns]



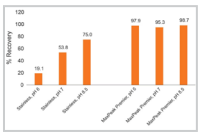
[Mass Confirmation and Purity Analysis of PCR Primer and Probe Reagents Using High Sensitivity Ion-pairing LC-MS]



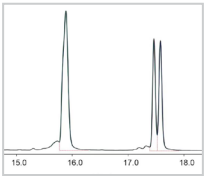
[Size and Purity Assessment of Single-Guide RNAs by Anion-Exchange Chromatography (AEX)]



[Best Practices for Oligonucleotide Analysis Using Ion-Pair Reversed-Phase (IP-RP) Liquid Chromatography – Columns and Chemistries]

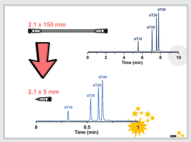


[Improved Chromatographic Analysis of Oligonucleotides with ACQUITY Premier Oligonucleotide BEH C_{18} Columns]

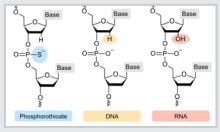


[Analysis of siRNA Drugs at Denaturing UPLC Conditions Using MaxPeak Premier Column Technology]

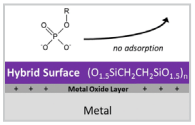
Waters Journal Articles



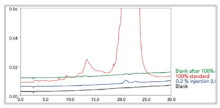
[High-Throughput Chromatographic Separation of Oligonucleotides: A Proof of Concept Using Ultra-Short Columns]
A. Chem. **2023** 95, 27, 10448-10456



[The impact of low adsorption surfaces for the analysis of DNA and RNA oligonucleotides]
J Chrom A **2022**, 1677, 463324



[Using Hybrid Organic–Inorganic Surface Technology to Mitigate Analyte Interactions with Metal Surfaces in UHPLC]
A. Chem. **2021**, 93, 14, 5773–5781



[Analysis of siRNA with Denaturing and Non-Denaturing Ion-Pair Reversed-Phase Liquid Chromatography Methods]
LCGC North America, **2023**, 41, 2, 60–66

Notes

Ordering Information

Information on the tools you need to comprehensively characterize an oligonucleotide can be found below.

RPLC

ACQUITY™ Premier Oligonucleotide BEH™ C_{18} 1.7 µm Columns

Format	Diameter	50 mm	100 mm 130 Å	150 mm	50 mm	100 mm 300 Å	150 mm
Standard Column	2.1 mm	186009484	186009485	186009486	186010539	186010540	186010541
VanGuard™ FIT Column	2.1 mm	186010685	186010686	186010687	186010754	186010755	186010756
VanGuard FIT Cartridge 3/pk	2.1 mm		186010696			186010772	

XBridge™ Premier Oligonucleotide BEH C_{18} 2.5 µm Columns

Format	Diameter	50 mm	100 mm 130 Å	150 mm	50 mm	100 mm 300 Å	150 mm
Standard Column	2.1 mm	186009836	186009837	186009838	186010542	186010543	186010544
	4.6 mm	186009901	186009902	186009903	186010545	186010546	186010547
VanGuard FIT Column	2.1 mm	186010688	186010689	186010690	186010757	186010758	186010759
	4.6 mm	186010691	186010692	186010693	186010760	186010761	186010762
VanGuard FIT Cartridge 3/pk	2.1 mm		186010694			186010773	
	4.6 mm		186010695			186010774	

AEX

Anion Exchange Columns

	Dimension	P/N
Protein-Pak Hi Res Q Column	4.6 x 100 mm	186004931
Gen-Pak FAX Column	4.6 x 100 mm	WAT015490

HILIC

GTxResolve Premier BEH Amide 300 Å

	Diameter	20 mm	50 mm	100 mm	150 mm
Standard Column	2.1 mm	186011248	186011249	186011250	186011251

VanGuard FIT Column	2.1 mm	–	186011252	186011253	186011254
VanGuard Fit Cartridge (3/pk)			186011255		

SEC

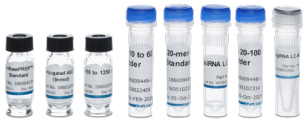
ACQUITY and XBridge SEC Columns

	Diameter	150 mm	300 mm	Guard
ACQUITY Premier Protein SEC 125 Å , 1.7 µm	4.6 mm	186006505	186006506	186006504
ACQUITY Premier Protein SEC 250 Å , 1.7 µm	4.6 mm	186009963	186009964	186009969
XBridge Premier Protein SEC 125 Å , 2.5 µm	7.8 mm	186009159	186009160	186009158
XBridge Premier Protein SEC 250 Å , 2.5 µm	7.8 mm	186009961	186009962	186009969

REAGENTS AND ENZYMES

	P/N
IonHance HFIP, 10 mL	186010781
RapiZyme MC1, 10,000 Units	186011190
RapiZyme Cusativin, 10,000 Units	186011192

OLIGONUCLEOTIDE STANDARDS



	P/N
MassPREP Oligonucleotide Standard	186004135
Lipid conjugated ASO LC-MS Standard	186010747
ssDNA 20-mer LC-MS Standard	186009451
siRNA LC-MS Standard	186010598
sgRNA LC-MS Standard	186011357
ssDNA 10 to 60 Ladder	186009449
ssDNA 20-mer LC-MS Standard	186009451
ssDNA 20 to 100 Ladder	186009448

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waters.com/GTxResolveSEC
waters.com/GTxAEX
waters.com/GTxResolveHILIC
waters/com/GTxStandards

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