Need to Achieve Confident Oligonucleotide Characterization and Purification?

Accurately analyze purity and impurities, determine sequences, and more with next-generation technologies from Agilent.

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Synthetic oligonucleotides are short nucleic acid chains (DNA or RNA) of approximately 20-30 nucleotides. They're chemically synthesized, usually modified to achieve pharmacological effects, and their applications include PCR primers, DNA sequencing, gene silencing and more. Today, oligos are popular because of their promise in treating previously incurable diseases.

Despite oligos' diversity, most of them are synthesized through the same four-step cyclical process, which is based on phosphoramidite chemistry. In this process, one nucleotide is added onto the sequence at the end of each cycle until the full target length is reached. However, imperfections in the manufacturing process can lead to the formation of impurities in the sample. For example, shortmers (missing one or more nucleotides) or longmers (including more than the intended number) are possible, as are incomplete deprotections, oxidation, nuclease degradation, and other impurities.

For labs aiming to characterize a synthetic oligo sample, Agilent offers various ready-made technologies including dedicated columns, instrumentation, and software solutions. Agilent AdvanceBio Oligonucleotide columns are designed specifically for reversed phase methods with a high pH range, and the Agilent 1290 Infinity II Bio LC System can mitigate bio-oxidation and the non-specific binding of oligo to the capillary surface for improved peak shape.

For purity and impurity analysis, MS is a powerful tool and Agilent recommends the 6230 LC/TOF system. For sequence confirmation, the Agilent 6545XT AdvanceBio LC/Q-TOF is recommended. Both of these instruments are easy to tune with a single click, and five orders of in-spectra dynamic range enable low abundance impurity detection, even for coelution. Given the complexity of the sample, an efficient software algorithm is paramount. Agilent MassHunter BioConfirm 12.0 software streamlines impurity and sequence confirmation.

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Oligo Target Plus Impurities (TPI) Workflow

The oligo target plus impurities (TPI) workflow begins with acquiring the MS1 data of the sample. Then, in the background, a TPI list is created based on the predefined sequence(s) including full length product and a series of relevant impurities. The MS data is then matched to that list, to identify those full-length target as well as relevant impurities.

For targeted analysis where the impurity type is known, it's recommended to use the Find By Formula (FBF) algorithm. For untargeted impurity analysis, Maximum Entropy Deconvolution is recommended. It will find all peaks and then match those against the impurity list. The results can be color coded by custom criteria for easy review at-a-glance.

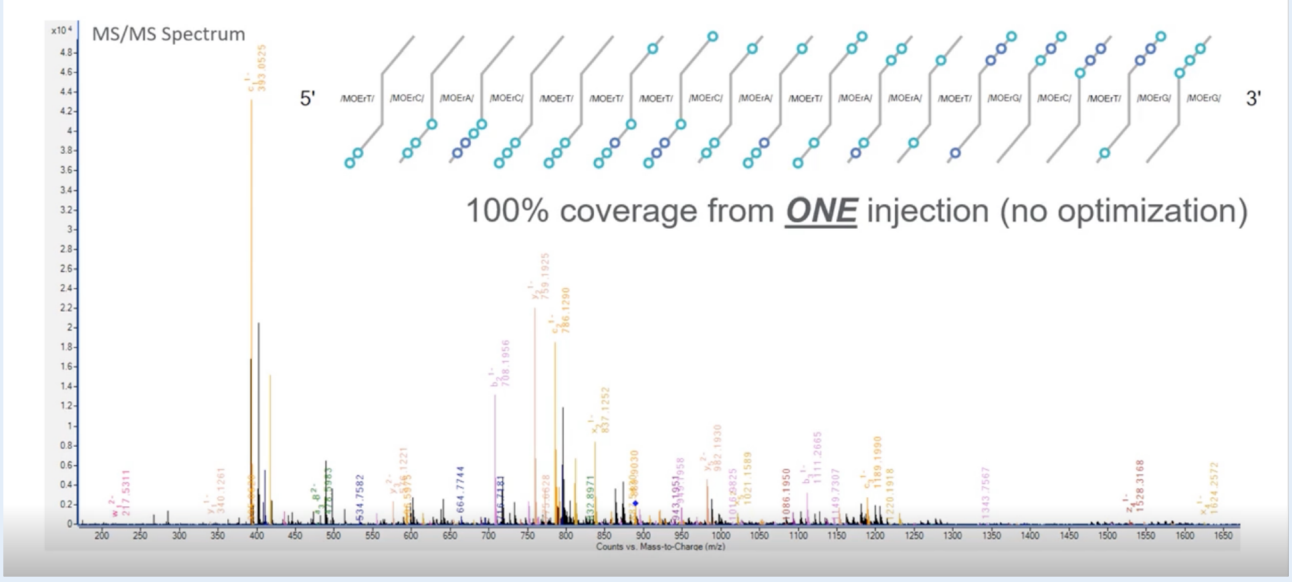
Once the purity of the sample is determined, how can labs be sure that the full length product is in its correct sequence? This is where the sequence confirmation workflow of the MassHunter BioConfirm 12.0 comes in. First, the oligo sample must be fragmented via MS/MS data acquisition. The resulting pool of oligo fragments is then matched to a predicted fragments database. The final result is annotated using a fragment confirmation ladder.

Confirmation of an impurity's sequence is sometimes also required, as it can provide insights that can help improve the conditions of the synthesis or process. Again, the sequence confirmation workflow is able to run impurity peaks through the MS/MS sequence confirmation workflow for more information.

More complex samples, like highly modified samples, will benefit from the Oligonucleotide Chemical Data Dictionary (CDD). With this tool, labs can customize the nucleotide, linker, and modification for their specially designed samples to be used in the subsequent TPI and sequence confirmation workflows. As shown in **FIGURE 1**, the sequence confirmation results show 100 percent sequence coverage based on the IP-RP-LCMS method. This was achieved with only one injection without parameter optimization.

Finally, this workflow is compliant to 21 CFR Part 11 and Annex 11, and MassHunter offers technical controls to meet

FIGURE 1: Sequence Data for Heavily Modified ASO by IP-RP-LCMS



compliance requirements. Agilent also offers data integrity, instrument qualification, computer system validation, consulting, and validation starter kits. For more information, download the Agilent white paper entitled "Support for Title 21 CFR Part 11 and Annex 11 Compliance: Agilent MassHunter for LC/TOF and LC/Q-TOF Systems."

HILIC Oligonucleotide Analysis

Hydrophilic interaction liquid chromatography (HILIC) is an alternative method to reversed-phase chromatography for analyzing oligonucleotides. Utilizing a polar stationary phase, this method elutes compounds in order of their increasing polarity. An aqueous layer is formed on the surface of each particle, and the analytes of the organic mobile phase are partitioned from the aqueous surface layer.

The current methodology for oligonucleotide analysis utilizes ion-pairing reversed-phase (IP-RP) LC. The first downside to this is that the fluoroalcohols utilized in this method, such as HFIP, are high cost compared to other mobile phase additives, and are subject to being banned in Europe in the near future. Second, the alkylamines used in this method, such as triethylamine (TEA), will contaminate the instrument, requiring labs to have a dedicated HPLC and mass spectrometer for this method.

When running HILIC methods, versus reversed-phase methods, there are a few considerations to take into account. First, when deciding on a sample solvent, it's best to use a high organic sample solvent or a performance optimizing injection sequence (POISe) if changing the sample pre-preparation is not ideal. Mobile phase selection and the ionic strength of the mobile phase are also important considerations, and Agilent tests show that the highest success for oligo applications comes with ammonium acetate and acetonitrile mobile phases with an ionic strength between 25 to 30 millimolar. HILIC stationary phases require longer equilibration times compared to reversed-phase stationary phases; thus, it is recommended to address this early in method development.

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Current HILIC Methodology: Agilent Application Notes

There are several methods that can help labs implement a HILIC workflow for oligo sequencing.

The first utilizes the MS/MS capabilities of the 6545XT AdvanceBio LC/Q-TOF. This application is a non-ion-pairing method for oligo sequencing, and scientists were able to achieve 100 percent sequence coverage for all of the oligos tested. This method also boasts minimal optimization requirements.

Second, a full MS scan can be used for the characterization of oligonucleotides. The 15-minute LC/MS method provides chromatographic separation of a wide range of oligo samples, from 20 to 100 -mer, variable base mixtures, aptamers, and siRNA duplexes. MS-friendly ammonium acetate-based additives need little system maintenance or source cleaning, allowing for fast and easy switching between negative and positive mode applications. HILIC-Z columns enable excellent retention time stability, and the method was able to preserve higher-order oligo structures.

Finally, the Agilent RapidFire 400 high-throughput LC/MS system coupled with an Agilent 6545XT mass spectrometer offers high-throughput oligo characterization by sustaining cycle times as fast as 12 seconds per sample during data

acquisition. The HILIC method is simple to set up and requires no adjustments to the mobile phase. This method displayed the robustness, reproducibility, dynamic range, and sensitivity required for high-quality oligo characterizations.

Fast and Selective Purification and Analysis using Prep-LC Agilent 1290 Infinity II HPLC/MS+UV

INSTRUMENTATION

The Agilent 1290 Infinity II Preparative and Analytical LC/MSD system combines Prep LC-MS/UV with an analytical LC/MS in the same system controlled by Agilent OpenLab CDS Chemstation Edition C.01.10 [287]. The included preparative binary pump features titanium pump heads for pH and buffer tolerance, and can deliver up to 200 mL/min.

The integrated Agilent 1290 Infinity II Preparative Open-Bed Fraction Collector is a powerful tool that facilitates repeated purifications and automated clean-up between products with injection volumes ranging from 5 µL to 60 mL, making it amenable to both preparative and analytical applications. Conveniently, mass spectrometry detection can be used for both preparative and analytical runs, while the built-in LC/MS instrument is very useful for fraction analysis.

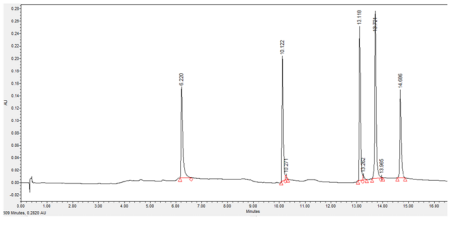
Preparative LC columns employed in the oligonucleotide purification study were the Agilent AdvanceBio Oligonucleotide columns with superficially porous particles, 4 µm, 21.2 × 150 mm, and the competitor W C18 column. The analytical LC columns were Agilent AdvanceBio Oligonucleotide columns with superficially porous particles, 4 µm, 4.6 × 150 mm for analytical internal analysis and the competitor W C18 column for UHPLC runs.

During a dibutylamine (DBA) separation, the negative charges of the oligo can interact with the amine functional group of the DBA ion pairing reagent, while the hydrophobic dibutyl groups are retained by the hydrophobic stationary phase on the particle surface. The interactions are strong between the sample and the particles covered in DBA ions, and the elution of the sample will be performed at a higher concentration (32 to 50 percent) of organic solvents compared to when using a less hydrophobic ion pairing reagent like triethylamine (TEA). By increasing the temperature, a very nice separation is possible without any other additions or a high pH (see **FIGURE 2**).

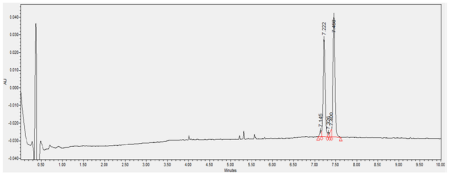
FIGURE 2: The DBA system in combination with selective columns have a high separation capability both in Analytical LC and Preparative LC

Analytical examples

Mix 6, 20, 35, 44 and 61 bases long ONs in16 min

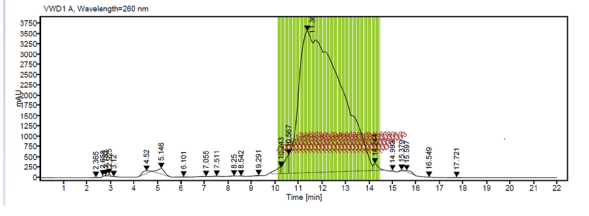


The 44 and 46 mer are well separated in 10 min



Preparative example;

With high purity +92 area % and yield :20-30 mer with a 45 µmole injection on a 30 x100 mm column



Several fractions with high purity, yield and concentration was obtained in this purification

Both the Analytical and the Preparative runs are performed with 75 mM DBA and 10 and 50 mM TRIS-HCl buffers at pH 8.0 and 8.3 respectively

As shown in the figure, the 1290 Infinity II Autoscale Preparative LC/MSD System can accommodate small scale analysis up to high loads without a problem. In the following examples, the sample used was a DNA oligo (20-61 nt) dissolved in synthesis buffers and water with a concentration of 1.4 to 6.2 mM. When using MS, it's possible to cut out fractions to yield a full-length product, or pure samples, with purity over 95 percent for several fractions (see **FIGURE 3**).

One of the best features of the 1290 Infinity II Autoscale Preparative LC/MSD System is that it's appropriate for both analytical and preparative work.

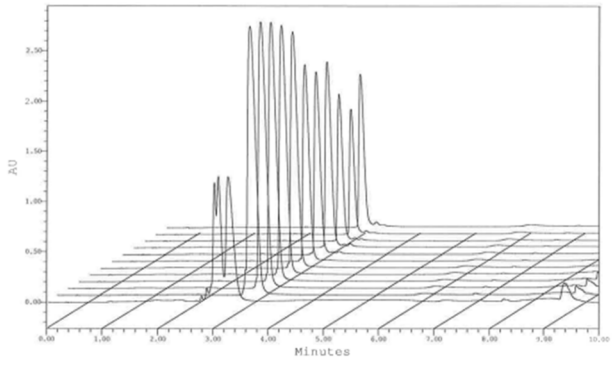
The temperature effects are shown in **FIGURE 4**, where the same sample has been injected at two different temperatures—around 25 °C and 60 °C. This variable not only yielded a change in the peak, but also demonstrates that chromatography runs better at higher temperatures. This is especially helpful for longer oligos, which need unfolding. Ultimately, there was a higher yield and fewer impurities with larger volumes at 60 °C than at ambient temperature.

One of the best features of the 1290 Infinity II Autoscale Preparative LC/MSD System is that it's appropriate for both analytical and preparative work. Via the open-bed collector, labs can reanalyze fractions without moving the samples, for fast and effective analysis of a large volume of samples.

In a test of an ASO sequence that was phosphorothioated in 19th position, the Agilent AdvanceBio column yielded a good peak and the 1290 Infinity II yielded super cutting of the product.

FIGURE 3: The purity of fractions selected with the MS is high

Results from a separate analysis of fraction on UPLC gives a purity of >> 95 area %

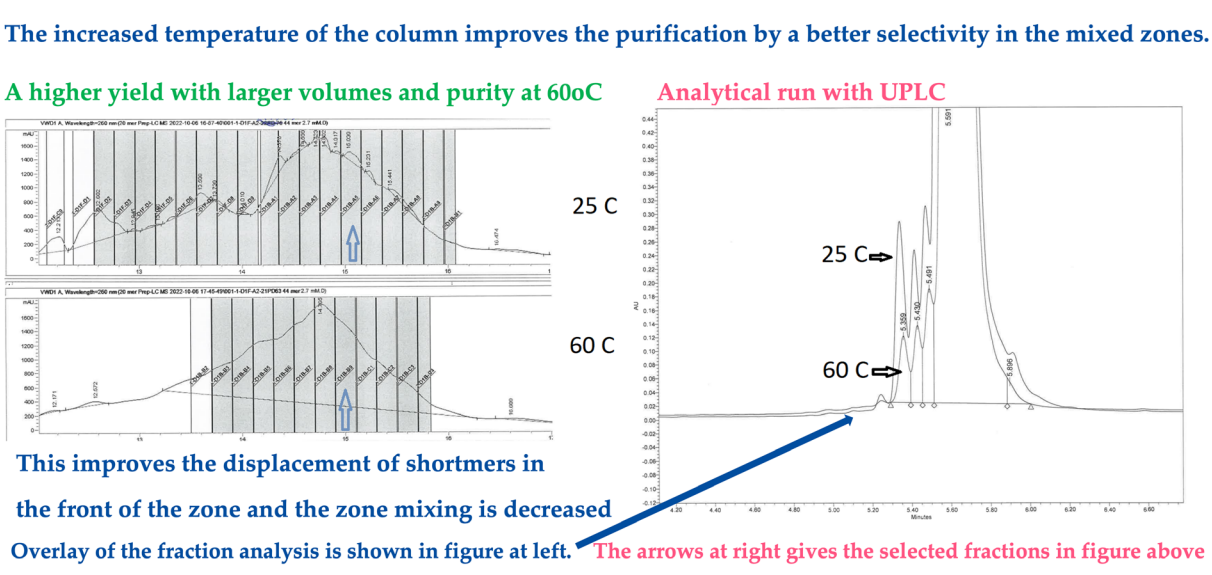


Summary of Results - Peaks over 50 % Area

	RT (min)	SampleName	Inj. ID	Vial	Height (µV)	% Area
1	3.462	22PD-105 Prep-LC MS fr A4	5895	1:C,1	2678481	99.93
2	3.459	22PD-105 Prep-LC MS fr A5	5913	1:C,2	2654902	99.92
3	3.450	22PD-105 Prep-LC MS fr A6	5917	1:C,3	2586073	99.41
4	3.443	22PD-105 Prep-LC MS fr A7	5921	1:C,4	2486619	99.28
5	3.451	22PD-105 Prep-LC MS fr A8	5925	1:C,5	2354504	98.76
6	3.474	22PD-105 Prep-LC MS fr A9	5929	1:C,6	1961711	98.15
7	3.482	22PD-105 Prep-LC MS fr B1	5933	1:C,7	1821059	97.45
8	3.483	22PD-105 Prep-LC MS fr B2	5937	1:C,8	1852547	97.64
9	3.504	22PD-105 Prep-LC MS fr B3	5941	1:D,1	1464388	97.50
10	3.526	22PD-105 Prep-LC MS fr B4	5945	1:D,2	1249248	96.66
11	3.501	22PD-105 Prep-LC MS fr B5	5949	1:D,3	1525555	95.94
Mean	3.476					
% RSD	0.75					
Min					1249248	

High yields (90 %) have been obtained in our studies on 20-30 mers using the DBA separation systems

FIGURE 4: A higher yield is achieved at elevated temperatures in separation of the shortmers from the FLP



Summary

Agilent offers myriad solutions for oligonucleotide analysis. Labs tasked with analyzing oligo purity will benefit from the high resolution 6230 LC-TOF combined with BioConfirm 12.0; together, these provide rich TPI results using multiple data mining techniques. If purity and sequence (for target or impurities) both need to be confirmed, high resolution 6545XT AdvanceBio LC/Q-TOF combined with BioConfirm 12.0 provide maximum performance and capabilities supporting both TPI and oligo sequencing workflows. Agilent supports both ion-pair reversed-phase and HILIC (non-ion-pairing) oligo methods on canonical and heavily modified synthetic oligo samples, and the BioConfirm 12.0 software is compliance-ready.

Agilent also has the instrumentation and materials needed to facilitate the move to HILIC workflows, which are ultimately capable of producing robust and sensitive oligo analysis, handling high throughput, and promoting fast and easy switching between negative and positive modes.

Finally, the Agilent 1290 Infinity II system in combination with the DBA method proves to be an excellent separation tool. Ultimately, this technology can help labs increase productivity thanks to high yield, increased purity, and shorter run times compared to other separation systems.

Learn more about Agilent end-to-end workflows for oligonucleotides: www.agilent.com/oligonucleotides

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