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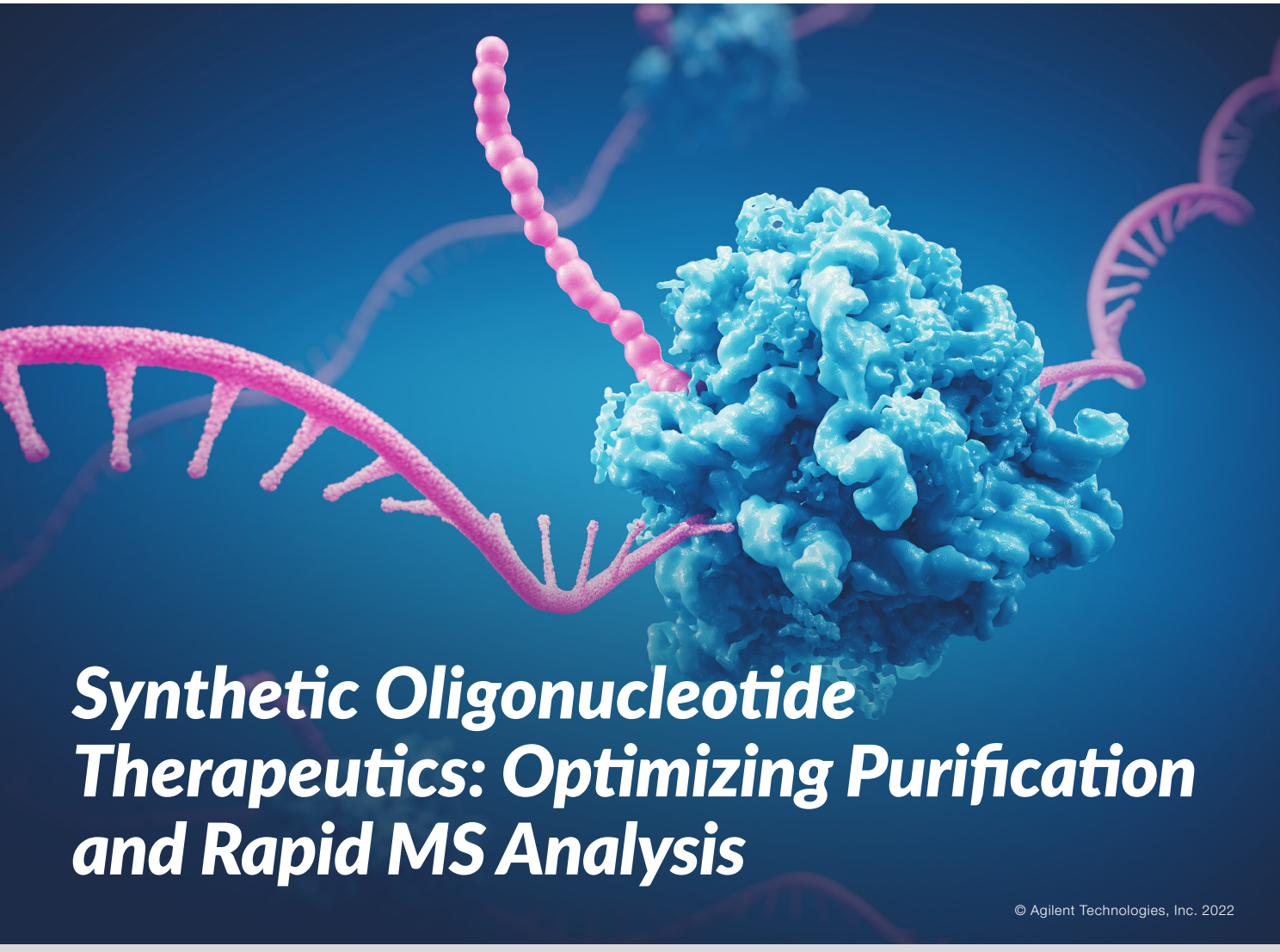

Synthetic Oligonucleotide Therapeutics: Optimizing Purification and Rapid MS Analysis

- Best Practices for Oligonucleotide Analysis and Purification
- Tips to Optimize Lab Scale Oligonucleotide Purification
- Purification of Single-Stranded RNA Oligonucleotides Using High-Performance Liquid Chromatography
- Ultra-fast Analysis of Intact Proteins and Oligonucleotides Using High-Throughput Mass Spectrometry

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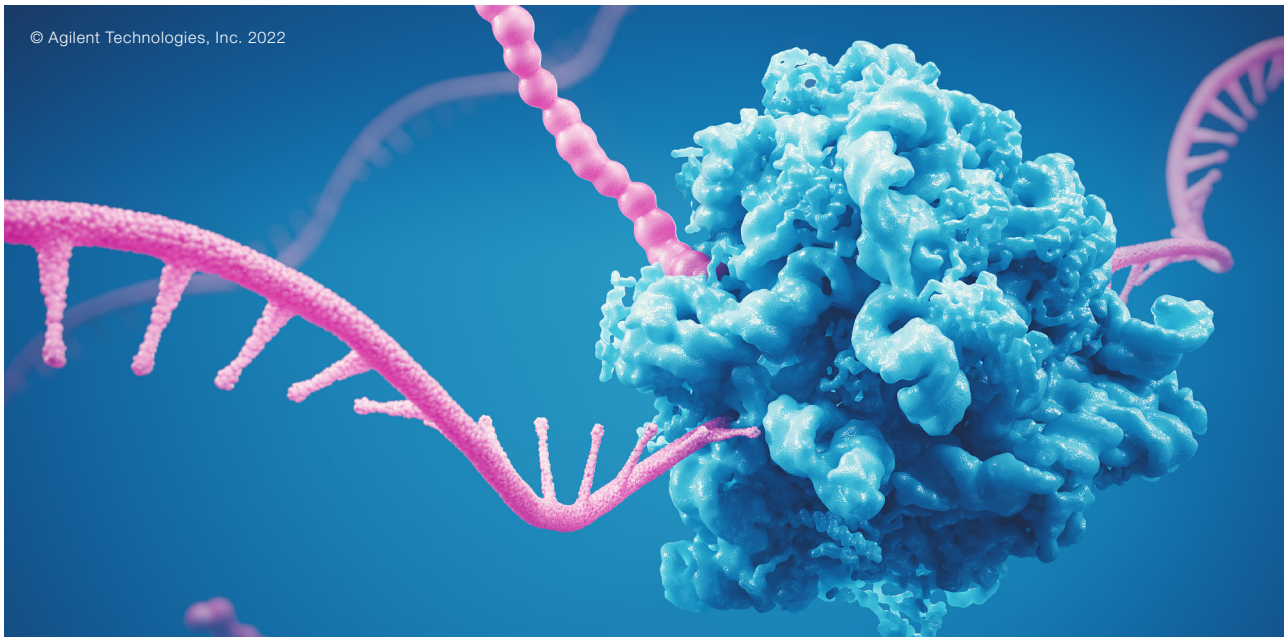

Best Practices for Oligonucleotide Analysis and Purification

Andrew Coffey, Ph.D., April Rachamim, and Matthew Turner, Ph.D.

Highlights from a recent roundtable discussion on oligonucleotide purification

Agilent Technologies experts Andrew Coffey, Ph.D.; April Rachamim; and Matthew Turner, Ph.D., appeared in a roundtable discussion, hosted by ChromAcademy, to review best practices surrounding oligonucleotide purification. Oligonucleotide analysis and purification techniques, method optimization parameters, and resources available to support laboratories were reviewed in detail by the panelists.

Oligonucleotide Purification Method Selection

"When looking at oligonucleotide purifications, it is important to consider what type of oligonucleotide is being purified across the diverse community of molecules," explained Matthew Turner, Ph.D., as he started highlighting the factors that need to be examined when selecting an oligonucleotide purification method.

"When looking at oligonucleotide purifications, it is important to consider what type of oligonucleotide is being purified across the diverse community of molecules."

Matthew Turner, Ph.D.
Biocolumns Product Manager
Biopharma Columns
Agilent Technologies

Turner suggested that oligonucleotide length, operating temperature, preference of chromatographic chemistry, and instrumentation should all be considered.

In regard to temperature, oligonucleotides may require purification at elevated temperatures in order to reduce secondary structure formation.

"You want to denature the oligonucleotides and make the peaks as sharp as possible to better separate the impurities, suggested Andrew Coffey, Ph.D. Coffey went on to explain that larger oligonucleotides are more challenging to assess, based on impurities and contaminants that are highly related to the full-length product and prone to co-elution.

Looking at the quantity of oligonucleotides is an additional consideration in order to determine the appropriate column size for downstream processing or experimentation.

"There is a wide range of column dimensions available to purify small quantities of oligonucleotides, up to a few grams, that don't require you to pack your column," said Coffey. "However, if you have a very large quantity of oligonucleotides, it's likely you will have to buy a stationary phase and load your column for process-scale purification."

April Rachamim added to the discussion on the packing of columns, highlighting the availability of prepacked columns up to 100 mm x 300 mm in size, offered by Agilent, as a solution to some common synthesis scale separations, accommodating purification without the need to pack a column without the expense of bringing your own packing technology in house, making large scale purification more accessible to smaller organizations.

ADDITIONAL RESOURCE

Oligonucleotide Purification Solutions: Agilent PLRP-S and PL-SAX HPLC columns

Method Optimization Parameters for Oligonucleotide Analysis and Purification

The next topic covered during the roundtable discussion was a review of parameters that need to be considered and evaluated for during oligonucleotide analysis and purification. Factors such as pore size, optimization of mobile-phase, temperature, and the gradient profile were highlighted as some of the top considerations by scientists.

"Choosing the right pore size is one of the most important factors," said Coffey, who went on to explain that larger pore size is associated with reduced surface area, leading to a decrease particle binding capacity, but also offering increased mass transfer associated with sharp peaks."

Customers are recommended to purchase analytical-sized columns with different stationary phases so researchers can validate the correct stationary phase as part of method optimization.

In terms of method development, separation is often challenging. The panelists discussed the importance of taking time to look at gradient profiles in addition to other parameters such as flow rates.

Tools for Effective Analysis of Oligonucleotides

"With any type of therapeutic, there are key aspects of the molecule, you want to verify," Turner stated, when responding to a question about quality attributes of importance with oligonucleotide therapeutics.

Turner explained the importance of ensuring that the final product results with minimal impurities. Common with other therapeutics as well, oligonucleotides have critical quality attributes. Verifying the final sequence is often assessed through liquid chromatography-mass

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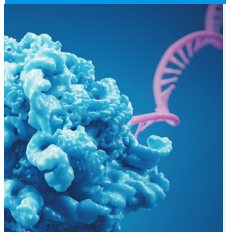
Andrew Coffey, Ph.D.
Senior Applications Scientist
Agilent Technologies

spectrometry (LC-MS), to look at impurity profiles.

"MS is essential because it's the only technique that can positively identify that you've made the right thing. There are several parts to the whole process, including adapting methods from LC-UV into something that is MS compatible. In many cases, the normal conditions that you would use for LC-UV techniques are not directly MS-compatible, so you may have to redevelop your methods," explained Coffey.

Impurities can also be a result of incomplete synthesis. The manufacturing process of mRNAs, as explained by Turner, typically uses an in-vitro, cell-free production compared to synthetic production used for small oligonucleotides. Therefore, cleanup and purification strategies must be personalized for a molecule's unique characteristics.

"There is a different set of process-related contaminants, such as contamination from the proteins or enzymes that are added to the reaction mixture, and they must be effectively removed or precipitated out of solution," said Turner.



ADDITIONAL RESOURCE

Agilent Oligonucleotide Chromatography Solutions

Agilent Technologies provides labs with a range of column chemistries to help scientists analyze impurities in oligonucleotide therapeutics.

Learn more here.

Oligonucleotide Purification: Setting up for Success

Size and quantity matter when purifying oligonucleotides, explained Coffey, when asked what factors need to be considered to optimize oligonucleotide purification.

"Agilent has prepacked columns that are 100 mm ID, but to go beyond that, you need to pack your own column," he said.

Ensuring that the same material is used, if moving from a prepacked to a process-scale column, is essential to optimizing the analysis. "You want to use the same material found in your prepacked column to pack your own process-scale column; then you can transfer things with much more ease," Coffey added.

Rachamim added the importance of having the same stationary phase available in larger particle sizes. "As you go up to a larger scale of purification, you need a particle that scales proportionately," said April. "Agilent has IEX and ion-pair reversed-phase chemistries available—from our analytical dimensions with PLRP-S and PL-SAX, semi-prep and prep, all the way to bulk material—this allows you to utilize the same optimized

chemistry regardless of the scale of production you are looking to achieve," Turner highlighted.

The polymeric chemistry and stability of PLRP-S and PL-SAX results allows for high pH and temperature compatibility, even simultaneously, which is useful for oligonucleotide purification.

Selecting the Right Products in a Growing Marketplace

With various products available for the analysis and purification of oligonucleotides, it is important to explore options and compare and contrast offerings. Working with molecules of large size can be challenging, making pore size a demand by scientists shopping for the best equipment.

"It's worth noting that the Agilent pore size is relatively unique in the marketspace," said Rachamim. "We can go up to a 4,000 Å pore," Rachamim explained, "which is helpful if customers are looking to do LC purification for these larger molecules."

"The key takeaway is to remember oligonucleotides are a range of molecules in terms of their attributes and size. As such, it's important to select a chemistry that is optimal for your molecule and purification goals, and which can be scouted and tested at analytical scale prior to scale up—that's what Agilent's PLRP-S and PL-SAX portfolios offer," concluded Turner.

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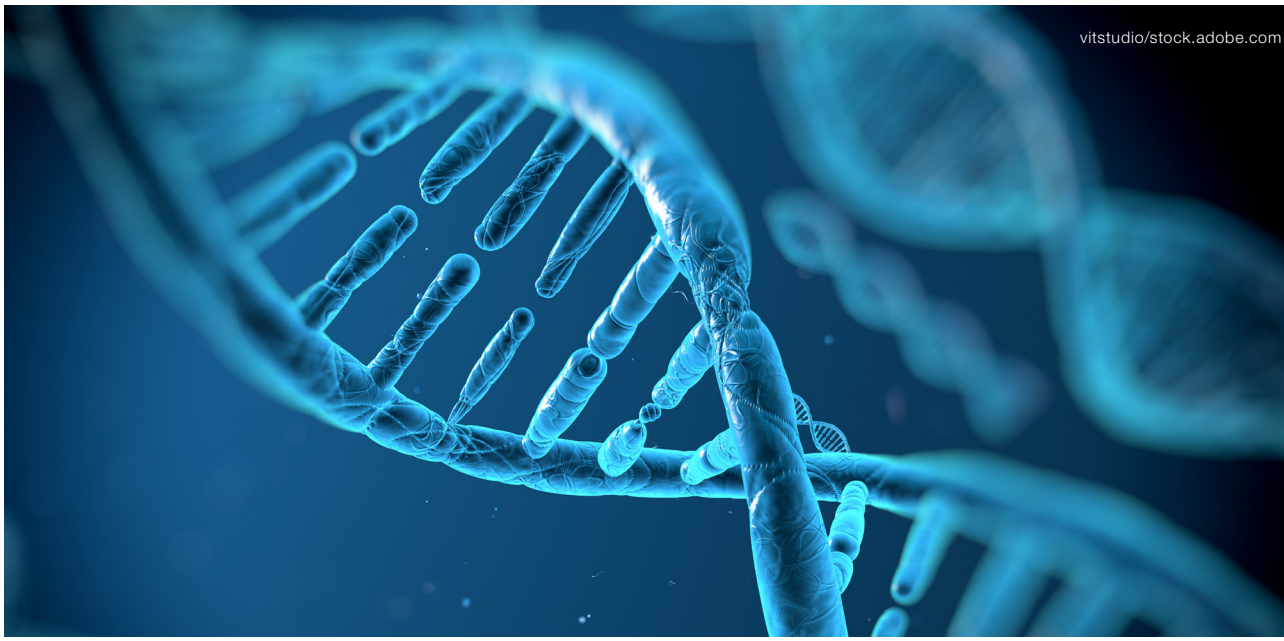
April Rachamim
Global Product Marketing Manager
Biocolumns & Bioconsumables
Chemistries & Supplies Division
Agilent Technologies

For more information on this important topic, visit www.agilent.com/chem/oligonucleotide-analysis.



Tips to Optimize Lab Scale Oligonucleotide Purification

By Matthew L. Turner, Ph.D.

Careful selection of separation media, mobile phase, and separation conditions can confer a critical edge in the analysis and purification of diverse DNA- and RNA-based therapeutics

INTRODUCTION

With the worldwide growth in the oligonucleotide therapeutic market, applying the appropriate tools for oligonucleotide purification and scale-up is essential. With the complex impurity profiles of synthetic oligonucleotides and varying sequence modifications and lengths, thoughtful optimization of HPLC methodologies is essential for final pure oligonucleotides for research, diagnostics, and therapeutics. Common challenges associated with oligonucleotide purification can be addressed using Agilent's suite of Bio LC column solutions, designed to address the complex needs of the market.

CHARACTERIZATION AND PURIFICATION OF OLIGONUCLEOTIDE THERAPEUTICS

Chromatographic separation is a powerful tool for the characterization and purification of oligonucleotide therapeutics,

but there are no one-size-fits-all solutions for this process. This is due to the vast range of nucleic acid-based products, which include both RNA and DNA, and range in length from short antisense oligonucleotides to multi-kilobase messenger RNA therapeutics.

The primary focus for separation in this context is on the negatively-charged phosphate backbone that is a universal feature of all nucleic acids. However, RNA molecules have a tendency to be more hydrophilic, with additional charge conferred by the 2'-hydroxyl group on their ribose ring. This can make separation on a standard anion-exchange or reversed-phase column more challenging, which is even more problematic given that preparations of RNA tend to have more impurities than DNA. Most therapeutic oligonucleotides also incorporate various chemical modifications that facilitate delivery, protect against nuclease-mediated degradation, or confer other advantageous properties. These modifications can also confound purification by altering the overall physicochemical properties of the biomolecule.

Additionally, both DNA and RNA sequences have the potential to form secondary structures that can further complicate size-based separation on an anion-exchange or ion-paired reversed-phase column. More generally, oligonucleotides tend to occupy a larger volume than globular proteins of the same molecular weight. This, in turn, impacts their interactions with porous chromatography media. For example, a 40-mer oligonucleotide with a weight of 12 kilodaltons (kDa) exhibits an elution time on a size-exclusion column that is comparable with that of a 50–60 kDa protein. This makes pore size an important consideration in choosing an appropriate chromatographic medium.

ADDITIONAL RESOURCE

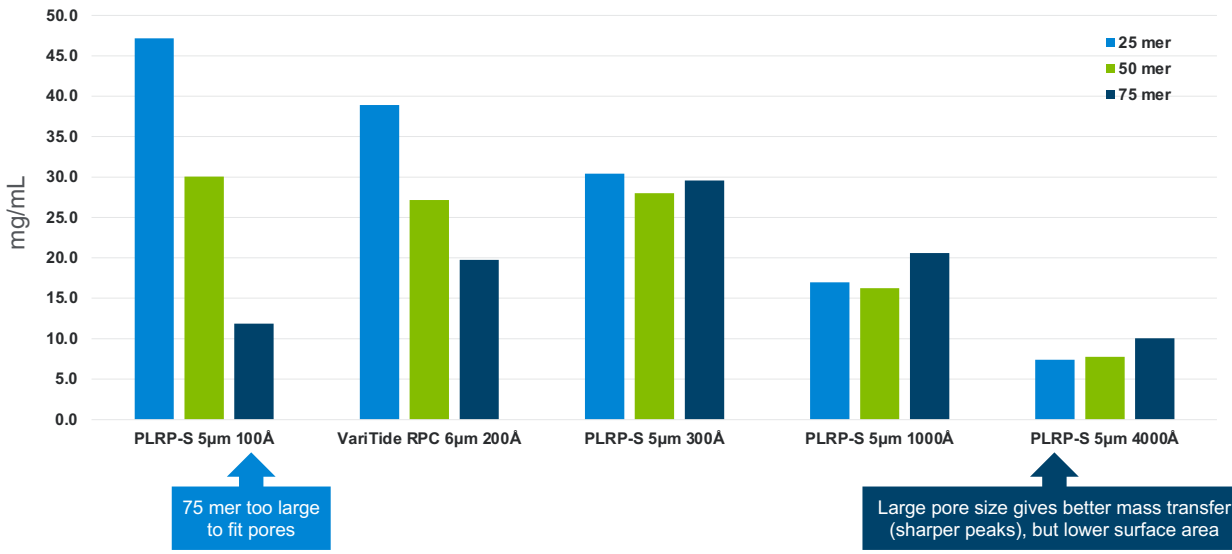
Watch Webinar On-demand

PORE SIZE AND OLIGONUCLEOTIDE BINDING CAPACITY

Dr. Andrew Coffey, at Agilent, ran a demonstration experiment for which he performed reversed-phase ion-pairing separation of oligonucleotides ranging from 25-100 nucleotides in length. Media with a pore-size of 1,000 Å yielded superior separation of full-length 75- and 100-mers from their associated impurities relative to media with a far smaller pore size of 100 Å. This reflects the difficulty that bulky, large oligonucleotides encounter with penetrating narrower pores, resulting in poorer separation.

But the choice of pore size must also be balanced against considerations of bead binding capacity. As demonstrated in FIGURE 1, media with smaller pores have a greater surface area and thus more binding capacity beneficial for optimizing yield

FIGURE 1: Effect of pore size on oligonucleotide binding capacity.



on a given column dimension. Therefore, selecting the largest pore available will not provide an ideal solution in terms of maximizing both product purity and yield. Such considerations are just some of the critical factors that go into developing an optimal workflow for the lab-scale purification and characterization of oligonucleotide therapeutics.

SELECTING THE PROPER PARTICLE

Agilent offers chromatography media with a range of different properties that may be advantageous for a given analysis or purification effort. For analytical workflows, non-porous Bio SAX particles can offer greater versatility for anion exchange-based separation by eliminating concerns over the diffusion behavior of different lengths of oligonucleotide.

Alternatively, users may opt to employ the superficially porous AdvanceBio Oligonucleotide media, which can offer improved resolution and diffusion for ion-paired reversed-phase separations utilizing its superficially porous particle.

Bio SAX UV Analysis of Oligonucleotides

Consider a simple demonstration with anion exchange-mediated separation of DNA and RNA ladders on a non-porous Bio SAX column. Using relatively simple separation conditions, with a 20 mM Tris buffer at pH 8.0 and a broad gradient of 0–100% 1M NaCl for elution, one can essentially achieve comparable charge-based separation of both RNA and DNA—for example, an RNA 17-mer exhibits an elution time that falls between that of a DNA 15-mer and 20-mer.

One can also tune the separation conditions with Bio SAX to achieve optimized elution of different oligonucleotide products. For example, by simply tuning the salt gradient while maintaining otherwise constant separation conditions, it becomes possible to achieve high-resolution separation of analytes ranging from a 25-mer DNA to a 105-mer RNA. This sharp peak resolution is particularly advantageous in the context of LC-UV analysis, where one can readily discriminate the peaks associated with the various impurities present in a given preparation (FIGURE 2).

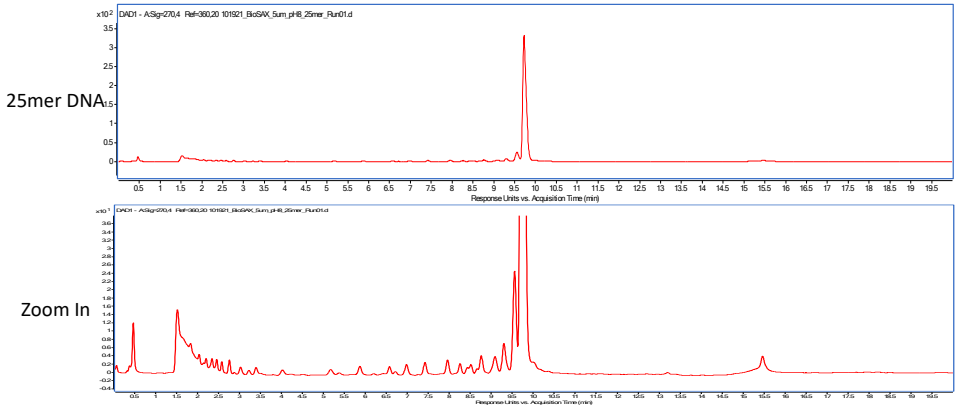
Bio SAX and Binding Capacity

Binding capacity is another important parameter to determine prior to purification or analysis, ensuring overloading of the oligonucleotide does not occur and load quantities are optimized. To determine the dynamic binding capacity (DBC), a common experiment called a breakthrough experiment is performed by measuring the concentration of oligonucleotide loaded at which point saturation occurs, and breakthrough emerges. Dr. Jordy Hsiao at Agilent conducted an assessment of Bio SAX particles of varying diameter in order to identify when these various media become saturated with 100-mer DNA in a 4.6 x 50 mm column. Since these particles are non-porous, their surface area, and, therefore, their binding capacity, diminished with increased particle size. Columns packed with 5-micron particles had a capacity of 6.7 mg/mL versus 2.7 mg/mL for a column loaded with 10-micron particles.

MAKE THE MOST OF YOUR MOBILE PHASE

Porous media are a better choice when the focus is on oligonucleotide purification rather than analysis. Agilent offers two popular options here: PLRP-S, for ion-paired reversed phase, and PL-SAX, for anion-

FIGURE 2: Bio SAX provides high resolution UV analysis of oligonucleotides.



Solvent A: 10mM Tris, pH 8.0 in water
Solvent B: 2M NaCl in solvent A
Column: Bio SAX 5 µm, 4.6 x 50 mm (P/N 5190-2468)
Column temp: 80°C
Flow rate: 1 mL/min
1 mg/ml samples: 1 µl injection
10 min gradients: 20-40% B

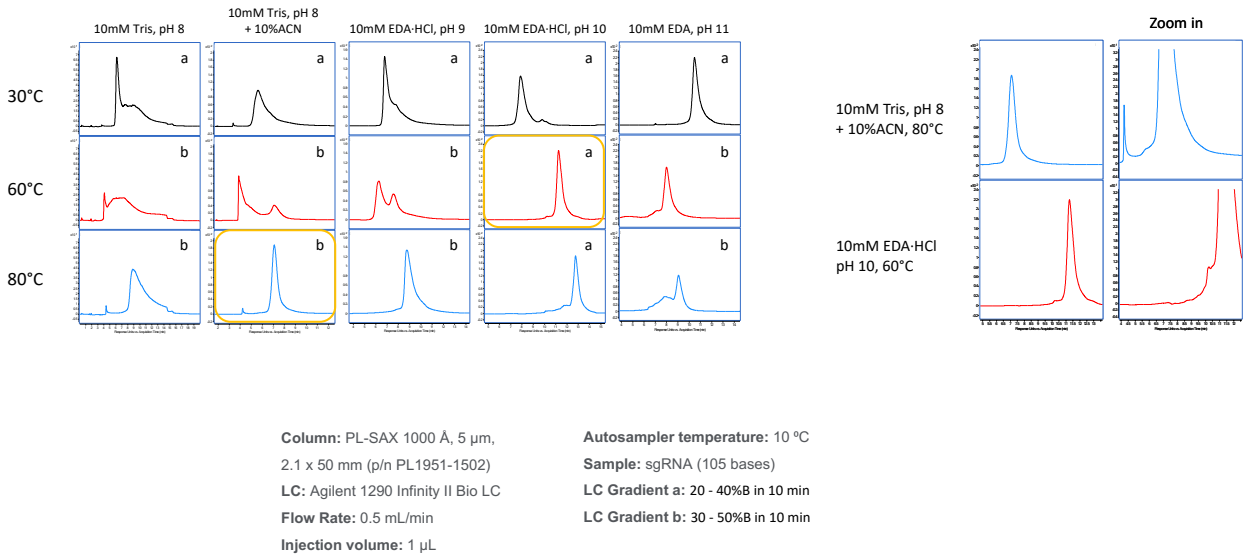
exchange. When Dr. Hsiao repeated the binding capacity experiment with PL-SAX particles with 1,000- Å pores, he observed a far greater binding capacity of 13.2 mg/mL for the same DNA 100-mer.

PL-SAX for Oligonucleotide Purification

Purification can require careful mobile-phase optimization, in terms of the choice of pH, temperature, buffer, elution salt, and organic modifiers. In a demonstration experiment with 25–100-mers separated on the same PL-SAX particles described above, a simple buffer based on 10 mM Tris, pH 8.0 performed well with smaller oligonucleotides but achieved poorer resolution of larger products. In particular, the 75- and 100-mers encountered issues with the formation of secondary interactions such as hairpins and self-dimerization. The inclusion of 10% acetonitrile as an organic modifier somewhat improved the outcome, but the best results were achieved in highly basic buffer conditions (10 mM NaOH, pH 12) that effectively disrupted the unwanted interactions.

Dr. Hsiao performed a similar experiment to optimize purification of a 105-mer guide RNA oligonucleotide, and again encountered issues with secondary interactions at baseline conditions of room temperature and pH 8.0. Due to the greater fragility of RNA relative to DNA, simultaneously increasing the temperature to 80 °C and the pH to 11 resulted in heavy degradation of the oligonucleotide product (FIGURE 3). After testing multiple different permutations of the purification process, it became clear that the best results came from somewhat milder regimens: either an 80 °C separation at pH 8.0 with acetonitrile added, or an

FIGURE 3: Applying experimental design to gRNA purification.



intermediate-temperature separation (60 °C) under slightly less basic conditions (pH 10). These results demonstrate the careful testing process that should go into mobile-phase optimization for a purification process.

Some oligonucleotides may benefit from very specific changes in protocol. For example, high alkaline buffer conditions have proven useful for the isolation of molecules with thiolated backgrounds, enabling robust purification of fully-phosphorothioated oligos from phosphate-based impurities.

IMPROVING PERFORMANCE WITH ION-PAIRING

The primary focus up to this point has been on anion-exchange workflows, but ion-paired reversed-phase chromatography is actually the most widely used method for oligonucleotide purification and is compatible with both UV-LC and mass spectrometric (MS) analysis.

As with anion-exchange, high temperature and pH conditions can provide an important edge in achieving robust separation of the desired oligonucleotide product from contaminants and failed sequences. Agilent's AdvanceBio Oligonucleotide silica-based media is highly stable under such conditions, achieving high reproducibility with minimal retention drift. And since the particles are superficially porous, they decrease back pressure and minimize the hindrance of diffusion of large oligonucleotides that may be observed with fully-porous particles.

AdvanceBio Oligonucleotide Column or Ion-Pairing

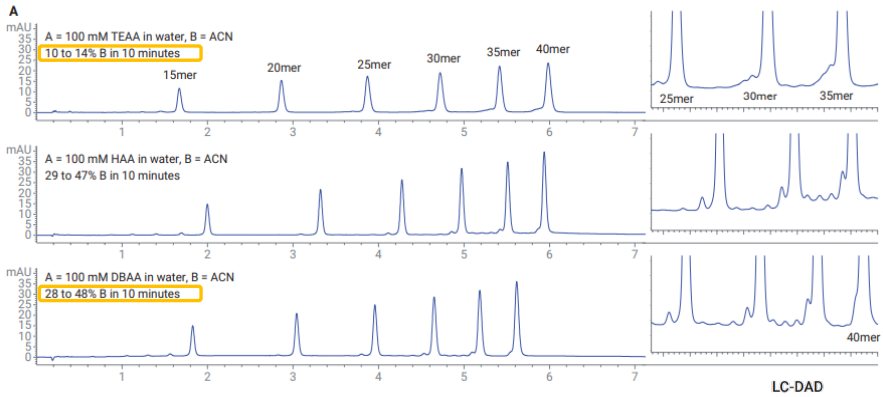
Agilent researchers performed a head-to-head comparison of separation of a DNA ladder and RNA ladder with the AdvanceBio Oligonucleotide media, with a 5–20% gradient of triethylamine acetate (TEAA) plus acetonitrile as an eluent. In contrast to the results described above for the same experiment performed with Bio SAX, the RNA sequences eluted considerably earlier than their DNA counterparts of similar length—a consequence of RNA's greater hydrophilicity. This means that an RNA-based standard should be employed while optimizing the separation for any RNA oligonucleotides with such columns. Tuning the gradient can also improve separation with RNA; the use of a 6–8% TEAA plus acetonitrile elution gradient effectively resolved 20-mer and 21-mer sequences whose peaks were overlapping after separation with the broader 5–20% gradient.

ADDITIONAL RESOURCE

Evaluation of Different Ion-Pairing Reagents for LC/UV and LC/MS Analysis of Oligonucleotides

Although TEAA is the most commonly-used ion-pairing agent, other alternatives are available. As demonstrated by FIGURE 4, a more hydrophobic agent such as hexylamine or dibutylamine might yield better outcomes. Separation of 15–40-mer oligonucleotides

FIGURE 4: Effect of hydrophobicity on oligonucleotide resolution and retention.



LC-DAD

Parameter	Value
Column	Agilent AdvanceBio oligonucleotide, 2.1 × 50 mm, 2.7 µm (p/n 659750-702)
Mobile Phase A and B	See *Results and discussion* section
Gradient	See *Results and discussion* section
Flow Rate	0.6 mL/min
Column Temperature	65 °C
Detection (DAD)	260/4 nm (reference 355/20 nm) Peak width >0.025 min (10 Hz)
Injection	2 µL (needle wash flush port, 3 seconds, methanol)
Injector Temperature	12 °C

Evaluation of Different Ion Pairing Reagents for LC/UV and LC/MS Analysis of Oligonucleotides (agilent.com)

on an AdvanceBio Oligonucleotide column required a relatively shallow gradient of 10% to 14% acetonitrile when TEAA was the ion-pairing agent. Achieving an equivalent retention time with the much more hydrophobic agent dibutylamine required a much higher concentration and broader gradient of TEAA (28% to 48%), but also conferred some benefits. The resolution was greatly improved through the use of dibutylamine. Furthermore, the use of higher concentrations of acetonitrile reduces the viscosity of the mobile phase and improves the denaturation of oligonucleotide secondary structures.

DIFFERENT ION PAIRING REAGENTS FOR OLIGONUCLEOTIDE PURIFICATION

Most MS-oriented workflows employ hexafluoroisopropanol (HFIP) as an organic counter-ion in combination with methanol as an organic modifier. This greatly improves the sensitivity of the subsequent MS analysis, but HFIP-based reagents are costly and typically must be prepared fresh for each new experiment. The selection of alternative ion-pairing agents can also be advantageous here. For example, by using dibutylamine rather than TEAA, one can achieve the same level of improved retention from lower concentrations of HFIP, without sacrificing MS sensitivity. In one comparison, an experiment performed with 15 mM DBA and 25 mM HFIP achieved comparable separation of RNA oligonucleotides relative to 15 mM TEAA and 400 mM HFIP, but with slightly improved resolution of larger oligonucleotides and reduced adduct formation in the MS spectra (FIGURE 5).

FIGURE 5: HFIP concentration considerations.

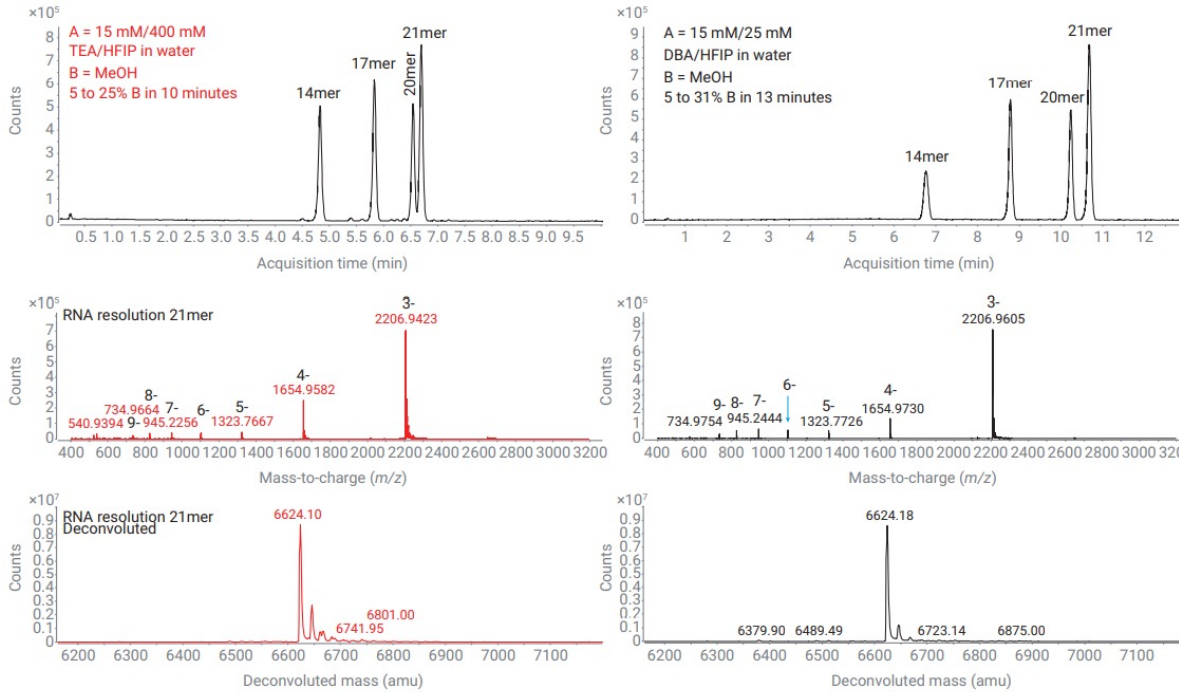


Figure 4. LC/MS results for RNA resolution standard analyzed with 15 mM TEA/400 mM HFIP reference condition (left) and with 15 mM DBA/25 mM HFIP alternative mobile phase (right). Top: BPC; middle: raw spectrum 21mer; bottom: deconvoluted spectrum 21mer. Mobile phase gradient: indicated in the figures; other method parameters: see the Experimental section.

Evaluation of Different Ion Pairing Reagents for LC/UV and LC/MS Analysis of Oligonucleotides (agilent.com)

CONCLUSION

Careful selection of separation media, mobile phase, and separation conditions can confer a critical edge in the analysis and purification of diverse DNA- and RNA-based therapeutics. Agilent's suite of Bio LC column solutions are specifically designed to address the complex needs of a rapidly growing and challenging market.

Matthew Turner, Ph.D.
Biocolumns Product Manager
Biopharma Columns
Agilent Technologies

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Trusted Answers

How To Meet Your Purification Goals

The right scale and the right pore size

Whether you're working on the bench or in manufacturing, Agilent PLRP-S and PL-SAX columns deliver a robust solution. What's more, they are available in pore sizes that are selective and appropriate for your unique oligonucleotide purification requirements, no matter if you're purifying small siRNA or large mRNA.

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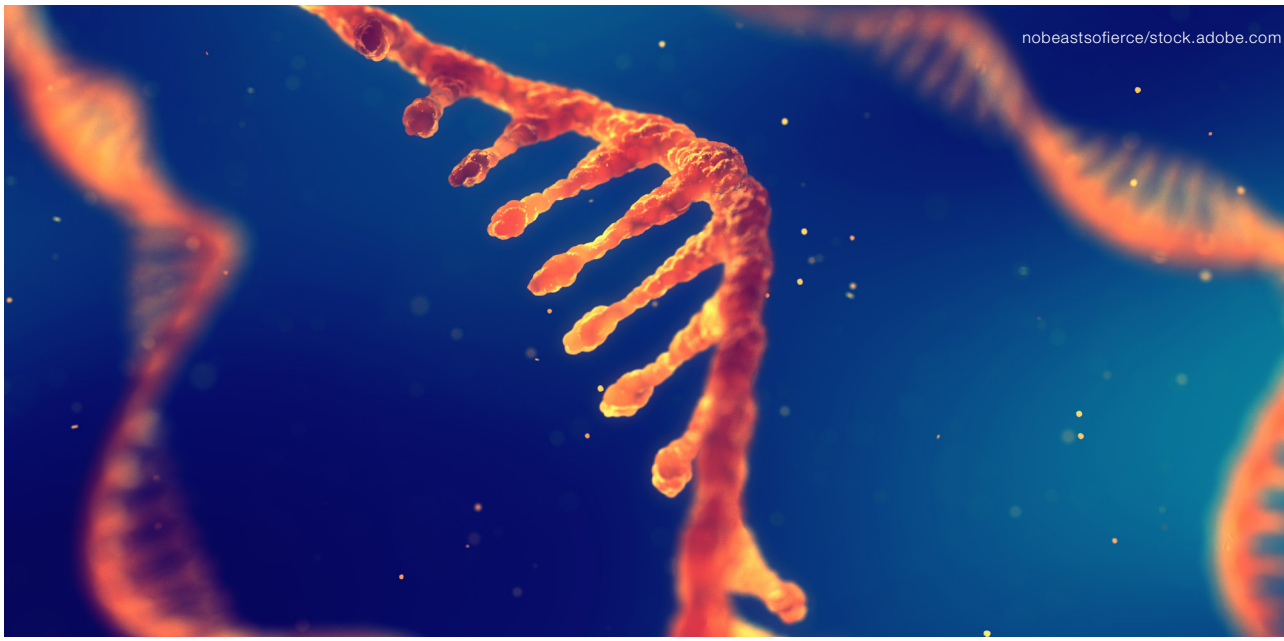


Purification of Single-Stranded RNA Oligonucleotides Using High-Performance Liquid Chromatography

By Florian Rieck

INTRODUCTION

Synthetic ONs are a class of compounds that have gained increasing interest over the last few years because of their use in biochemical research and as pharmaceuticals.[1] The process of synthesizing ONs has become much more efficient since its implementation in the 1980s. However, even with 99% coupling efficiency, a 25mer ON synthesis will yield less than 80% of the desired product. By-products are, for example, truncations of the target ON that have a sequence length of N–1, N–2, etc. Separating these impurities from the target molecule becomes more challenging with increasing sequence length.

An established separation method for ONs is reversed-phase HPLC. This technique employs separation columns with particles of 10 µm diameter or less, enabling high resolution of the analytes. By adding ion-pair reagents to the eluent, retention of the polar ONs on the nonpolar stationary phase is enabled. The ion-pair reagents interact with the charged phosphate backbone of the ONs and render them less polar with increasing sequence length. Elution and separation still require shallow solvent gradients of 1% slope or less, which can pose a challenge to HPLC instruments.

This application note demonstrates the separation and purification of a single-stranded RNA ON by preparative HPLC. The 1290 Infinity II Preparative LC System features a binary pump that is capable of precise gradient mixing even at high flow rates, which improves reproducibility and confidence in the results. Titanium pump heads are compatible with a wide pH range and salt concentration, stretching the range of applications that can be run on the instrument.

Method development is done at an analytical scale, then transferred to preparative conditions with added fraction collection. All experiments are conducted using PLRP-S columns, which are available in both analytical and preparative dimensions and thus enable easy scale-up and high reproducibility from analytical to preparative purification conditions.

EXPERIMENTAL

Instrumentation

The Agilent 1290 Infinity II Preparative LC System consisted of the following modules:

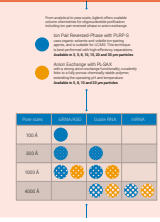
- Agilent 1290 Infinity II Preparative Binary Pump (G7161B)
- Agilent 1290 Infinity II Preparative Open-Bed Sampler/Collector (G7158B)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A)

Analytical method development and fraction reanalyses were conducted on an analytical system comprising the following modules:

- Agilent 1260 Infinity II Binary Pump (G7112A)
- Agilent 1260 Infinity II Vialsampler (G7129A)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A)

Columns

- Analytical column: Agilent PLRP-S 100 Å, 4.6 × 150 mm, 8 µm (part number PL1512-3800)
- Preparative column: Agilent PLRP-S 100 Å, 25 × 150 mm, 8 µm (part number PL1212-3800)



ADDITIONAL RESOURCE

Purification Your Way: siRNA to mRNA Infographic

Software

Agilent OpenLab CDS ChemStation edition for LC and LC/MS Systems, Rev. C.01.10 [239] or later versions

Chemicals

Hexylamine >99% for synthesis, acetic acid >99% ReagentPlus, and analytical-grade urea were purchased from Sigma-Aldrich (Taufkirchen, Germany). LC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak).

Mobile phase A was prepared freshly according to the following protocol: Prepare 450 mL of water, add 2.86 mL of acetic acid and 6.57 mL of hexylamine. Adjust pH to 7. Transfer solution to a 500 mL volumetric flask and fill to volume with water. For denaturing conditions, 10% (w/v) of urea was added to the buffer[2] to enhance the separation on preparative scale.

Sample

The sample to be purified was a crude synthetic, all-2'-O-methylated 22-mer oligonucleotide synthesized by Agilent NASD (Boulder, Colorado, USA) with the following sequence: 5'-aaaagcaccgacucggugccac-3'.

The sample was desalted and lyophilized before shipment. A fresh solution of 5 mg/mL in mobile phase A was prepared immediately before analysis.

RESULTS AND DISCUSSION

Analytical separation

The separation method was optimized on an analytical system using a sample concentration of 2 mg/mL. The optimized method was then scaled to preparative conditions using an HPLC method transfer calculator (TABLE 1).[3] As the mobile phase and column under preparative conditions were not amenable to temperature control, urea was added to the mobile phase to act as a chaotropic, or denaturing, agent.

FIGURE 1 shows a chromatogram of the separation under preparative conditions. A total sample amount of 20 mg on column was well separated. Fraction collection was set to peak-based, collecting time slices of nine seconds width. This fraction mode enabled targeted collection of the full-length product (FLP) peak, based on signal threshold and slope. The combination of peak-based collection with time slices divided the FLP peak into 11 slices. These can be analyzed with respect to purity and product content to decide which fractions need to be pooled to meet purity and yield demands of the purification process.

Fraction reanalysis was carried out on an analytical HPLC system using the same stationary phase. To increase resolution, separate possible aggregates, and accelerate the analysis, the column and solvent were thermostatted to 80 °C and the gradient adjusted accordingly (see TABLE 2). All fractions collected over the elution of the

FIGURE 1: Chromatogram (UV 260 nm) of a 4 mL (20 mg on column) injection on the preparative column. Blue bars represent fraction collection of 9-second time slices.

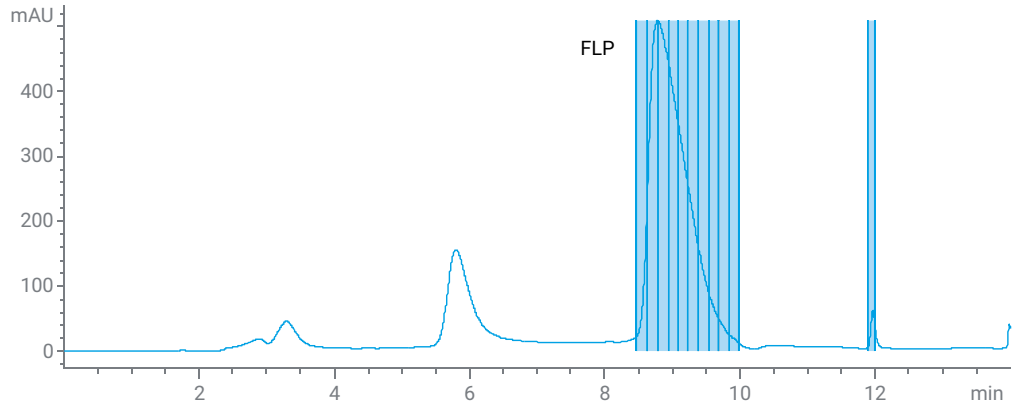


TABLE 1: Chromatographic parameters for purification.

Parameter	Value
Mobile Phase	A) 0.1 M Hexylammonium acetate in water, pH = 7.0, + 10% urea B) Acetonitrile
Flow Rate	30 mL/min
Gradient	Time (min) %B 0 32 0.26 32 10.10 42 11.09 100 12.07 100 13.06 32 Stop time: 14 min Post time: 1.5 min
Injection Volume	4,000 µL
Temperature	Ambient
UV Detection	260 nm No reference 5 Hz data rate
Fraction Collection	Peak-based from 7.0 to 12.0 min, collecting 9 s time slices UV threshold: 5 mAU UV upslope: 2 mAU/s UV downslope: 1 mAU/s

TABLE 2: Chromatographic parameters for fraction reanalysis.

Parameter	Value
Mobile Phase	A) 0.1 M Hexylammonium acetate in water, pH = 7.0 B) Acetonitrile
Flow Rate	1 mL/min
Gradient	Time (min) %B 0 28 8 36 9 100 10 100 11 28 Stop time: 11 min Post time: 6 min
Injection Volume	10 µL
Temperature	80 °C
UV Detection	260/4 nm Reference 360/100 nm 5 Hz data rate

FLP were reanalyzed and evaluated with respect to purity, measured by peak area percentage. FIGURE 2 shows a chromatogram overlay of all fraction reanalyses.

By injecting the crude sample and comparing peak areas at different dilutions with the fraction reanalysis, the FLP content in each fraction was calculated. With purity and content of the FLP determined, a pooling diagram and table can be created, which helps in judging which fractions to pool when a given purity or yield of the FLP needs to be achieved. FIGURE 3 shows the pooling diagram and table for the 11 fractions of the FLP. As expected, purity is highest in the center of the peak, whereas FLP mass is decreasing towards the tail. The pooling table quickly shows which fractions can be pooled if a minimum purity must be achieved and the yield is to be maximized. If, for example, the workflow requires minimum 99% purity, fractions 3 to 7 can be pooled and a yield of 56.3% be achieved.

ADDITIONAL RESOURCE

Dynamic Binding Capacity of Oligonucleotides on PLRP-S Columns and Stationary Phases

CONCLUSION

This application note demonstrates the purification of a short single-stranded RNA oligonucleotide by means of ion-pair reversed-phase chromatography. Separation conditions

FIGURE 2: Chromatogram overlay (UV 260 nm) of the reanalysis of 11 fractions collected over the FLP.

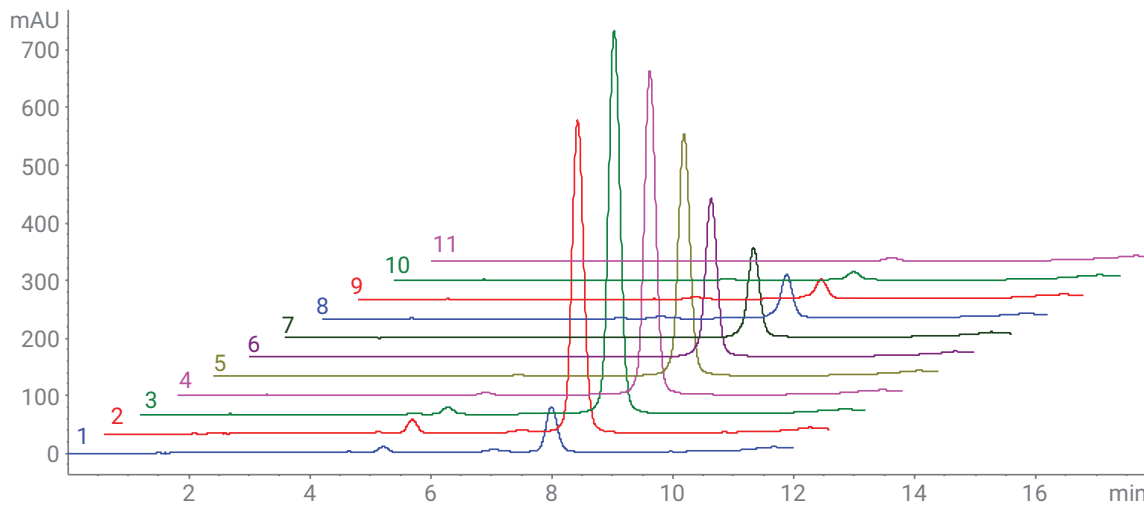
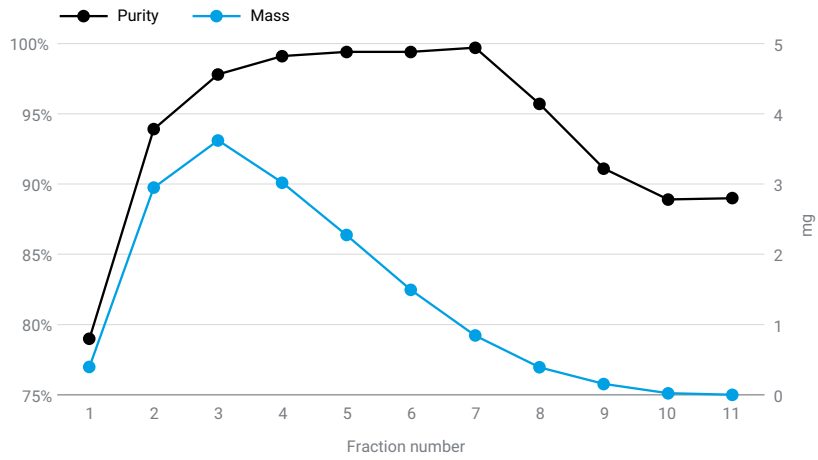


FIGURE 3: Pooling diagram and table for the 11 fractions of the FLP. Sorting fractions by purity, the pool to achieve highest yield at a given purity can easily be determined.



Fractions	Pool Purity	Pool Yield
7	99.7%	4.2%
6 to 7	99.6%	11.7%
5 to 7	99.5%	23.1%
4 to 7	99.4%	33.9%
3 to 7	99.1%	56.3%
3 to 8	98.5%	58.2%
2 to 8	97.9%	72.9%
2 to 9	97.0%	73.7%
2 to 10	96.1%	73.8%
2 to 11	95.4%	73.8%
1 to 11	93.9%	75.8%

were scaled up from analytical to preparative scale, using a 25 mm id column that enables high sample load. The Agilent 1290 Infinity II Preparative LC, comprising a combined autosampler/fraction collector, proved to be the ideal tool for large-scale injections and flexible fraction collection into tubes of various sizes. Collecting the target peak in time slices enabled specific pooling of the fractions by purity to meet workflow demands. Using this technique, a >99% pure fraction pool could be created with a yield of >56%.

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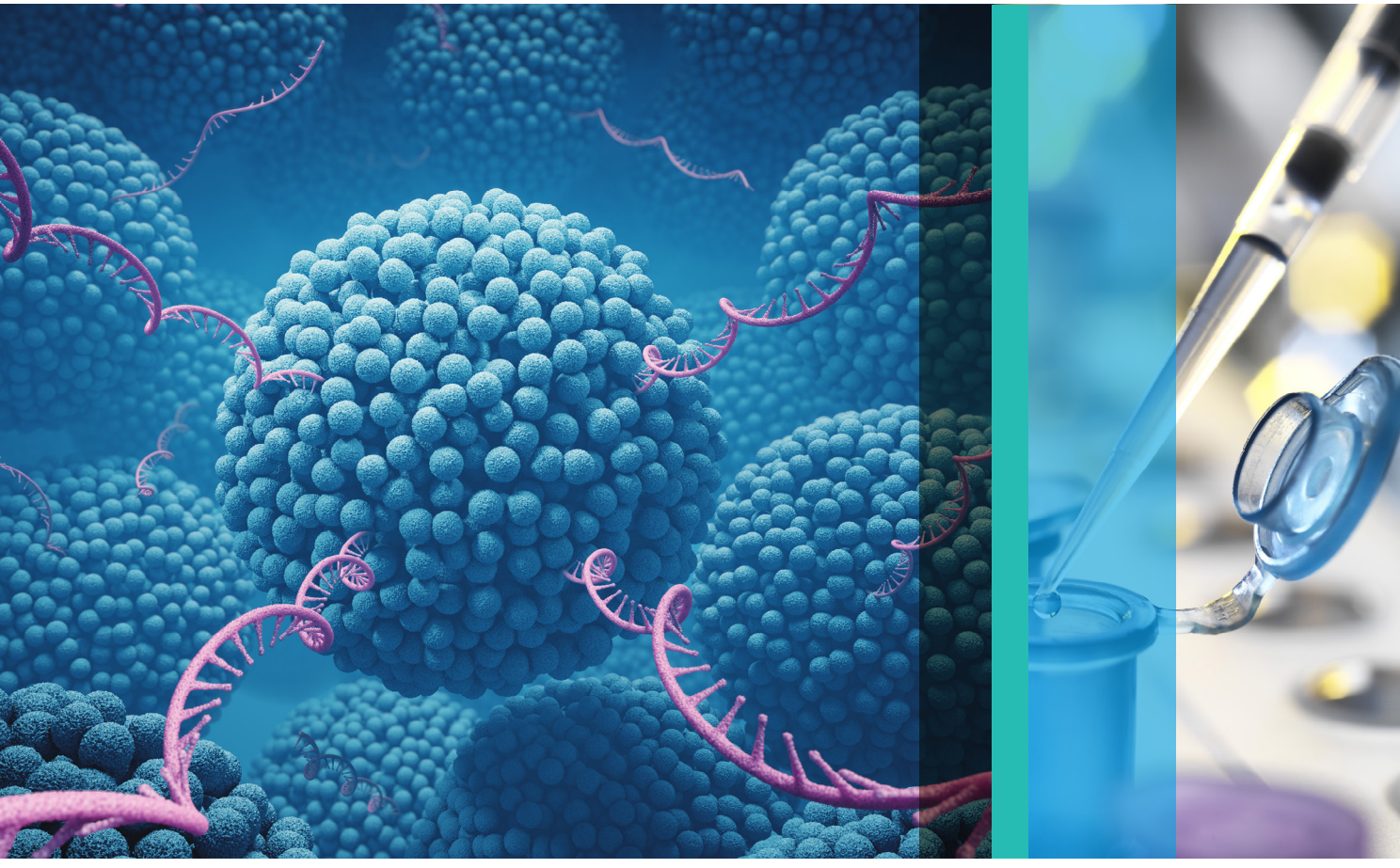

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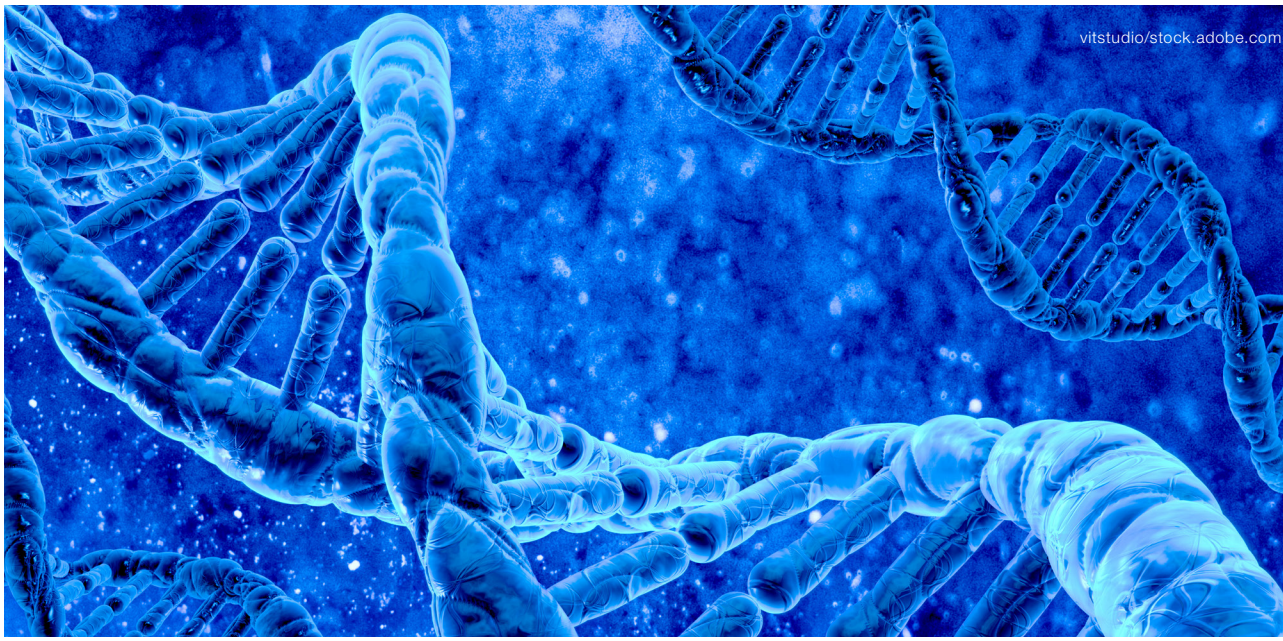


Ultra-fast Analysis of Intact Proteins and Oligonucleotides Using High-Throughput Mass Spectrometry

By Christopher Colangelo, PhD, MBA

Agilent's RapidFire 400 platform can dramatically boost the efficiency with which biopharmaceutical researchers can develop and perform robust sample purification and mass spectrometry workflows.

High-throughput sample analysis can be taxing for biopharmaceutical laboratories, testing the limits of what can be achieved with an individual instrument in a reasonable timeframe. Scaling up equipment capacity is one solution, but this is often not economically feasible—particularly in the context of high-performance, cutting-edge analytical systems.

RapidFire 400: A COMPLETE APPROACH TO HIGH-THROUGHPUT MS

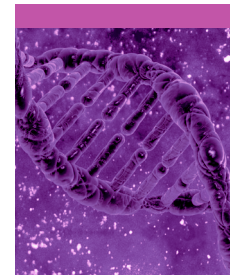
Agilent's RapidFire 400 autosampler is designed to help alleviate this strain by maximizing throughput in mass spectrometric analytical workflows while also minimizing the cost per sample.

The RapidFire 400 achieves greater speed by employing an online solid-phase extraction (SPE) system, which enables rapid cleanup and desalting of specimens, and can make use of a wide range of cartridge chemistries that facilitate the characterization of diverse analytes in complex matrices ranging from cell culture medium to blood to fermentation products. These chemistries include C4 resins for oligonucleotides, 18 resins for peptides or proteins, and hydrophilic interaction liquid chromatography (HILIC) columns for the characterization of glycans.

This instrument can also manage large numbers of samples, with a temperature-controlled storage unit that can accommodate up to 138,240 samples in 1,536-well plates. The hardware is combined into a relatively compact and mobile package, which can readily be relocated to enable easy connection to liquid chromatography (LC) and mass spectrometry (MS) instrumentation.

RapidFire ANALYSIS IN 5 STEPS

Sample preparation is a five-step process. The sample is first aspirated, and then loaded onto the SPE cartridge and washed with an aqueous buffer. Some samples may benefit from an additional wash step at this point, including interferent-rich matrices such as blood or plasma. The analytes of interest are then eluted from the cartridge with an organic buffer, after which the cartridge is re-equilibrated. The system can accommodate 12 SPE cartridges at once, each of which can be reused up to 2,000 times before needing to be replaced. The full five-step process typically requires less than 10 seconds per sample, but this can be further streamlined to just two seconds by employing 'blaze mode', which bypasses the use of the SPE cartridge and is therefore ideally suited for already-purified samples.



ADDITIONAL RESOURCE

Agilent Oligonucleotide Chromatography Solutions

USER-FRIENDLY ANALYZER SOFTWARE FOR RAPID RESULTS

Agilent has also developed user-friendly Analyzer software that allows researchers to rapidly assess their results as MS experiments progress. The program generates a clear visual readout of each plate, tracking which wells have already been processed and which are still in the queue, and allowing users to select individual wells for closer investigation. The resulting data can be readily exported in a variety of formats, enabling simple integration with existing laboratory information management systems (LIMS).

HIGH-PERFORMANCE PROTEIN AND PEPTIDE CHARACTERIZATION

As an initial demonstration of the RapidFire 400's performance with protein and peptide analytes, Application Scientist Caroline S. Chu, PhD, used the system in conjunction

with the Agilent 6230 Time of Flight (TOF) LC/MS system to analyze various preparations of the National Institute of Standards and Technology (NIST) reference monoclonal antibody. These included intact antibody, a preparation that had been reduced with Tris(2-carboxyethyl) phosphine (TCEP) to decouple the heavy and light chains, and a batch that was digested with the Genovis FabRICATOR enzyme to separate the antibody's Fab and Fc domains.

First, the intact antibody was subjected to TOF analysis in triplicate over a wide range of concentrations, with a blank injection of water between each sample (**FIGURE 1**). The data revealed a clear linear response with no detectable carryover, even at the highest antibody concentration. Analysis of the raw spectra and subsequent deconvolution of those spectra with the maximum entropy algorithm further confirmed the reproducibility and linear scale of the peaks obtained at concentrations ranging from 5 to 500 ng/µL intact antibody. The TCEP-reduced and FabRICATOR-digested monoclonal antibody preparations yielded similarly robust performance, with a concentration-dependent linear response and clearly defined peaks of appropriate mass for each of the isolated protein domains.

In a follow-up experiment, Agilent researchers used the same instrumental workflow to perform peptide mapping of bovine serum albumin (BSA) in both its standard and methylated proteoform. They obtained excellent coverage using a variety of SPE cartridges. For example, the C18 cartridge yielded an average sequence coverage greater than 75% from a triplicate analysis of a 2 pmol/µL tryptic digest of BSA (FIGURE 2). C8 and

FIGURE 1: Intact NIST Monoclonal Antibody: Triplicate Injections 5ng/µL-500ng/µL Deconvoluted by Maximum Entropy.

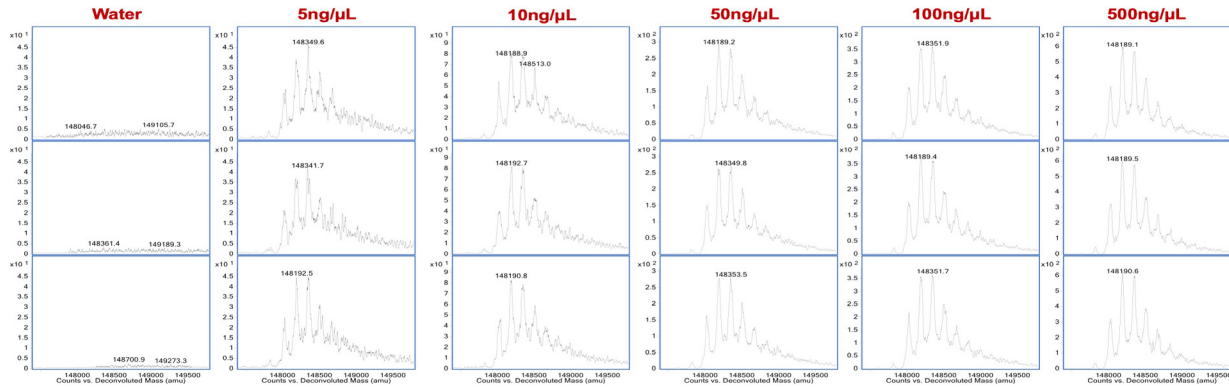
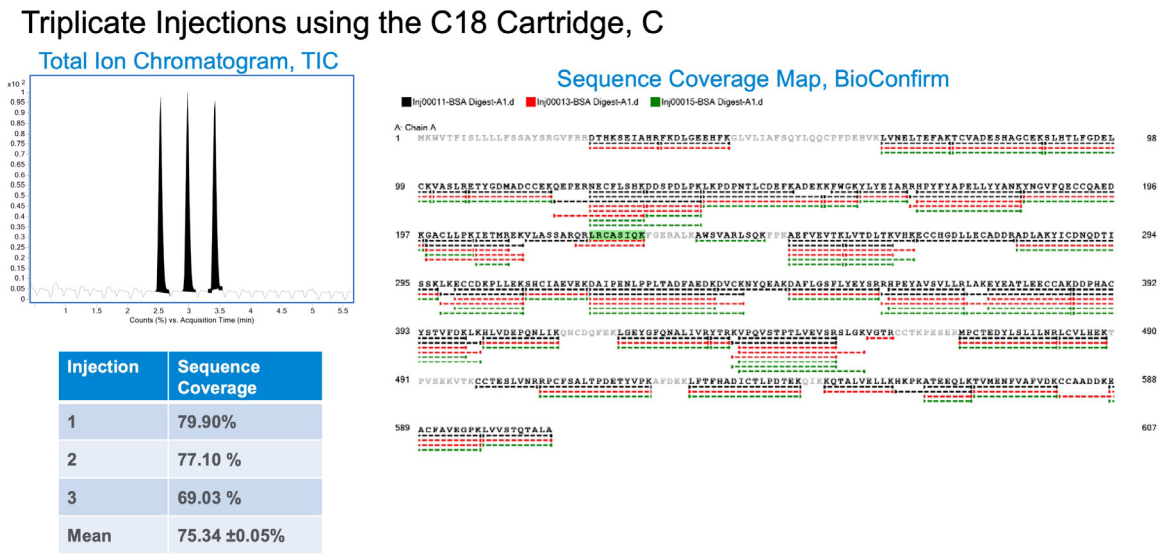


FIGURE 2: RapidFire TOF Analysis of BSA Digest, 2pmol/µL.



Injection	Sequence Coverage
1	79.90%
2	77.10 %
3	69.03 %
Mean	75.34 ±0.05%

C4 cartridges both yielded an average of more than 70% coverage, while a custom cartridge containing LRPS—a ring-shaped polymeric structure that binds to both peptides and proteins—exhibited a slightly lower sequence coverage of 65%. In all four scenarios, the level of coverage should be more than sufficient to quickly determine whether one has the correct protein sequence with a workflow that only requires 10 to 12 seconds per sample.

ACCELERATING OLIGONUCLEOTIDE ANALYSIS

The rapid rise of gene therapies and other genetic medicines such as short interfering RNAs, antisense oligonucleotides, and CRISPR/Cas9-based interventions is creating unprecedented demand for ultra-high quality oligonucleotide preparations.

These are challenging to analyze for several reasons. In addition to assessing the purity of the product and determining how much represents full-length product, one must also account for a range of chemically altered nucleotide building blocks that can also give rise to additional, unwanted byproducts. These include more than a dozen commonly used modifications that tune oligonucleotide characteristics including target affinity, specificity, and resistance to degradation by endogenous nucleases.

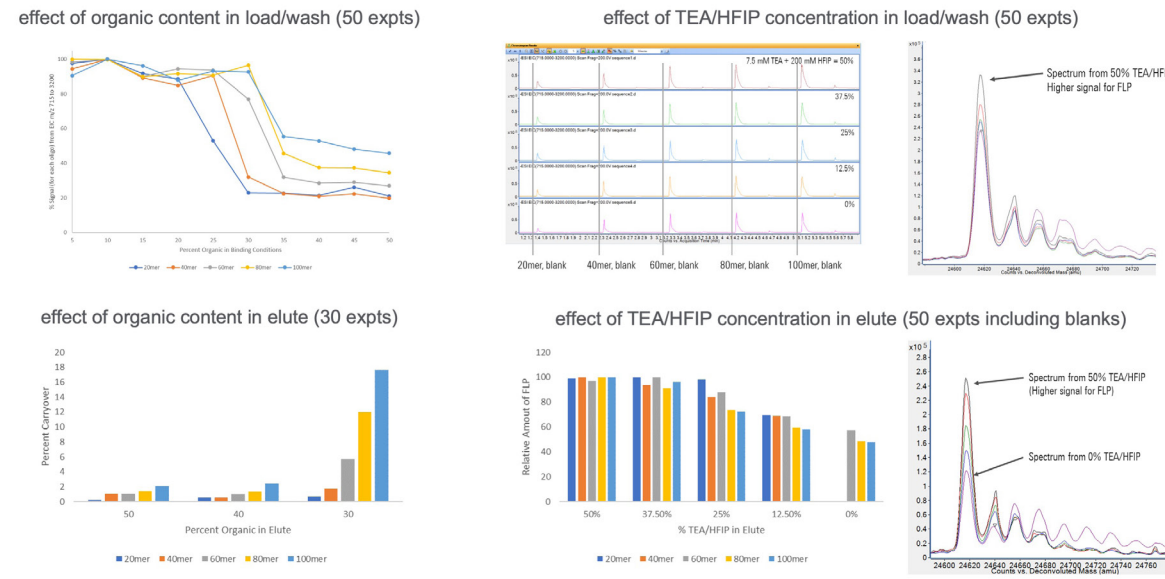
LC-MS analysis is a useful means for sorting through these various byproducts and assessing product purity, but this is also a challenging and time-consuming task. In the early days of this approach, it could take up to 50 minutes per run to

characterize an oligonucleotide sample.(1) Current LC-MS offerings have accelerated this process considerably, such that a 2.6 minute LC process is sufficient to comprehensively assess the purity of a 25-mer oligonucleotide preparation. Even so, this analytical process remains challenging to automate and therefore time-consuming to perform at scale.

The RapidFire system can be a powerful asset in this scenario, particularly when coupled to a quaternary pump system, which allows users to control the influx of four different reagents in parallel. For example, such a pump can selectively introduce varying concentrations of different solvents or ion-pairing reagents—a popular tool for LC-based separation of oligonucleotides. This is especially valuable during the method development process, when users are looking to identify optimal conditions for their separation process.

As a demonstration, application scientist Peter Rye, PhD coupled the RapidFire 400 to a quaternary pump to assess how different combinations of water and methanol with the ion-pairing reagents triethylamine (TEA) and hexafluoroisopropanol (HFIP) affected the SPE-based separation of oligonucleotides ranging from 20 to 100 nucleotides in length. These results, demonstrated in FIGURE 3, revealed that organic solvent concentrations of less than or equal to 20% will generally result in good product retention during the load and wash phases, while higher concentrations will lead to significant losses. Accordingly,

FIGURE 3: RapidFire Method Development for Oligos.



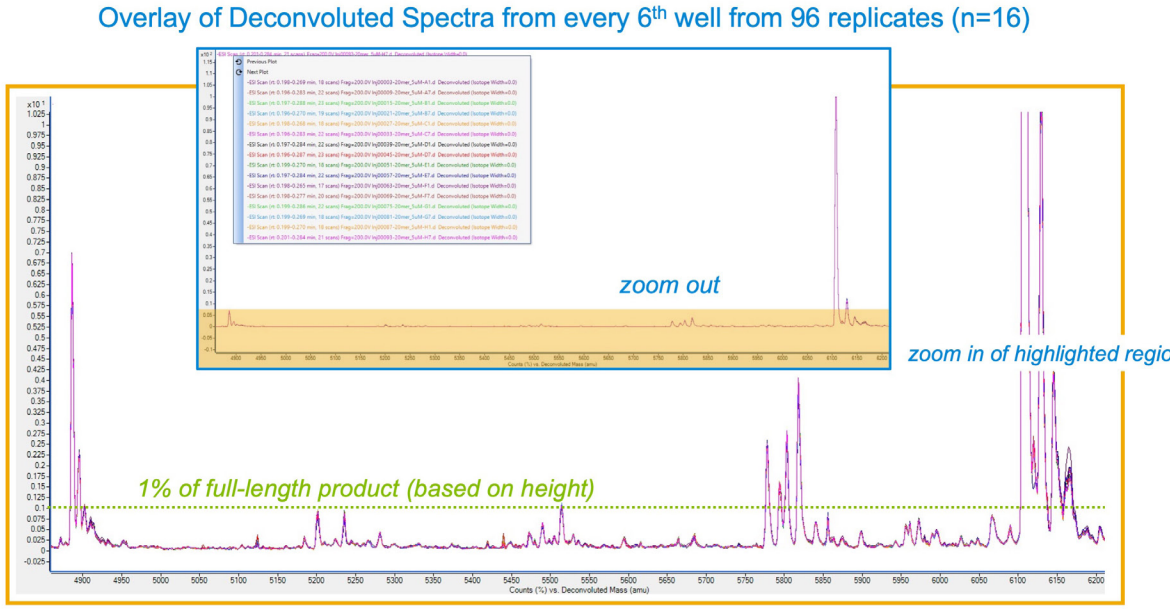
higher organic concentrations (40% to 50%) deliver best performance during the elution stage, with minimal carryover observed. High concentrations (50%) of ion-pairing reagents are also beneficial, leading to extremely efficient isolation of full-length product. Further experiments enabled the determination of optimal load, wash, and elute times—and critically, this entire method development process, encompassing 300 experiments, could be completed in just one day.

This optimized workflow yielded consistently robust separation. The spectra collected from 16 different wells in a 96-replicate plate overlapped closely and were essentially indistinguishable (FIGURE 4), with unwanted byproducts present at 1% of the concentration of the full-length oligonucleotide. Equally robust performance can be observed across the full range of oligonucleotide lengths tested, with minimal impurities in the final output despite the absence of an LC separation step. Data from each 96-well plate could be collected in the space of half an hour.

SOFTWARE TOOLS TO ALLEVIATE THE DATA ANALYSIS BOTTLENECK

Agilent also offers software tools to alleviate the data analysis bottleneck. This is normally a multi-step process, in which users extract and deconvolute their spectra, followed by painstaking database search based on the mass data to identify the various impurities

FIGURE 4: RapidFire/MS of Oligos - Reproducibility and Dynamic Range.



and byproducts present in their preparation. With the Agilent Bioconfirm 12 software, users need only input each sample's name, location, and sequence and specify the impurities that the program should be looking for. Bioconfirm software analyzes the sample data and generates annotated spectra in which both the full-length product and impurities are clearly identified and labeled. These data can further be imported into the Bioviewer software, which generates a summary table and color-coded map of the sample purity obtained from every single well on each plate.

From start to finish, the entire process can take as little as three hours for a 384-well plate—1.5 hours for the sample preparation and MS analysis, and then another 1.5 hours to set up and complete the computational analysis. This means that high-quality data can be generated from more than 5,000 sample a day.

CONCLUSION

The examples highlighted throughout this article demonstrate the performance and efficiency gains that can be achieved by incorporating the RapidFire 400 into an MS analytical workflow. However, this is only scraping the surface. With nearly 80 known peer-reviewed publications and many more conference posters and presentations describing studies based on the RapidFire system, new users have access to ample resources for getting started in finding out how to leverage this system's capabilities in their own projects.

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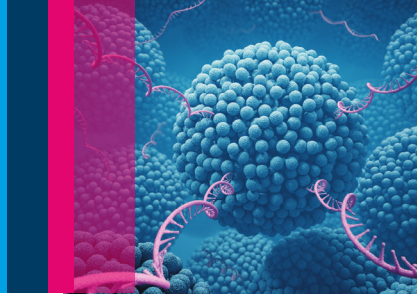
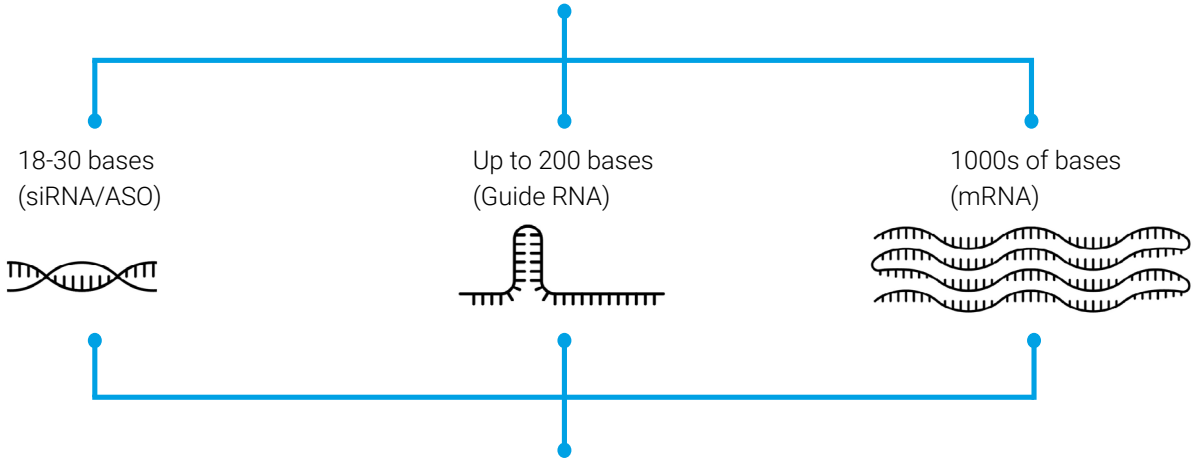
Purification Your Way: siRNA to mRNA



Synthetic Oligonucleotides
DNA/RNA



From analytical to prep scale, Agilent offers scalable column chemistries for oligonucleotide purification including ion pair reversed-phase or anion exchange.

Pore sizes	siRNA/ASO	Guide RNA	mRNA
100 Å	●		
300 Å	●	●	
1000 Å	● ●	● ●	
4000 Å		● ●	● ●

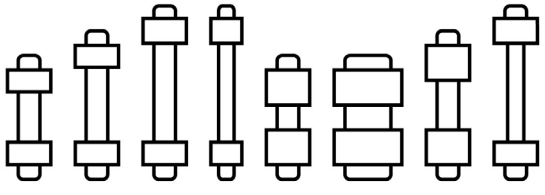
Ion Pair Reversed-Phase with PLRP-S
uses organic solvents and volatile ion-pairing agents, and is suitable for LC/MS. This technique is best performed with high-efficiency separations.

Available in 3, 5, 8, 10, 20 and 30 µm particles

Anion Exchange with PL-SAX
with a strong anion exchange functionality, covalently links to a fully porous chemically stable polymer, extending the operating pH and temperature.

Available in 5, 8, 10 and 30 µm particles

Agilent recognizes the diversity of the oligonucleotide landscape, offering the optimal chemistry and pore size, no matter your molecule size or purification scale.



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