[ CONSUMABLES GUIDE BOOK ]

# Purifying Oligonucleotides

## High Efficiency Preparative Chromatography to Improve Yields and Turnaround Time

Waters™

# Understanding Oligonucleotide Purification

## Oligonucleotide Structures and Modifications

Synthetic oligonucleotides are short nucleic acid sequences. They are chemically synthesized and typically 20–30 residues long but can sometimes be up to 100 nucleotides in length. They can be used both therapeutically and diagnostically. For accurate base pairing and recognition, it is critical that they are made with the correct sequence and free of impurities.

These so-called "oligos" are composed of deoxyribose or ribose sugars connected to nucleobases, such as adenine (A), thymine (T), cytosine (C) and guanine (G) or uracil (U). Many therapeutic oligos and oligo reagents are modified. These modifications are fundamental for improvements in nuclease stability, efficacy of delivery and detection sensitivity. Among several chemical modifications and structural adjustments to oligonucleotides, it is Phosphorothioate (PS) backbone modification, Locked Nucleic Acids (LNA), 2´-O-methyl and 2´-fluoro, capping, PEGylation etc. that are common.

## Need for Purification

### Identification

- Rapid identification of synthetic modified oligos
- Chemical modifications like PEGylation, conjugation to other chemical moieties etc.

### Purification

- Synthesis- and process-related impurities
- Challenging base modifications

### Purity Requirements

- Purity range for synthetic oligonucleotides depends on the goal of purification
- In vivo screening (>95%)
- In vitro screening (≥ 85%)

## Essential Strategies

**Reversed Phase Chromatography (RP)** is effective for separating oligonucleotides with different length and sequences, and conjugates such as GalNAc & lipid conjugated moieties. **Ion-pairing (IP-RP)** is the go-to choice for high resolution separations of modified and unmodified oligos especially in complex mixtures.

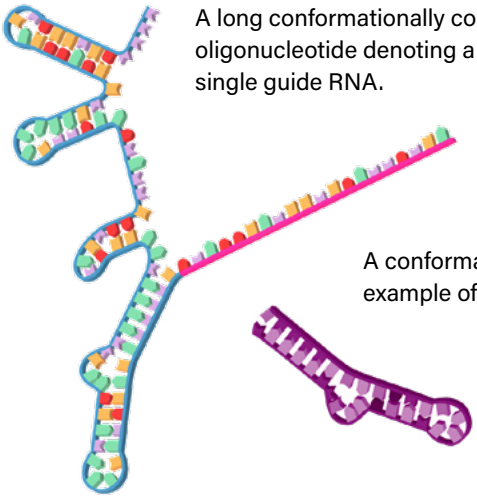
**Anion-Exchange Chromatography (AEX)** is useful for separation of oligonucleotides based on their charge differences, such as single-stranded vs. double-stranded DNA. Oligonucleotides with high GC content are strong candidates for purification using AEX chromatography.

**Size Exclusion chromatography (SEC)** can also be used as a desalting step after IP-RP. It can also be used to size select different lengths and conformers. Impurities generated during synthesis of larger Oligos, usually are shorter than the target oligo thus can be separated by size exclusion chromatography.

## Oligonucleotide Structures

A single stranded oligonucleotide, like an antisense oligonucleotide (ASO), PCR primer, or fluorescent probe

Double stranded oligonucleotide representative of a small interfering RNA (siRNA)



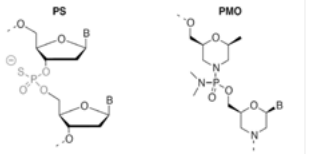
A long conformationally complex oligonucleotide denoting a CRISPR single guide RNA.

A conformationally example of an aptamer

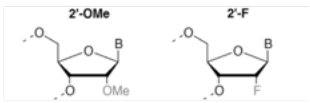
## Oligonucleotide Modifications

The following provides select modifications as reported in Pharmaceutics. 2022 Feb; 14(2): 260. CC-BY.

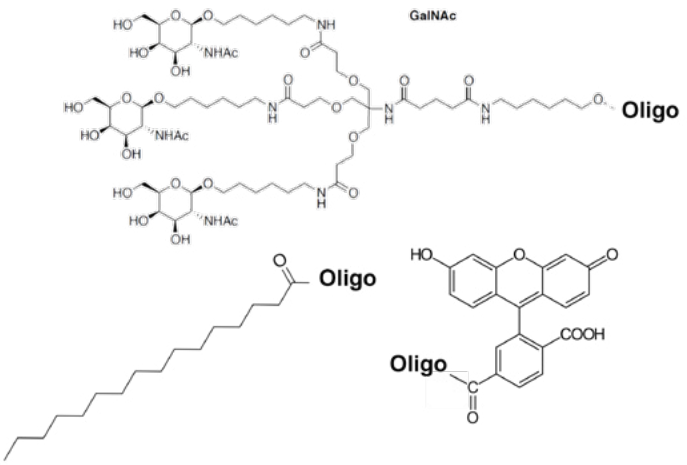
Backbone Modifications




Sugar Modifications



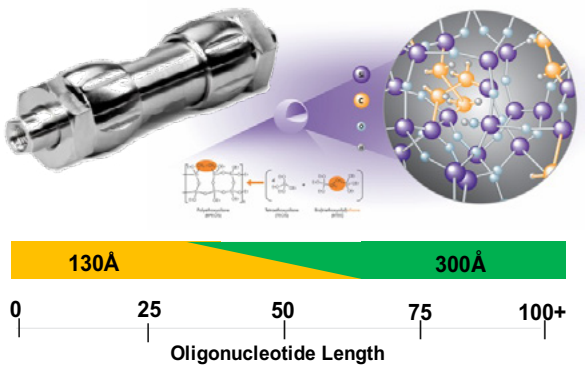

Conjugates

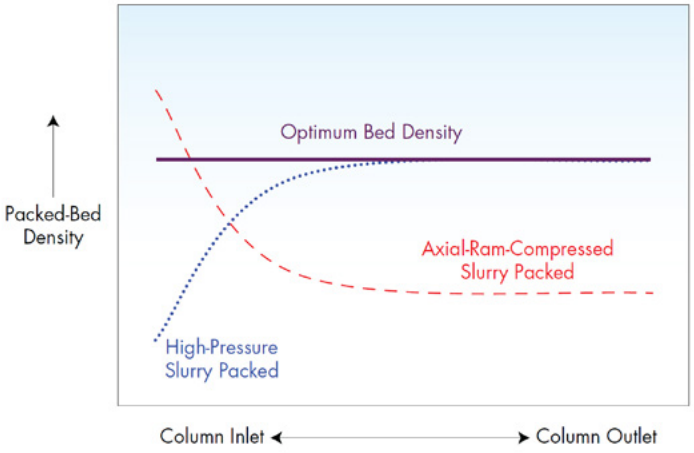


# Tools and Strategies for Successful Purification

## Successfully Applying IP-RP Purifications

High resolution purification of oligos from their process and product related impurities is most easily established using ion pair reversed phase LC. Since oligos are hydrophilic, they will not inherently retain to the hydrophobic alkyl chains or polymeric sorbents used as stationary phases for reversed phase columns. Consequently, retention is achieved through the use of an ion pairing reagent, typically an alkyl amine. The protonated amine will ion pair to the negatively charged backbone of the oligonucleotide. Preparative flow HPLC with flow rate capabilities up to 150 mL/min are used to readily apply this form of chromatography to columns with inner diameters ranging from 10 to 100 mm wide. Mass directed fraction collection can also be applied with specialized AutoPurification Systems.




*Effect of column packing procedures on bed density, as reported in Waters Application Note 720001939.*


*The LC Prep AutoPurification System provides the flexibility of high-throughput parallel runs for selective mass-directed fraction collection from hundreds of samples.*

## Stationary Phase Considerations

Waters batch certified Bridged Ethyl Hybrid (BEH™) Technology for oligo separations ensures extra performance, reproducibility and scale-up for purification. High temperature and high pH are prevalent strategies for minimizing secondary interactions in oligonucleotide separation and purification. BEH Particle Technology withstands harsh conditions such as high pH, temp and additives that usually are required for Oligonucleotide work.

Oligonucleotides and nucleic acids vary in size and structure, ranging from just a few bases to thousands. Selecting the appropriate pore size is essential for efficient mass transfer of the oligonucleotide into the pore structure. The chart to the left helps visualize the selection of pore size for oligonucleotides with different lengths.

## Optimal Bed Density (OBD) Packing

For prep LC columns with smaller aspect ratio (length/diameter), high pressure slurry packing with small particles often fails to achieve the ideal bed density in well designed analytical columns. Excessive axial compression at the inlet can cause particle breakage, channeling and reduced local bed permeability. Carefully designed OBD™ Columns deliver improvements in column lifetime, efficiency, peak shape, and low back pressure.

## Challenges to Address

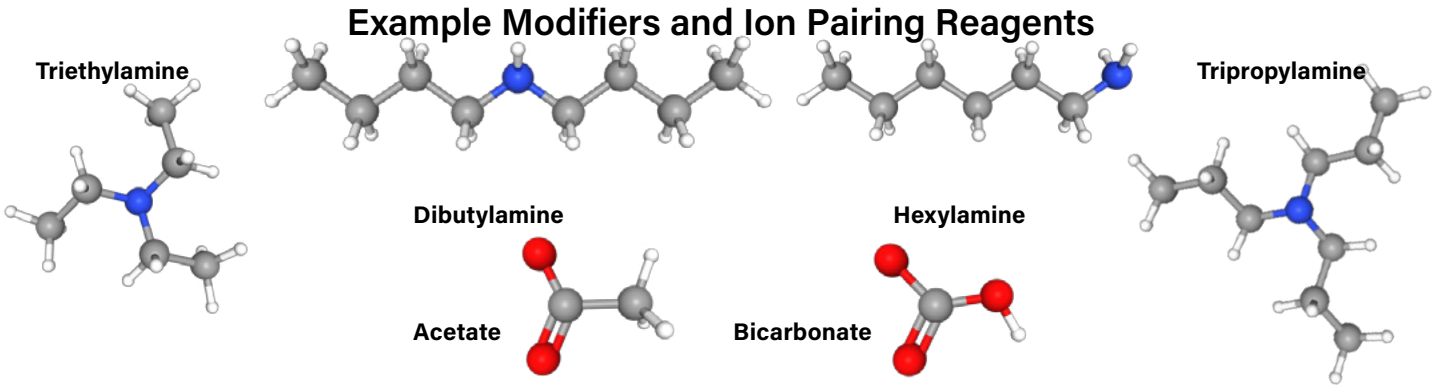
- Loading capacity
- Low resolution
- Unspecific binding
- Solubility
- Low purity
- Irreproducibility batch to batch and column to column
- Long elution time
- Need for a different selectivities
- Struggles with column secondary interactions and passivation
- Few starting point methods for newly emerging conjugates and modifications

# Method Considerations

## Mobile Phase Options

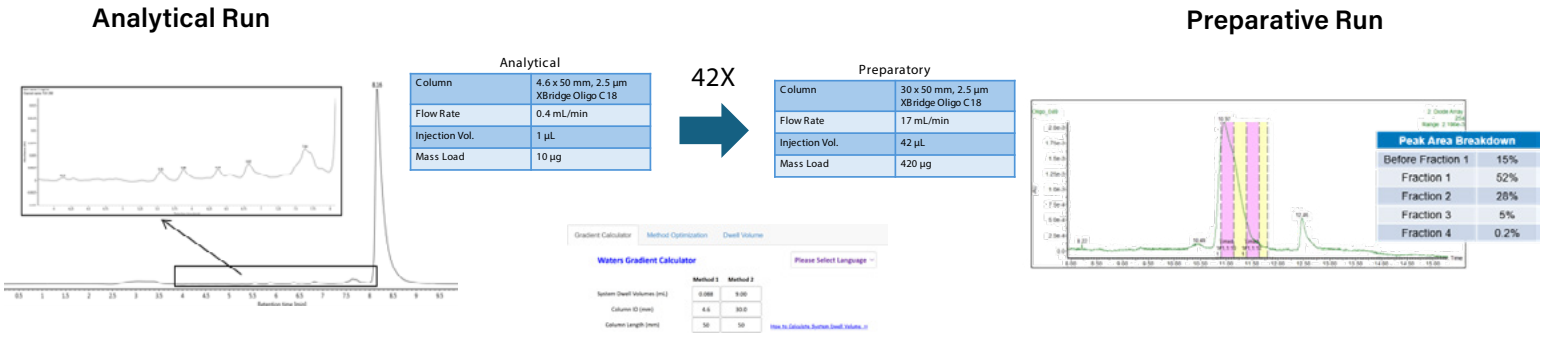
Ion-pairing reagents in the mobile phase increases the hydrophobicity of charged components. The reagent interacts with the charged groups in the oligonucleotide, reducing their impact on overall hydrophobicity. The type and concentration of the ion-pairing reagent influence retention mechanism and selectivity. Acetonitrile and methanol are most frequently applied eluents, though ethanol could also be used in some situations.

Depending on the type of oligonucleotides a range of alkylamines with different hydrophobicity can be used. To selectively separate oligos based on their length, more hydrophobic alkylamines such as TPA (tripropylamine), HA (hexylamine) or DBA (dibutylamine) can be considered. TEAA (triethylammonium acetate) or HAA (hexylammonium acetate) are volatile and can be removed from a collected fraction by evaporation or lyophilization. Triethylammonium bicarbonate presents yet another interesting volatile mobile phase option.




## Scalability

Scalability while sustaining the chromatography profile is crucial for separation of critical impurities from a target compound. During oligonucleotide synthesis, deletion sequences often occur, resulting in the presence of full-length products alongside impurities, such as n-1,




HPLC w/ TEAA modifier
XBridge Oligonucleotide
BEH 130Å $C_{18}$ 2.5 µm 4.6 x 50 mm Column
Flow rate: 0.8 ml/min
Column temp: Ambient

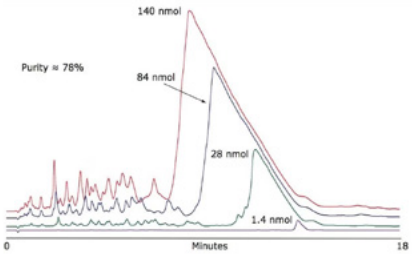
Mass Load: 420 µg
XBridge Oligonucleotide BEH $C_{18}$
130Å 2.5 µm OBD Prep 30 x 50 mm Column

Time-Based Fractionation
Productivity = ~1mg of purified material / hour

## Loading Capacity

There are several important parameters to consider when it comes to optimizing your loading capacity. Column efficiency, pore size and particle size play significant roles. Larger pore size columns allow for better mass transfer and sharper peak shapes of larger analytes but having reduced surface area means they can exhibit lower loading capacity. Most importantly, a wide range of column IDs are available to optimize your purification runs.

| ID (mm) | Loading (mg) |
|---|---|
| 4.6 | ≤4 |
| 10 | ≥5–15 |
| 19 | ≥15–<200 |
| 30 | ≥200–<350 |
| 50 | ≥350–<600 |




*Mass loading studies performed on a 4.6 mm ID column, as reported in Waters Application Note 720002602EN.*

## System Considerations

Your choice of preparative LC system depends on the goal, and quantity of sample to be purified. Options include a semi-prep, lab scale prep, pilot or process scale instrument. Waters provides semi-prep and lab-scale preparative LC systems that allow auto purification configurations and ensure analytical scale up to prep scale chromatography with optimized fluidic designs, minimized dispersion and low carryover.

**Up to 5 mL/min**

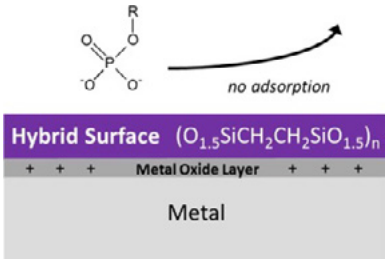
## Detector Directed Fractionation and Impurity Analysis

Mass spectrometry (MS) directed fraction collection in preparative runs, enables maximization of targeted compound recovery. Diode Array/ultraviolet-visible directed fraction collection can also be applied, albeit for less selective triggering. A streamlined and compatible fraction collection strategy improves overall lab productivity.

**Up to 10 mL/min**

## Column Hardware and MaxPeak Premier High Performance Surfaces

Oligonucleotides phosphodiester groups can adsorb to the metals found in column bodies and frits. Using low adsorption column hardware can improve peak shape by minimizing chelation, leading to more reliable retention and more consistent chromatographic parameters. XBridge Premier Oligonucleotide BEH $C_{18}$ OBD Prep columns are manufactured with MaxPeak™ HPS vapor deposited components to enhance peak shape and improve recovery.

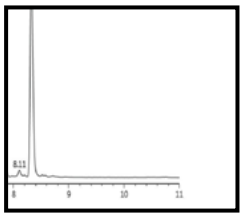



## Cost-effective purification

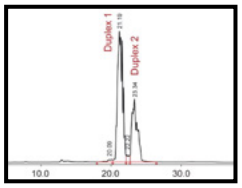
Most older generation prep columns are made with particles ranging from 7 to 50 µm in diameter primarily due to limitations in column technology as well and instrument performance at the time. Prep columns with smaller particle size and optimized packing technology enhance the separation of impurities from unmodified, modified and conjugated oligonucleotides, providing higher reproducibility.

XBridge Oligonucleotide BEH $C_{18}$ 5 µm Prep Columns combined with OBD Technology offer extended column lifetimes and reduced the need for multiple purification passes.

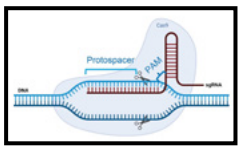
# Application Notes



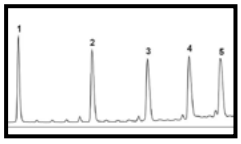
Increasing the Productivity of Oligonucleotide Purification through Column Scaling and Method Optimization



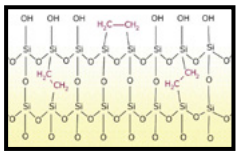

Analysis of siRNA Duplexes at Non-Denaturing UPLC Conditions Using MaxPeak Premier Column Technology



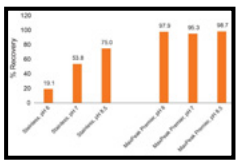

CRISPR Single Guide RNA Characterization by IP- RP-LC-MS with a Premier Oligonucleotide BEH 300 Å $C_{18}$ Column



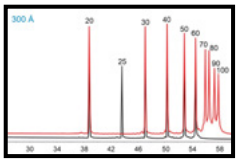

Improved Peak Recovery and Peak Shape of Oligonucleotides Using Waters Premier Columns



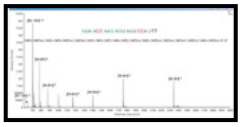
Best Practices for Oligonucleotide Analysis Using Ion-Pair Reversed-Phase (IP-RP) Liquid Chromatography – Columns and Chemistries



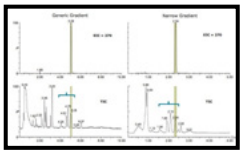
Improved Chromatographic Analysis of Oligonucleotides with ACQUITY Premier Oligonucleotide BEH $C_{18}$ Columns



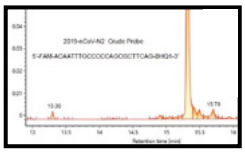

Enhanced Resolution for Longer Oligonucleotide Analytes With a MaxPeak™ Premier Oligonucleotide BEH $C_{18}$ 300 Å



Analysis of Oligonucleotide Impurities on the BioAccord System with ACQUITY Premier

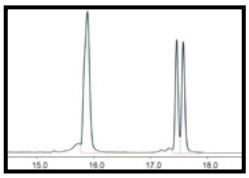


Evaluating the Tools for Improving Purification Throughput



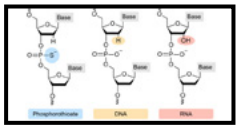
Mass Confirmation and Purity Analysis of PCR Primer and Probe Reagents Using High Sensitivity Ion-pairing LC-MS


Optimum Bed Density [OBD™] columns: Enabling Technology for Laboratory-Scale



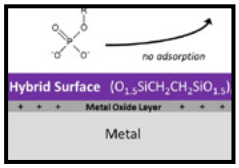
Analysis of siRNA Drugs at Denaturing UPLC Conditions Using MaxPeak Premier Column Technology

# Waters Journal Articles and Other Key Resources



The impact of low adsorption surfaces for the analysis of DNA and RNA oligonucleotides.

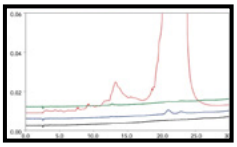
J Chrom A 2022, 1677, 463324




Using Hybrid Organic–Inorganic Surface Technology to Mitigate Analyte Interactions with Metal Surfaces in UHPLC

A. Chem. 2021, 93, 14, 5773–5781

www.waters.com/prepcalculator



Analysis of siRNA with Denaturing and Non-Denaturing Ion-Pair Reversed-Phase Liquid Chromatography Methods

LCGC North America, 2023, 41, 2, 60–66

# Ordering Information

## The columns, standards, and reagents that can help you purify your oligonucleotides.

### XBridge™ Oligonucleotide BEH $C_{18}$ OBD Prep 5 µm Columns

| Diameter | | C. Guard | 50 mm | 100 mm | 150 mm | C. Guard | 50 mm | 100 mm | 150 mm |
|---|---|---|---|---|---|---|---|---|---|
| | Pore Size | 130 Å | | | | 300 Å | | | |
| Standard Column | 10 mm | 186011150[1] | 186011151 | 186011152 | 186011153 | 186011168[1] | 186011169 | 186011170 | 186011171 |
| | 19 mm | 186011154[2] | 186011155 | 186011156 | 186011157 | 186011172[2] | 186011173 | 186011174 | 186011175 |
| | 30 mm | 186011158[3] | 186011159 | 186011160 | 186011161 | 186011176[3] | 186011177 | 186011178 | 186011179 |
| | 50 mm | – | 186011162 | 186011163 | 186011164 | – | 186011180 | 186011181 | 186011182 |

*[1] Requires Prep Guard Holder P/N 289000779*
*[2] Requires Prep Guard Holder P/N 186008745*
*[3] Requires Prep Guard Holder P/N 186006912*

### XBridge Premier Oligonucleotide BEH $C_{18}$ OBD Prep 5 µm Columns

| Diameter | | C. Guard | 50 mm | 100 mm | 150 mm | C. Guard | 50 mm | 100 mm | 150 mm |
|---|---|---|---|---|---|---|---|---|---|
| | Pore Size | 130 Å | | | | 300 Å | | | |
| Standard Column | 10 mm | 186011205[1] | 186011206 | 186011207 | 186011208 | 186011216[1] | 186011217 | 186011218 | 186011219 |

*[1]Requires MaxPeak™ Prep Guard Holder P/N 186011548*

### XBridge Oligonucleotide BEH $C_{18}$ OBD Prep 130Å 2.5 µm Columns

| Diameter | | 50 mm |
|---|---|---|
| Standard Column | 10 mm | 186008212 |
| | 19 mm | 186008962 |
| | 30 mm | 186008963 |
| | 50 mm | 186008964 |

*For custom made products, contact a Waters representative.*

### XBridge Oligonucleotide BEH $C_{18}$ Analytical Columns for Method Development Screening

| Diameter | | 50 mm | 100 mm |
|---|---|---|---|
| | | 130 Å | 300 Å |
| Standard Column 2.5 µm | 4.6 mm | 186003953 | |
| Standard Column 5 µm | 4.6 mm | 186011165 | 186011183 |

## For more information about oligonucleotide analytical testing, please visit waters.com/Oligo

**waters.com/GTx**

**waters.com/Oligo**

**waters.com/OligoRPLC**

**waters.com/OligoWorks**

**waters.com/GTxResolveHILIC**

**waters.com/GTxStandards**

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