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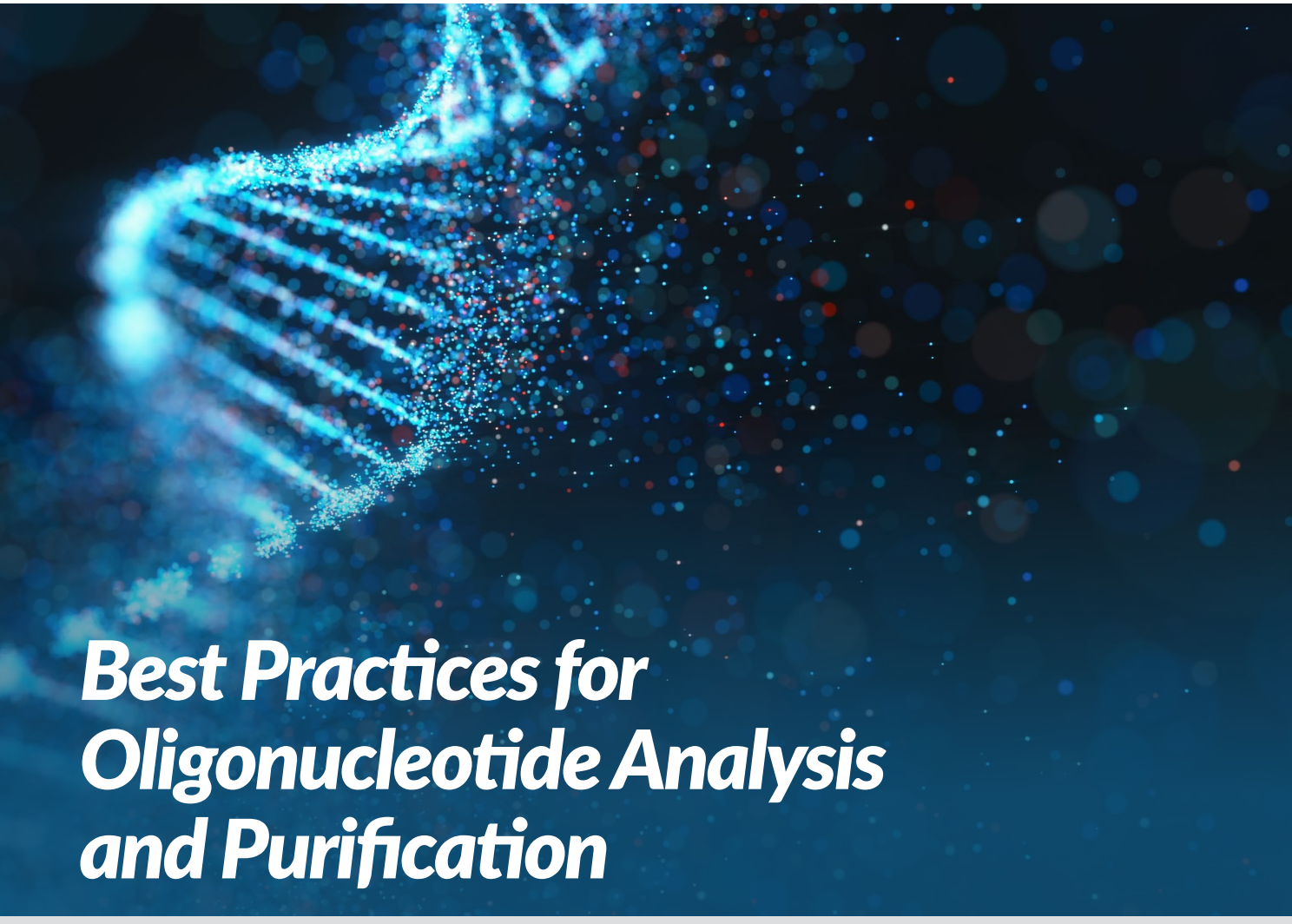

Best Practices for Oligonucleotide Analysis and Purification

A Fireside Chat: What Labs Need to Know About Oligonucleotide Purification

This custom ebook is sponsored by Agilent and presented in partnership with *LCGC's CHROMAcademy*.

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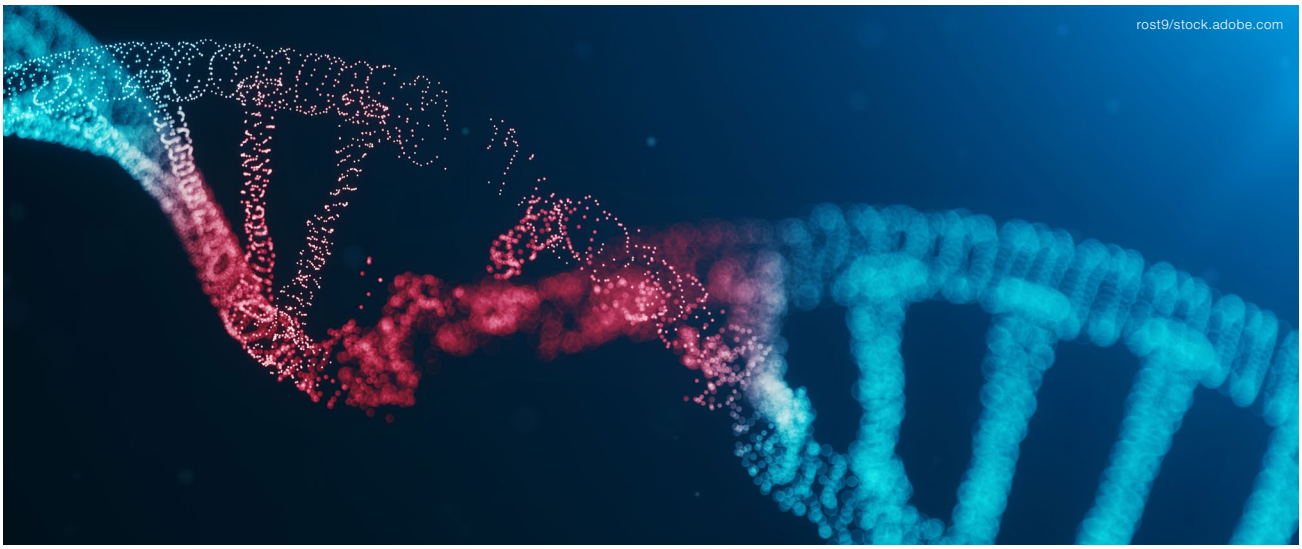

A Fireside Chat: What Labs Need to Know About Oligonucleotide Purification

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

Experts discuss oligonucleotide analysis and purification techniques.

ChromAcademy sat down with folks from Agilent Technologies—Andrew Coffey, Ph.D., senior applications scientist; April Rachamim, global biocolumns product marketing manager; and Matthew Turner, Ph.D., biocolumns product manager—to discuss oligonucleotide analysis and purification techniques, method optimization parameters, and what Agilent can offer that's different from the competition.

CHROMACADEMY: **What are common oligonucleotide purification techniques, and what factors should be examined when selecting an oligonucleotide purification method?**

TURNER: When looking at oligonucleotide purifications, it is important to consider what type of oligonucleotide is being purified across the diverse community of molecules. What lengths of oligonucleotide are you working with? What chromatographic chemistries do you prefer and have the most experience with for purification? Are you typically working with low-pressure instrumentation and

methodologies and prefer ion exchange (IEX), or are you looking for high-performance liquid chromatography (HPLC) solutions and have familiarity with reversed phase or ion-pair reversed phase?

COFFEY: For oligonucleotides purification, ultimately, you need to know where you're going to end up or what scale you're going to operate at. 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. However, if you have a very large quantity of oligonucleotides, then it's likely you will have to buy a stationary phase and load your column for process-scale purification. Preparative LC columns that are already packed include both IEX or ion-pair reversed phase.

Another factor to consider is operating temperature: Oligonucleotides generally require purification at elevated temperatures. You want to denature the oligonucleotides and make the peaks as sharp as they can be to better separate the impurities. The type of impurity is another factor: During the process of synthesizing oligonucleotides, you end up with deletion sequences, so you might have your full-length product but with impurities that are n - 1, n - 2, n - 3, etc., as well as other contaminants. So, the bigger the oligonucleotide, the more challenging it becomes.

RACHAMIM: In terms of the Agilent solution, many of the stationary phases we offer for this type of analysis come in prepacked columns up to 100 mm x 300 mm. So, you can go up to a large scale before you need to pack your columns.

CHROMACADEMY: **Oligonucleotides encompass a range of molecules, such as sRNA, ASO, gRNA, and mRNA. Given the variability in size, what method optimization parameters should be considered and evaluated for oligonucleotide analysis and purification?**

COFFEY: The important thing to appreciate is if you have a large molecule like an oligonucleotide, it has to fit into the pores of the stationary phase, so choosing the right pore size is one of the most important factors. There are two things to bear in mind. First is the larger the pore, the easier it is for the oligonucleotide to get in and out of the particle and the sharper the peaks. Second, there's a downside, and that is the larger the pore size, the lower the surface area, and potentially the lower the capacity of the particles, so finding the right combination is important. We recommend customers who are purifying oligonucleotides buy analytical-sized columns with different stationary phases—particle size, pore size, etc.—and then they can validate the correct stationary phase as part of their method optimization.

Once you have the right stationary phase, the second thing to keep in mind is there are a number of parameters to consider in terms of method development: mobile-phase optimization, temperature, the gradient profile, etc. Oftentimes, getting the separation is challenging—closely eluting

impurities requires a shallow gradient, so you're spending time looking at flow rates and gradient times. Gradient profiles are as important as choosing the right mobile-phase composition.

CHROMACADEMY: **What key quality attributes are of importance with oligonucleotide therapeutics, and what tools are effective in their analysis?**

TURNER: With any type of therapeutic, there are key aspects of the molecule itself you want to verify, i.e., ensure that the final product is the therapeutic entity of interest with minimal and identifiable impurities. Just as with other therapeutics, oligonucleotides have critical quality attributes (CQAs) such as verifying the final sequence that's been created from synthesis. In many cases, this is done with liquid chromatography-mass spectrometry (LC-MS) methodologies, as Andy pointed out, looking at the impurity profiles in the crude synthesis material, e.g., n – 1 failure sequences, loss of bases, or incomplete phosphorothioate backbone substitutions. These are also impurities created as a result of incomplete synthesis that must be characterized and effectively purified with your validated method.

Agilent has a range of column chemistries, including ion-pair reversed phase or anion exchange being the most commonly used, while some are also looking at other methodologies such as size-exclusion chromatography (SEC) for analyzing single-stranded versus double-stranded impurities while others are considering hydrophilic interaction chromatography (HILIC) chemistries as an alternative for MS-compatible characterization.

COFFEY: If there are residual impurities, you have to identify them and work out how to minimize them. The objective is to make the target molecule as pure as possible. As Matt said, 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. You may also have to use a long, slow gradient to get the best resolution and the highest confidence in your data. But CQAs for mRNA are slightly different from others.

TURNER: Yes, because mRNA's manufacturing process typically uses an in-vitro, cell-free production of significantly larger molecules compared to the chemical synthesis of smaller oligonucleotides; the strategies for cleanup and purification must be adapted to the 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. Contaminating DNA, whether it be from a plasmid or cDNA template, need to be removed through a DNase treatment, leaving

Agilent PLRP-S						
	5 µm	8/10 µm	10-15 µm	15-20 µm	30 µm	50 µm
100Å	✓	✓	✓	✓		✓
300Å	✓	✓	✓	✓		✓
1000Å	✓	✓			✓	✓
4000Å	✓	✓			✓	
Agilent PL-SAX						
	5 µm	8/10 µm	10-15 µm	15-20 µm	30 µm	50 µm
1000Å	✓	✓			✓	
4000Å	✓	✓			✓	

small DNA fragments that are subsequently separated through cleanup or purification.

CHROMACADEMY: **What factors should be considered to optimize for success in the purification of oligonucleotides when moving from gram to kilogram scale?**

COFFEY: When you're talking about purifying large quantities of oligonucleotides, that can mean using a prepacked column that you bought off the shelf—Agilent has prepacked columns that are 100 mm ID, but to go beyond that, you need to pack your own column. And so, in some instances, if you buy the stationary phase from a vendor, the actual grade of the material will be different from the stationary phase in a prepacked column, which is not ideal. 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. Ensuring you have the same quality stationary phase throughout the process.

The facility at Agilent, where we manufacture a lot of these stationary phases, can produce on a very large scale. That material can then be packed into smaller columns to do the initial development. Let's not forget when you're going through this process, there are several stages in scaling up. You don't go straight from gram to multikilogram scale; you'll go through various phases. It's equally important to know when you use a stationary phase in a column that you can use it again at a later date for another run and without contamination—making sure you can effectively sanitize the material and store it safely without risking contamination for subsequent processes is very important. Overall, there are a lot of things to consider, but the availability of the stationary phase in the scale that you need is among them.

RACHAMIM: The necessity of having the same stationary phase available in larger particle sizes is critical. As you go up to a larger scale of purification, you need a particle that scales proportionately.

TURNER: As Andy pointed out, when you scale up you don't want to have to change the manufacturing chemistry and methods if possible. Agilent has IEX and ion-pair reversed-phase chemistries that are 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. Along with particle- and pore-size availability, the polymeric chemistry of PLRP-S and PL-SAX, and their inherent stability, affords you high pH and temperature compatibility commonly used for the purification of oligonucleotides.

CHROMACADEMY: There are many competitors in this space. What makes the Agilent offering unique, and why should customers choose your products?

RACHAMIM: As the nucleic acid market grows, it's worth noting that the Agilent pore size is relatively unique in the marketspace. Historically, there have been a lot of challenges—working with molecules this size and the fact we can go up to a 4,000 Å pore is helpful if customers are looking to do LC purification for these molecules. Matt mentioned the temperature and pH stability of the polymeric media is unrivaled when you compare it to silica-based media, so you start with the biggest available diversity of method parameters.

TURNER: 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.

For more information on this important topic, register for our webcast "Tips to Optimize Lab Scale Oligonucleotide Purification."

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