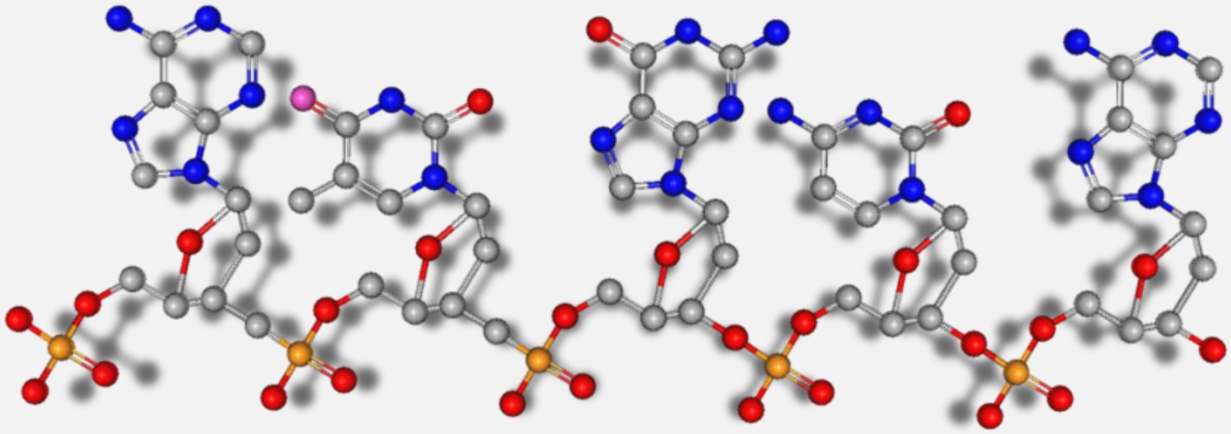
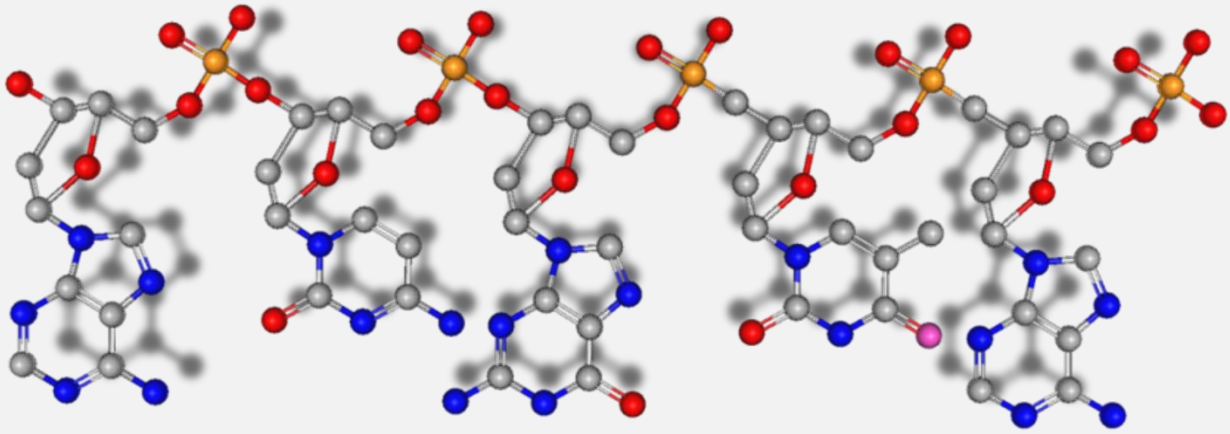
**SIELC Technologies, Inc.**
Wheeling, IL 60090 USA
**P. 847-229-2629** **F. 847-655-6079**
mail@sielc.com www.sielc.com

SIELC



# OLIGONUCLEOTIDES

## BY HPLC



# Oligonucleotide Analysis Background

Oligonucleotides are short sequences of nucleotides—the fundamental building blocks of DNA and RNA—that play a vital role in modern biotechnology, life sciences, and medical research. Typically consisting of 2 to 50 bases, these synthetic molecules are indispensable tools in a variety of applications, including genetic testing, molecular diagnostics, gene editing, and therapeutic development. Their precise ability to target specific genetic sequences makes them invaluable in areas such as antisense therapy, RNA interference (RNAi), and CRISPR-based gene editing.

However, analyzing oligonucleotides presents unique challenges due to their complex structure and physicochemical properties. Their highly charged nature, stemming from multiple phosphate groups, often results in strong interactions with stationary phases, complicating chromatographic separation. Additionally, variations in sequence length, base composition, and chemical modifications can impact retention and resolution, making it difficult to achieve consistent separation. The presence of impurities—such as truncated or extended sequences and modified byproducts—further necessitates high-resolution methods to ensure accurate differentiation and quantification. Moreover, achieving reproducibility and precision can be demanding, as minor fluctuations in analytical conditions can significantly influence retention times and peak shapes. Overcoming these challenges requires innovative techniques and optimized methodologies designed specifically for oligonucleotide analysis.

We recently introduced an innovative approach to liquid chromatography, the Bridge Ion Separation Technique (BIST™), which provides an effective solution for the analysis of multicharged molecules like oligonucleotides. This advanced technology facilitates efficient separation by utilizing a mobile phase containing some amount of organic modifier and a doubly charged positive ion (Figure 1). The positive ion forms a "bridge" between the negatively charged stationary phase and the negatively charged oligonucleotides, enabling precise and reliable separation of these complex molecules.

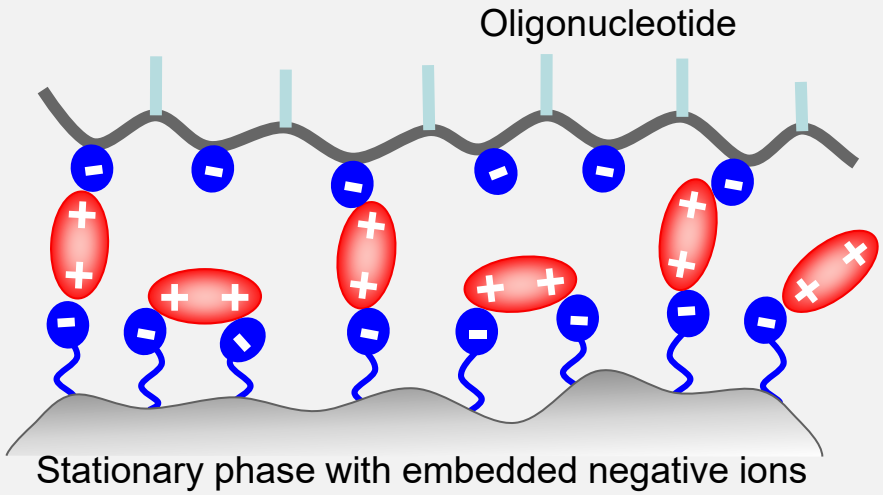



**Fig. 1.** Non-solvated doubly charged positive ions forming a bridge to hold oligonucleotides on the column surface

# BIST™ Explained

When doubly charged ions are present in the mobile phase (MP), the polarity of the stationary phase surface can switch. For example, if the surface is negatively charged and doubly charged positive ions, such as diamines or inorganic ions like $Mg^{2+}$ and $Ca^{2+}$, are present in the mobile phase, the net charge of the surface can become positive (Figure 2).

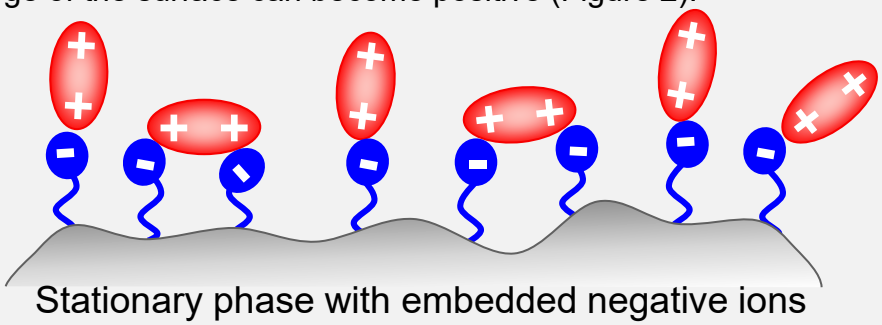



**Fig. 2.** Stationary Phase with negatively charged surface with positive net charge

However, this occurs only when the mobile phase has a relatively low concentration of water. When water is the main component of the mobile phase (Figure 3), it forms a solvation shell around each ion, preventing the surface charge from switching.

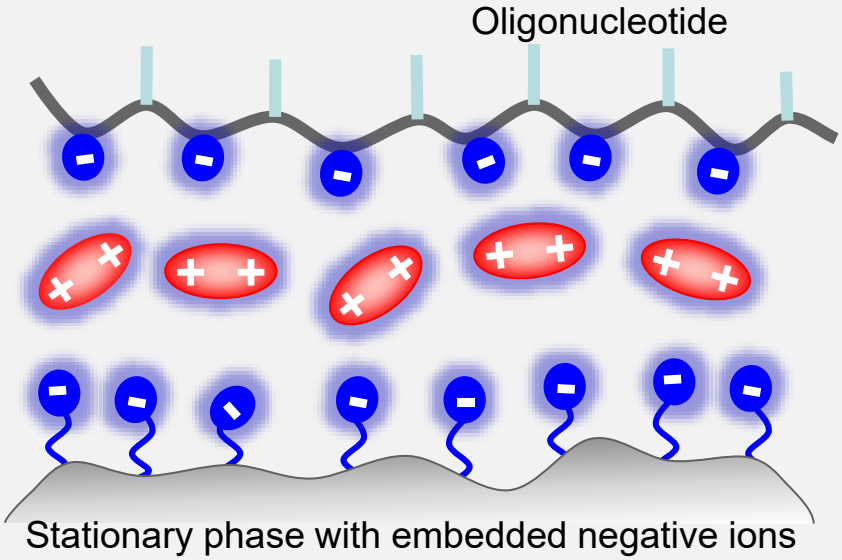
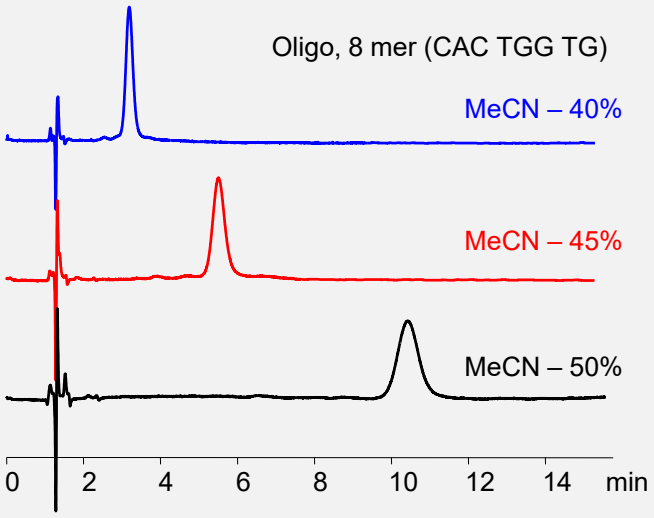



**Fig. 3.** Solvation of doubly charged ions affects the degree of bridge formation and oligonucleotide retention

The essence of this phenomenon is that the net charge of the surface can switch from negative to positive (or vice versa) simply by changing the concentration of water in the mobile phase. This effect can occur with either a negatively charged or a positively charged surface, as long as the mobile phase contains a buffer with doubly charged ions of opposite charge to the stationary phase. When the surface switches polarity, an analyte with a charge similar to the initial surface charge can be retained on this phase. The doubly charged buffer component acts as a bridge between the surface and the analyte. This phenomenon has introduced a novel way of controlling the retention of charged molecules through their interaction with the column's stationary phase, achieved by adjusting the relative amount of water in the mobile phase.

By precisely controlling the water content in the mobile phase, the solvation of doubly charged ions is regulated, directly influencing the degree of bridge formation between the analyte and the stationary phase. This interaction, in turn, affects the retention behavior of the analyte. The key advantage of BIST™ lies in its ability to easily and effectively control the retention of multicharged compounds, such as oligonucleotides, by managing this phenomenon (Figure 4).




| | |
|---|---|
| **Column:** | BIST AC |
| **Column size:** | 4.6 × 100 mm, 5 µm |
| **Mobile phase:** | MeCN/$H_2O$ |
| **Buffer:** | TMEDA Formate – 20 mM pH 4.0 |
| **Flow rate:** | 1.0 mL/min |
| **Detection:** | UV 260 nm |
| **Temperature:** | 30ºC |

**Fig. 4.** Effect of water concentration on oligonucleotide retention on BIST AC

This mechanism is not feasible unless the buffer provides doubly charged ions. The example below (Figure 5) demonstrates that when the buffer cation changes from 2+ (TMEDA) to 1+ (TEA), the oligonucleotide interaction shifts from retention (5a) to repulsion (5b). This highlights the critical role of the cation's double charge in forming the “bridge” and influencing the overall behavior and interaction of the oligonucleotide within the system.

| | |
|---|---|
| **Column:** | BIST AC |
| **Column size:** | 4.6 × 100 mm, 5 µm |
| **Mobile phase:** | Gradient MeCN - 50-5%, in 10 min |
| **Buffer:** | pH 4.0 - 20 mM |
| **Flow rate:** | 1.0 mL/min |
| **Detection:** | UV 260 nm |
| **Temperature:** | 30ºC |

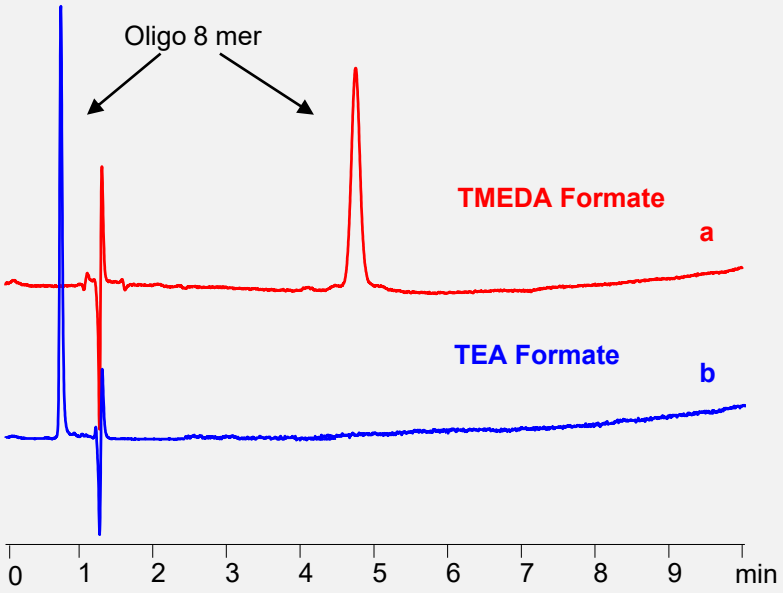
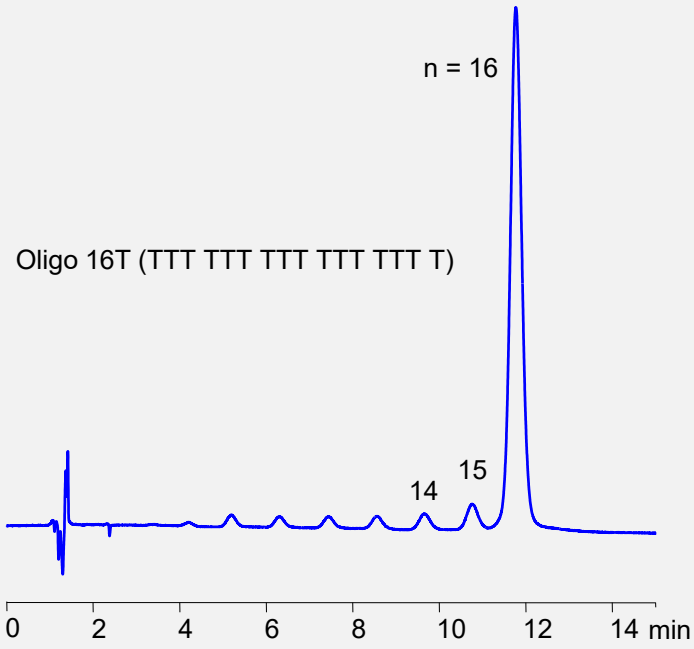



**Fig. 5.** Effect of buffer cation charge number on oligonucleotide retention

# BIST AC

BIST™ is specifically designed for gradient methods, providing a superior solution for separating complex mixtures of multicharged compounds. Traditional ion-exchange and isocratic techniques often face challenges in resolving compounds with minimal charge differences, such as those differing by only one or two charges.

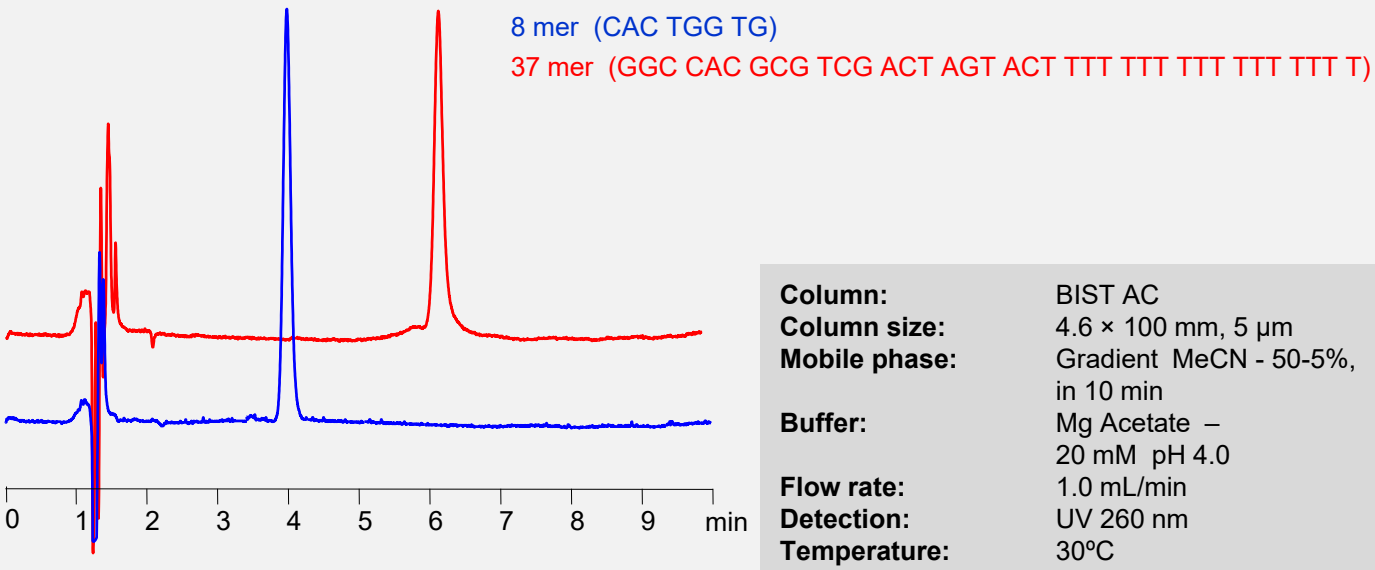
BIST™ overcomes these limitations by offering enhanced selectivity and precision. By employing gradient methods with a controlled increase in water content during the run, BIST™ enables the efficient separation of multicharged compounds, such as oligonucleotides, even when charge differences are minimal (Figure 6).




| | |
|---|---|
| **Column:** | BIST AC |
| **Column size:** | 4.6 × 100 mm, 5 µm |
| **Mobile phase:** | Gradient MeCN - 50-35%, in 15 min |
| **Buffer:** | TMEDA Acetate – 20 mM pH 4.0 |
| **Flow rate:** | 1.0 mL/min |
| **Detection:** | UV 260 nm |
| **Temperature:** | 30ºC |

**Fig. 6.** Retention of 16T oligonucleotide and its shorter byproducts on BIST AC

The BIST™ method can utilize various modifiers with double positive charges (Figure 6). Magnesium acetate serves as a convenient buffer modifier for the mobile phase when a volatile mobile phase is not necessary (Figure 7). This flexibility allows for optimized separation under different analytical conditions.

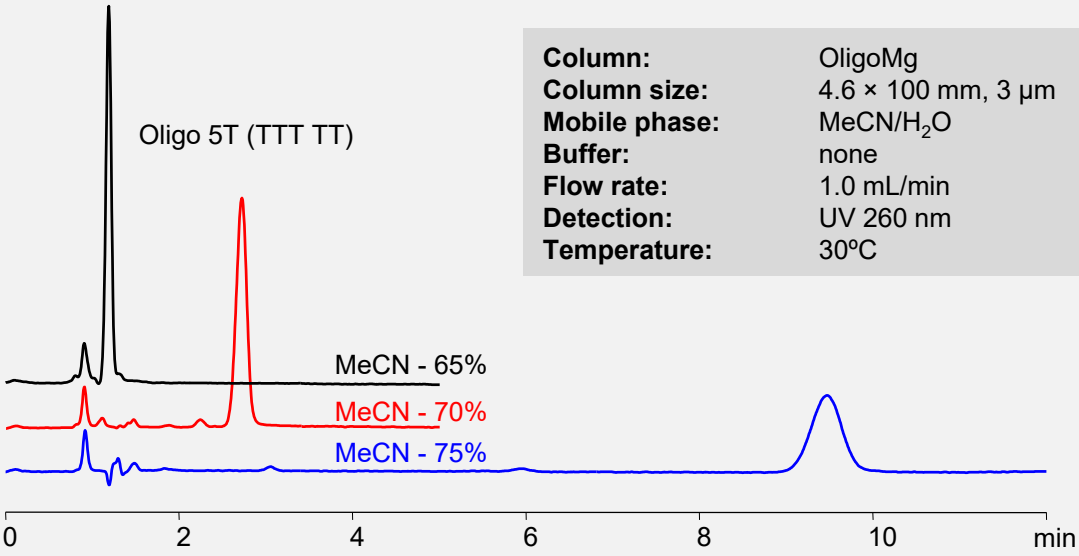



| | |
|---|---|
| **Column:** | BIST AC |
| **Column size:** | 4.6 × 100 mm, 5 µm |
| **Mobile phase:** | Gradient MeCN - 50-5%, in 10 min |
| **Buffer:** | Mg Acetate – 20 mM pH 4.0 |
| **Flow rate:** | 1.0 mL/min |
| **Detection:** | UV 260 nm |
| **Temperature:** | 30ºC |

**Fig. 7.** Separation of different oligonucleotides with $Mg^{2+}$ as a doubly charged modifier

# OligoMg

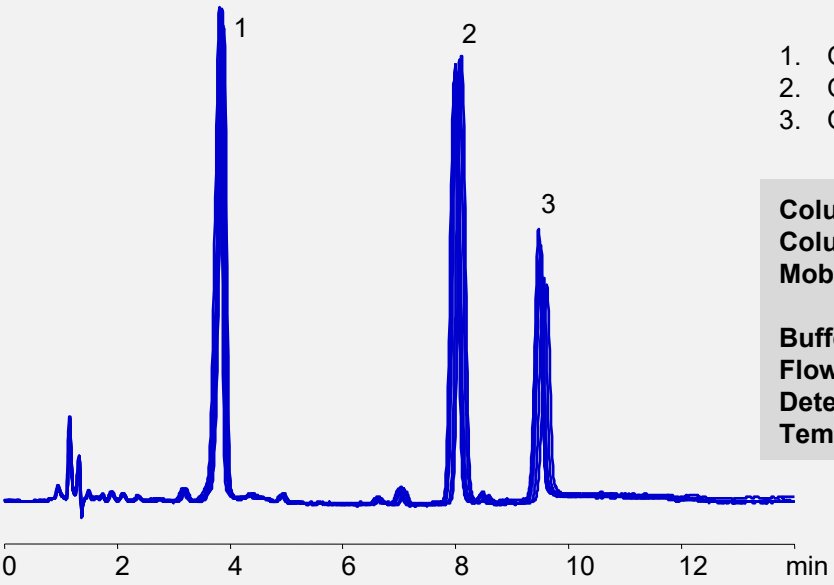
Our innovative work with the Bridge Ion Separation Technique (BIST™) has led to a groundbreaking discovery: doubly charged positive ions can be dynamically loaded onto the stationary phase, eliminating the need for continuous addition through the mobile phase. This advancement inspired the creation of the OligoMg column (Figure 8).




| | |
|---|---|
| **Column:** | OligoMg |
| **Column size:** | 4.6 × 100 mm, 3 µm |
| **Mobile phase:** | MeCN/$H_2O$ |
| **Buffer:** | none |
| **Flow rate:** | 1.0 mL/min |
| **Detection:** | UV 260 nm |
| **Temperature:** | 30ºC |

**Fig. 8.** Effect of water concentration on oligonucleotide retention on OligoMg

Once loaded with these ions, the OligoMg column operates seamlessly with a simple $H_2O$/MeCN mobile phase, enabling hundreds of injections without compromise (Figure 9). The ion-free mobile phase ensures that the bridging ions remain stably bound to the stationary phase, providing consistent and reliable performance. This stability allows the column to deliver precise and efficient separation, even over repetitive analyses, making it an ideal choice for oligonucleotide applications.




1. Oligo 5T (TTT TT)
2. Oligo 5A (AAA AA)
3. Oligo 5C (CCC CC)

| | |
|---|---|
| **Column:** | OligoMg |
| **Column size:** | 4.6 × 100 mm, 3 µm |
| **Mobile phase:** | Gradient MeCN - 75-55%, in 10 min |
| **Buffer:** | none |
| **Flow rate:** | 1.0 mL/min |
| **Detection:** | UV 260 nm |
| **Temperature:** | 30ºC |

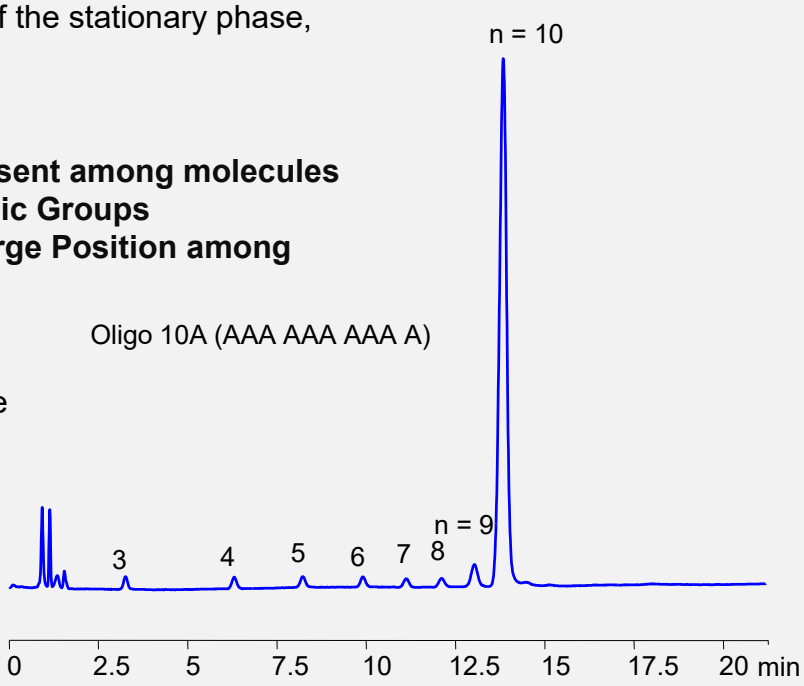
**Fig. 9.** 50 consecutive injections of a mixture of three oligonucleotides

# How BIST™ improves Selectivity

BIST™ interaction happens on the surface of the stationary phase, which offers very high selectivity toward:

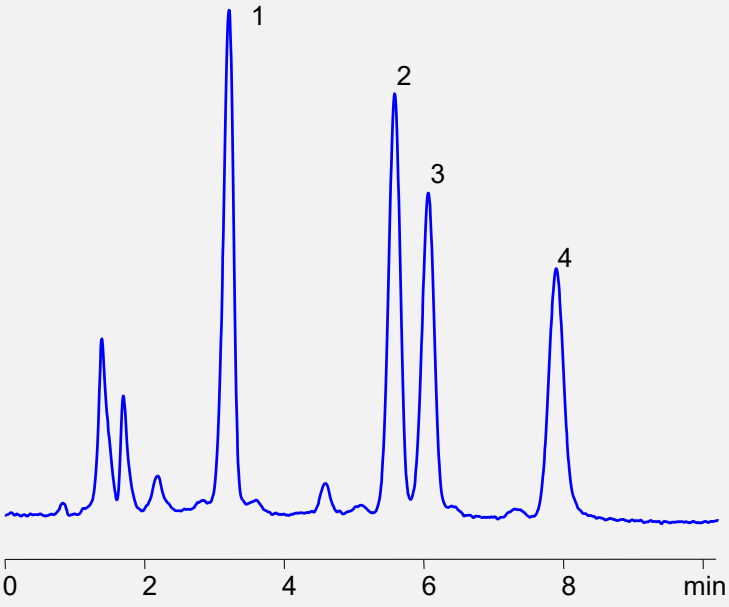
- **Structural Isomers**
- **Variations in the Number of Charges Present among molecules**
- **Differences in the Strength of Acidic/Basic Groups**
- **Variances in Geometry and Relative Charge Position among molecules**

In general, the retention of oligonucleotides correlates with the number of nucleobases in the sequence. This aligns with the bridge formation phenomenon, where longer molecular chains possess more negative charges available for bridging, resulting in increased retention times. Additionally, the geometry of oligonucleotide molecules significantly influences retention and bridge formation. Beyond geometry and chain length, the strength and composition of the nucleobases within the sequence also play a critical role. This is evident from the varying retention times observed for sequences of identical length but differing nucleobase compositions (Figures 11 and 12).




| | |
|---|---|
| **Column:** | OligoMg |
| **Column size:** | 4.6 × 50 mm, 2.1 µm |
| **Mobile phase:** | Gradient MeCN - 75-50%, in 10 min |
| **Buffer:** | none |
| **Flow rate:** | 0.5 mL/min |
| **Detection:** | UV 260 nm |
| **Temperature:** | 30ºC |

**Fig. 10.** Retention of 10T oligonucleotide and its shorter byproducts




1. Oligo 3A3T (AAA TTT)
2. Oligo 6A (AAA AAA)
3. Oligo 3A3C (AAA CCC)
4. Oligo 3A3G (AAA GGG)

| | |
|---|---|
| **Column:** | OligoMg |
| **Column size:** | 4.6 × 150 mm, 3 µm |
| **Mobile phase:** | Gradient MeCN - 65-45%, in 10 min |
| **Buffer:** | none |
| **Flow rate:** | 1.0 mL/min |
| **Detection:** | UV 260 nm |
| **Temperature:** | 30ºC |

**Fig. 11.** Retention differences of 6-mer oligonucleotides

# How BIST™ improves Selectivity

**SP6 Upstream** – ATT TAG GTG ACA CTA TAG (6T, 6A, 2C, 4G)
**pET 3'** – CTA GTT ATT GCT CAG CGG (6T, 3A, 4C, 5G)
**BGH Reverse** – TAG AAG GCA CAG TCG AGG (2T, 6A, 3C, 7G)

| | |
|---|---|
| **Column:** | OligoMg |
| **Column size:** | 4.6 × 150 mm, 3 µm |
| **Mobile phase:** | Gradient MeCN - 65-40%, in 15 min |
| **Buffer:** | none |
| **Flow rate:** | 1.0 mL/min |
| **Detection:** | UV 260 nm |
| **Temperature:** | 30ºC |

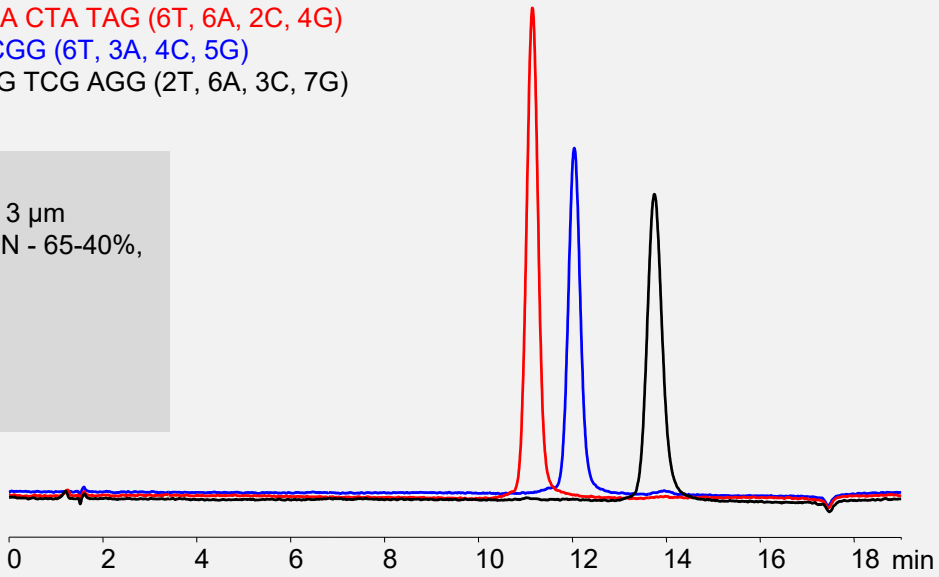



**Fig. 12.** Separation of 18-mer oligonucleotides

17 mer – **Bluescript KS** – TCG AGG TCG ACG GTA TC (4T, 3A, 4C, 6G)
18 mer – **SP6 Upstream** – ATT TAG GTG ACA CTA TAG (6T, 6A, 2C, 4G)
20 mer – **3' RACE PCR** – GGC CAC GCG TCG ACT AGT AC (3T, 4A, 7C, 6G)
22 mer – **SP6 Promoter** – TAC GAT TTA GGT GAC ACT ATA G (7T, 7A, 3C, 5G)

| | |
|---|---|
| **Column:** | OligoMg |
| **Column size:** | 4.6 × 150 mm, 3 µm |
| **Mobile phase:** | Gradient MeCN - 65-40%, in 15 min |
| **Buffer:** | none |
| **Flow rate:** | 1.0 mL/min |
| **Detection:** | UV 260 nm |
| **Temperature:** | 30ºC |

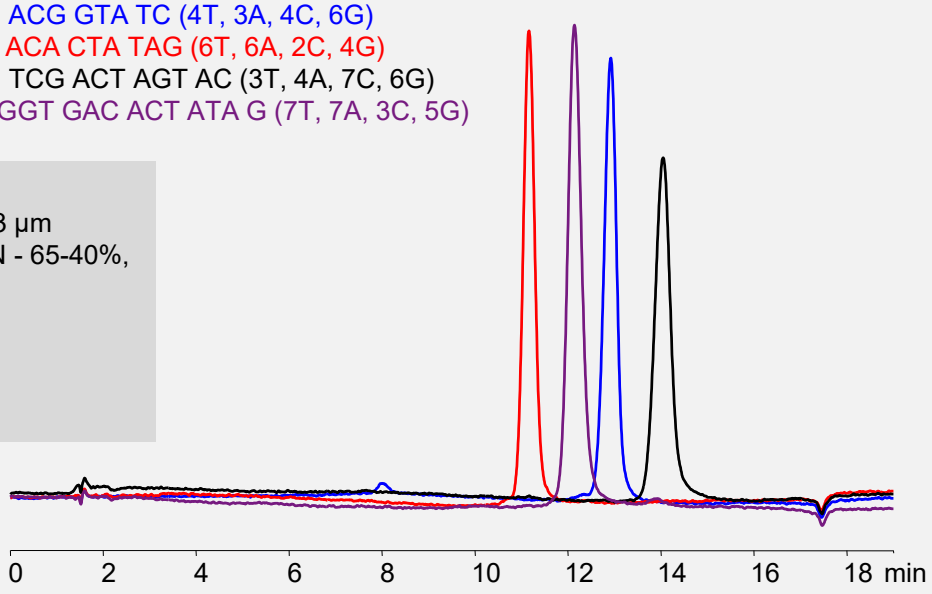



**Fig. 13.** Separation of oligonucleotides of different lengths

Although the length of the oligonucleotide and the overall number of charges are the most important factors influencing retention, in certain cases, the strength and composition of the nucleobases within the sequence can have a more significant impact. For example, in the case shown above (Figure 13), the elution order of four oligonucleotides of different lengths does not correlate with their size. This demonstrates how various factors influence selectivity and highlights the sensitivity of BIST™ technology, which can separate closely related compounds by taking advantage of even the smallest differences.

# Summary

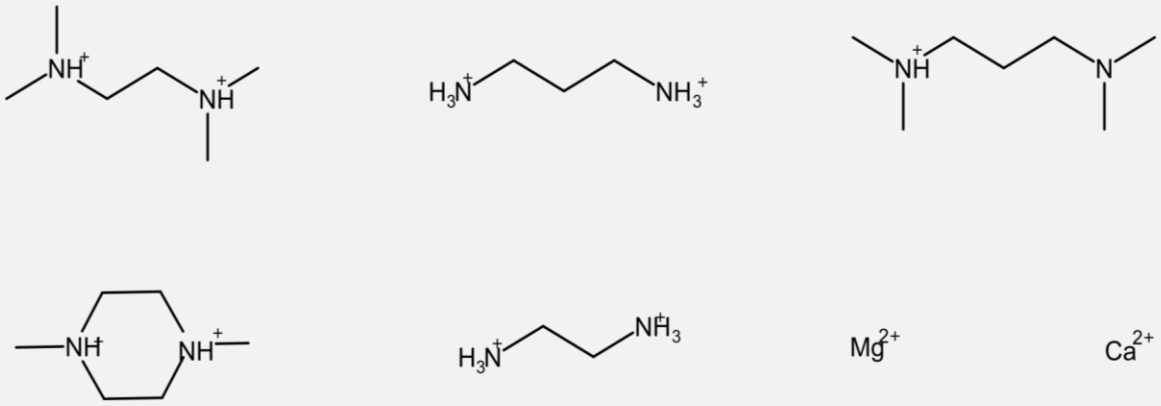



**Fig. 14.** Examples of some doubly charged ions compatible with BIST™

| Column Name | Technology | Buffer in MP |
|---|---|---|
| BIST AC | BIST™ | YES |
| OligoMg | BIST™ | NO (preloaded) |

The power of the BIST™ mechanism lies in its ability to achieve complex separations with simple mobile phases, using low-concentration buffers or even no buffer at all (e.g., with the OligoMg column). Multicharged compounds, such as oligonucleotides, are challenging to separate using ion-exchange (IE) chromatography due to their strong interactions with the oppositely charged stationary phase. This typically requires the use of extremely high buffer concentrations to drive the ion-exchange process. However, these high buffer concentrations can lead to higher mobile phase viscosity and potential salt formation in system components. With BIST™ technology, oligonucleotides can be efficiently retained and analyzed by precisely controlling the amount of water in the mobile phase, offering a simpler and more effective separation approach.

## Ready to Transform Your Oligonucleotide Analysis?

Let's make innovation happen together! Whether you have questions or are ready to get started, we're here to assist.

**SIELC Technologies, Inc.**

**www.sielc.com email: mail@sielc.com ph. 847-229-2629 fax 847-655-6079**

**Copyright © 2024 by SIELC Technologies Corporation. The information and disclosures contained herein were originated by and are the property of SIELC Technologies Corporation and may not be copied, distributed, republished, translated, uploaded, posted, transmitted, stored, or otherwise used in any shape, manner, or form by any means without the specific prior written permission of SIELC Technologies Corporation. Modification or use of this material for any reason shall be construed as a violation of the property rights of SIELC Technologies Corporation. SIELC Technologies Corporation reserves all rights, including without limitation all patent, proprietary design, manufacturing, reproduction, use, advertising, and sales rights thereto, and to any articles disclosed herein, except to the extent such rights are granted to others by law or by SIELC Technologies Corporation. LIPAK™ is a trademark of SIELC Technologies Corporation.**

**All data were obtained in SIELC Technologies labs.**