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Application Note

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

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This is an Application Brief and does not contain a detailed Experimental section.

Abstract

Waters presents some critical column and chemistry best practices for performing successful oligonucleotide analysis by ion-pair reversed-phase (IP-RP) liquid chromatography. Ensuring high quality oligonucleotides, either in diagnostic applications or as therapeutic entities, relies on robust analytical methods. For example, in response to the global COVID-19 pandemic, PCR-based diagnostic kits have been developed to detect SARS-CoV-2 genetic code in novel coronavirus patients. Accurate viral detection via PCR requires high-quality oligonucleotide primers and probes. Additionally, oligonucleotides are being investigated as therapeutics (including mRNA-based vaccines) for the treatment and prevention of COVID-19.

Benefits

- Waters BEH-based C_{18} particles enable high pH and high temperature oligonucleotide separations in LC or LC-MS compatible buffers
- Batches are QC tested with MassPREP Oligo Standard to ensure consistency
- Different particle sizes and column dimensions for preparative or analytical applications
- Three step method development of <5-minute IP-RP oligonucleotide separations

Introduction

In this application brief, Waters provides a short list of best practices to characterize oligonucleotides by ion-pair reversed-phase (IP-RP) liquid chromatography. These best practices cover separation temperature, pH optimization, pore size selection, mobile phase choice, rapid method development considerations, purification guidelines, and popular buffer recipes. Following these best practices for oligonucleotide analysis helps ensure robust methods, enabling the delivery of consistently high quality oligonucleotides for therapeutic or diagnostic applications.

The COVID-19 pandemic serves as an example of the importance of oligonucleotides in diagnostic applications. Timely diagnosis of SARS-CoV-2 viral infection remains paramount to successfully managing the novel coronavirus pandemic. As a result, many firms have developed in vitro diagnostic tests to detect the presence of SARS-CoV-2 genetic material. Of the emergency use authorizations (EUA) for in vitro diagnostic tests in the United States, most of them are PCR-based (e.g. qPCR, RT-PCR) tests which detect SARS-CoV-2 viral genetic code.[1] Diagnostic kits indicated for the molecular detection of viral genetic code rely on oligonucleotides as primers and probes during PCR amplification and detection. As a result, analytically assessing the quality and ensuring appropriate purity is of principle importance for a successful diagnostic test.

Results and Discussion

Waters created a short list of best practices that have emerged as a result of reviewing method and product development resources for IP-RP oligonucleotide separations. We discuss 6 key practices below.

1) Perform oligonucleotide separations at elevated pH and temperature for best results.

i. Elevated temperature prevents the oligonucleotide secondary structure from impacting retention (60 °C). Elevated temperature ensures that DNA/RNA secondary structure does not affect the separation. For CG rich or G-rich oligonucleotides with high degree of secondary structure, it may be necessary to increase column temperature to 80 or 90 °C.

ii. High pH buffers (pH ≥ 7) are commonly used in oligonucleotide separations (e.g. TEAA).

iii. TEA-HFIP was found to be a robust mobile phase offering superior LC-MS sensitivity and resolution across various sized single stranded oligonucleotides.[2]

iv. The high pH used for IP-RP oligonucleotide separations renders most common silica-based stationary phases unsuitable.

v. Waters BEH sorbent technology lends itself well for oligonucleotide separation due to its high pH stability and temperature tolerance.

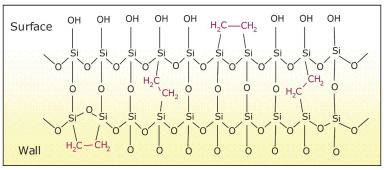


Figure 1. Schematic structure of BEH sorbent. Hydrolytic stability is achieved by bridging ethyl groups. For oligonucleotide analysis, the surface of sorbent is alkylated by C_{18} functional groups.

For more detailed information, see Waters Application Note

HPLC and UPLC Columns for the Analysis of Oligonucleotides. 720002376EN.

2) Choose a relevant pore size for your oligonucleotide separation. Choosing the correct pore size

enables appropriate analyte diffusivity resulting in the best interaction between the oligonucleotide and the ligand. Improved ligand interactions improve peak shape.

i. 130 Å pore size is ideally suited for single stranded oligonucleotides (2-100 mers).

ii. 300 Å pore size allows for efficient separation of both single stranded oligonucleotides and longer dsDNA fragments.

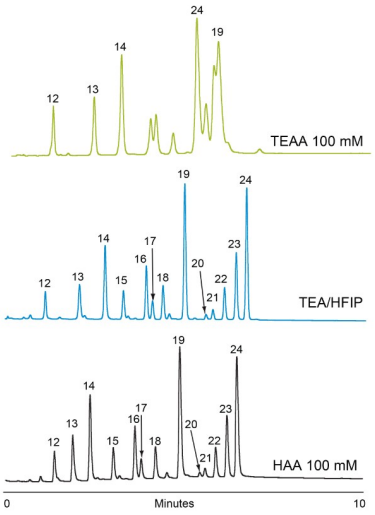
3) Choose an appropriate mobile phase. See section 6 for buffer recipes.

i. Triethylamine/hexafluoroisopropanol (TEA/HFIP) is MS compatible and has impressive resolving power. Higher TEA/HFIP buffer concentrations improve separation performance. Lower concentrations improve MS sensitivity.

ii. Hexylammonium acetate (HAA) also offers exceptional resolution and MS compatibility, however, the MS compatibility of HAA is less than that of TEA/HFIP. Use of HAA may result in better separation of labeled oligonucleotides and longer oligonucleotides (>35-mer) compared to TEA/HFIP. This may be relevant if performing only LC analysis.

iii. Fresh TEA/HFIP and HAA/HFIP mobile phases are critical to good separations. These semi-volatile mobile phases can gradually lose their separation strength and MS spectra become contaminated with alkali ion adducts. For robust day-to-day results, make mobile phases daily or in limited quantities. Upper limit of mobile phase usability is one week.

iv. Both TEA and HFIP should be prepared in a fume hood, use Waters APC solvent bottle caps to prevent gassing out, and if possible, use a snorkel above the system.



LC system:	ACQUITY UPLC with ACQUITY UPLC PDA
Sample:	5'-CCC CTT GGT TAA CCA AGG TTC CAA-3'
Column:	ACQUITY UPLC OST C_{18}, 2.1 × 50 mm, 1.7 µm
Column temp.:	60 °C
Flow rate:	0.2 mL/min
Detection:	260 nm, 20 points/second
Mobile phases:	
Top trace:	A: 100 mM TEAA, pH 7.0 B: 20% ACN in A Gradient: 45–55% B in 10 min
Middle trace:	A: 15 mM TAE, 400 mM HFIP, pH 7.9 B: 50% MeOH in A Gradient: 30–47% B in 10 min
Bottom trace:	A: 100 mM HAA, pH 7.0 B: 50% ACN in A Gradient: 56–78% B in 10 min

Figure 2. Separation of heteromeric oligonucleotides. The type of ion-pairing mobile phase influences the selectivity of separation. With "weak" IP systems such as TEAA both hydrophobic and ion-pairing interactions participate in separation. With "strong" ion-pairing systems (TEA/HFIP, HAA) the oligonucleotide separation is mostly driven by their charge/length.

For more detailed information, see the below Waters Application Notes

- UPLC/MS Separation of Oligonucleotides in Less than Five Minutes: Method Development. 720002387EN.
- Evaluation of Alternative Ion-pairing Reagents in the Analysis of Oligonucleotides with the ACQUITY QDa Detector. 720005830EN.

- Hexylammonium Acetate as an Ion-Pairing Agent for IP-RP LC Analysis of Oligonucleotides. 720003361EN.

4) Quick three step method development for <5-minute IP-RP oligonucleotide separations.

i. Identify suitable initial gradient strength or start with a scouting gradient. With 15 mM TEA/400 mM HFIP ion pairing system, an example scouting gradient may be:

- Start at 20% MeOH, perform at 1%/min MeOH gradient with 0.2 mL/min flow rate (0.2 mL/min is useful to enhance column efficiency for macromolecules).

ii. Adjust gradient slope to achieve desired separation (shallower gradients increase resolution, but increase time needed for analysis). Adjust the starting percentage of MeOH to reduce the time of analysis as needed. Extend the MeOH gradient time as needed until the target oligonucleotide and impurities are eluted. High organic flush is then inserted to elute highly retained components (often non-oligonucleotide components) and minimize carryover. Target oligonucleotides should elute during the gradient and not in the high organic flush.

iii. If speed is important, speed up the separation by increasing the flow rate while proportionally reducing gradient time (constant gradient column volume). Selectivity of separation should not change while minimal loss in resolution can be observed. When driving towards sensitivity or a need for optimal resolution, use lower flow rates.

- 0.4 mL/min is a good compromise between speed and analytical performance. For high-throughput separations a 0.8 mL/min flow rate is recommended (1-3 min separation time, 2.1 mm column I.D).
- For more detailed method development considerations for XBridge OST Columns (e.g. starting gradient slopes, buffer formulations, and column considerations) see Waters 715001476.

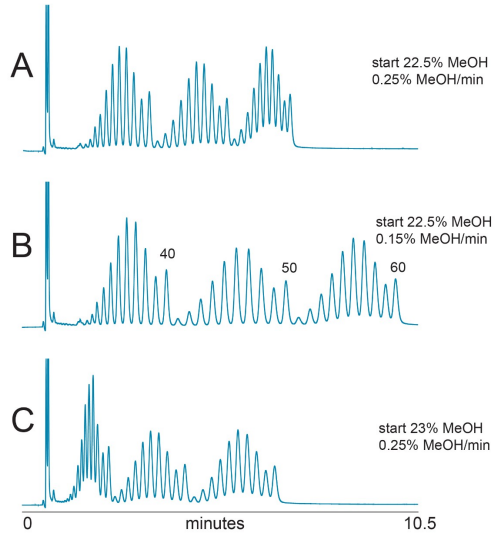


Figure 3. Separation of 30 to 60 nt oligodeoxythymidines using 2.1 x 50 mm, 1.7 µm ACQUITY UPLC OST C_{18} Column.

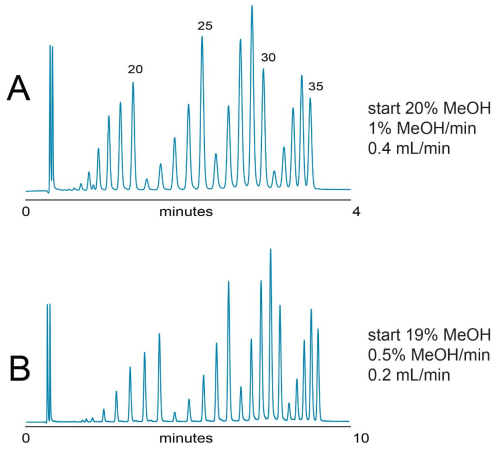


Figure 4. Separation of 15 to 35 nt oligodeoxythymidines.

For more detailed information, see te below Waters Application Notes

- UPLC/MS Separation of Oligonucleotides in Less than Five Minutes: Method Development. 720002387EN.
- UPLC Separation of Oligonucleotides: Method Development. 720002383EN.
- XBridge OST C_{18} Method Guidelines. 715001476.

5) Guidelines for oligonucleotide purification using XBridge Oligonucleotide BEH C_{18} Columns.

i. XBridge Oligonucleotide BEH C_{18}, 130 Å Columns are the preferred offering for detritylated oligonucleotide purifications due to the availability of column sizes designed to meet lab-scale isolation requirements.

ii. The choice of XBridge Oligonucleotide C_{18} Column dimension and operating flow rate depends primarily on the scale of the synthesis reaction mixture.

- For example, a 4.6 × 50 mm column containing XBridge Oligonucleotide BEH C_{18}, 130 Å, 2.5 µm material is an excellent selection when oligonucleotide mass loads are less than or equal to 0.2 µmol.
- Selection of the appropriate column size for oligonucleotide sample load is recommended to maximize component resolution and recovery of the target product from non-desired failure sequences. See Table 1 for mass loading guidelines.

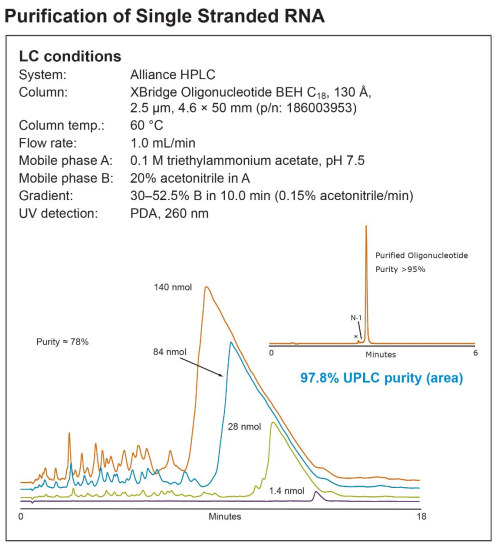


Figure 5. HPLC purification of a synthetic 21-mer oligonucleotide. Sample concentration was 2.8 nmol/µL, with on-column loading ranging from 1.4 to 140 nmol.

Column (mm)	Approx. mass load (µmoles)*	Flow rate (mL/min)
2.1 x 50	0.04	0.2
4.6 x 50	0.20	1.0
10.0 x 50	1.00	4.5
19.0 x 50	4.00	16.0
30.0 x 50	9.00	40.0
50.0 x 50	25.00	110.0

**Values are only approximates and vary depending on oligonucleotide length, base composition, and "heart-cutting" fraction collection method used.*

Table 1. XBridge OST C_{18} Column selection guide for detritylated oligonucleotide purification.

6) How to make select IP-RP buffers (1 liter)

i. Perform all work in a fume hood.

ii. Filter all mobile phases through a solvent compatible, 0.45 µm membrane filter and store in bottles that are clean and particulate free.

- These are all recipes for *mobile phase A*. Mobile phase B is generally a mixture of organic (e.g. ACN, MeOH) mixed with mobile phase A at a suitable percentage (e.g. 20-50%). The higher concentration buffer (15 mM TEA/400 mM HFIP) can be used for separations involving G-rich oligonucleotides. The lower strength buffer (8.6 mM TEA/100 mM HFIP) is often enough for routine detriylated oligonucleotide LC-MS applications.

Step	0.1 M TEAA	8.6 mM TEA/ 100 mM HFIP	15 mM TEA/ 400 mM HFIP	25 mM HAA
1	Add 5.6 mL of glacial acetic acid into 950 mL of water and mix well	Add 16.8 g (10.4 mL) of HFIP into 988.4 g of water and mix well	Add 67.17 g (41.56 mL) HFIP into 956.36 g of water and mix well	Add 995 g of water into 1 L flask
2	Slowly add 13.86 mL of TEA	Slowly add 1.2 mL of TEA	Slowly add 1.52 g (2.08 mL) of TEA	Add 1.43 mL of 99% acetic acid
3	Adjust pH to 7 ± 0.5 by careful addition of acetic acid	pH = 8.3 ± 0.1	pH = 7.9 ± 0.1	Add 3.283 mL of hexylamine (pH 7.0–7.5 depending on accuracy (add ~40 µL of hexylamine to adjust pH to 8.5
4	Adjust final volume to 1 L with water			Adjust final volume to 1 L with water

Conclusion

In support of customers working on oligonucleotides, Waters offers these critical best practices to ensure consistent high-performance IP-RP liquid chromatographic separations for oligonucleotide analysis. Robust methods are critical to delivering high-quality oligonucleotides in diagnostic or therapeutic applications. For example, high-quality oligonucleotide primers and probes are essential for accurate PCR-based diagnostic assays, including those for COVID-19.

References

1. In Vitro Diagnostic EUAs - Test Kit Manufacturers and Commercial Laboratories Table [Internet]. Washington (DC). FDA. [cited 2020 Jun 1]. Available from: https://www.fda.gov/medical-devices/emergency-situations-medical-devices/emergency-use-authorizations#covid19ivd

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