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Analysis and Purification of Oligonucleotides by Anion Exchange and Ion-Pairing Reversed Phase Chromatography

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This Whitepaper ...

...is written for all chromatographers who deal with the analysis and/or purification of oligonucleotides. The focus is on anion exchange chromatography (AEX) and ion pair reversed phase chromatography (IP-RP).
For both modes, the main aspects are the selection of a suitable stationary and mobile phase as well as the optimum temperature.
Of course, the special features of the large-scale process are also taken into account. If this aspect is of particular interest to you, simply follow the icon:

In this whitepaper, you are guided through the following aspects:

Introduction to Therapeutic Oligonucleotides
by Valentina D'Atri, PhD, University of Geneva

Separation Modes in Liquid Chromatography for Therapeutic Oligonucleotides

Anion Exchange Chromatography

Ion-Pairing Reversed Phase Chromatography

Important Considerations for Loadability and Scale-Up

1. Introduction

By Valentina D'Atri, PhD (University of Geneva, Institute of Pharmaceutical Sciences of Western Switzerland (ISPSO), Geneva, Switzerland)

Therapeutic oligonucleotides (TOs) are synthetic DNA or RNA oligomers designed to target specific genes or transcriptional pathways with the purpose of treating a wide range of diseases, including cancer, genetic disorders, and viral infections [1, 2, 3].

Under the name of TOs lies a wide range of different nucleic acid structures including e.g. antisense oligonucleotides (ASOs) as splice-switching oligonucleotides (SSOs), gapmers, antagomirs, and agomirs (miRNA mimetics), small interfering RNAs (siRNAs), and aptamers [4, 5].

The different nucleic acid structures of TOs imply different functions and mechanisms of action: i) ASOs are designed to bind to specific RNA sequences and inhibit the expression of disease-causing genes. They can be used to silence the expression of oncogenes, viral genes, or genetic mutations that cause inherited diseases. ii) siRNAs are designed to bind to specific mRNA sequences and induce degradation of the mRNA, thereby inhibiting the expression of specific proteins, while iii) aptamers are designed to bind to specific proteins and alter their functions. Aptamers and siRNA can be used to inhibit the activity of viral proteins or proteins that are overexpressed in cancer cells [6].

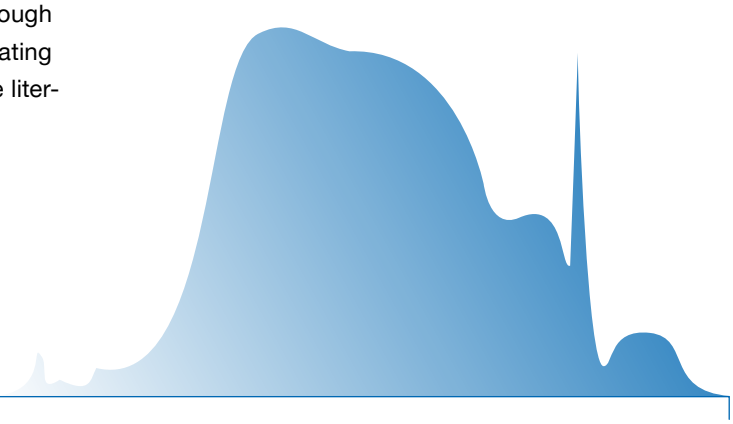
According to their size and complexity, TOs can be generally classified in short single-stranded oligonucleotides (ASOs, SSOs, gapmers, antagomirs, and agomirs), short double-stranded oligonucleotides (siRNA), and large single-stranded ONs (aptamers). Short oligonucleotides can be produced by solid-phase synthesis, while enzymatic in vitro transcription (IVT) is preferred for larger oligonucleotides [7].

The solid-phase synthesis of therapeutic oligonucleotides is a complex cyclic process that involves several steps, including detritylation, coupling, and capping nucleotides of the growing oligomer, purification, and characterisation. The oligonucleotides must be synthesised with high accuracy and purity, and they must be characterised to ensure that they are of the desired length and sequence [8]. Indeed, several impurities can be formed at each step of the synthesis, mainly consisting in shortmers (n–1, n–2), longmers (n+1), abasic oligonucleotides, and partially deprotected or detritylated oligonucleotides [8, 9, 10, 11, 12, 13].

In addition, TOs can be chemically modified to enhance their stability, targeting ability, or other properties. Chemical modifications can be introduced at several sites of the nucleotide, namely heterocyclic nucleobase, sugar moiety, and phosphodiester linkage, as well as at the backbone level (sugar and phosphodiester) [14, 15]. Some of the most common chemical modifications include: phosphorothioate (PS, replacing one of the oxygen atoms in the phosphate backbone of the oligonucleotide with a sulphur atom), which increases the stability and nuclease resistance of the oligonucleotide); sugar modification such as 2'-O-Methyl (2'OMe), 2'-O-Methoxyethyl (2'MOE), and Locked Nucleic Acid (LNA), which increase the stability and nuclease resistance of the oligonucleotide and also increases its ability to bind to its target; and the phosphorodiamidate morpholino backbone (morpholino), a synthetic alternative to the natural nucleotides having an aminoglycoside-like structure that increases nuclease resistance, stability and target binding ability [14, 15]. These are just a few examples, but there are many other types of chemical modifications that have been developed and are being researched for use in therapeutic oligonucleotides [14, 15].

In addition to chemical modifications, synthetic ligands and carriers can be used for targeted delivery, with oligonucleotides conjugated to GalNAc or encapsulated in lipid nanoparticles (LNPs) being the most used delivery approaches [16, 17, 18, 19].
Obviously, the different levels of chemical complexity imparted by the chemical modifications and the addition of delivery ligands/carriers result in a wider diversity of impurities and structural variability that needs to be addressed.
Concerning the regulatory aspects, TOs are considered at the interface between small molecule drugs and biologics, as they are manufactured like new chemical entities but also share properties with biologics. For this reason, they do not benefit from clear guidelines, although suggestions on the classification of impurities originating from the manufacturing process can be found in the literature [8].

A classification of common starting material and process-related impurities observed in oligonucleotide manufacturing has been recently published by Rupp and Cramer with the goal to guide a chemistry, manufacturing, and controls (CMC) chemist in oligonucleotide therapeutic development [20]. Of note, most of the impurities require advanced analytical instrumentation to allow their identification and quantification as they are closely related to the parent compound.
In this context, approaches based on liquid chromatography (LC) have undoubtedly a pivotal role in the impurity characterisation and purification of oligonucleotides and the advancement of CMC strategies to better monitor these impurities. Specifically, anion exchange (AEX) and ion-pairing reversed phase chromatography (IP-RP) techniques can be addressed as reference approaches.



1.1. Separation Modes

Anion Exchange (AEX) Chromatography

Separation Principle

The separation of oligonucleotides via anion exchange chromatography is based on the direct interaction of the negative charged sugar-phosphate backbone with the positive charged modification of the AEX stationary phase. This charge-charge interaction leads to the retention of the target molecule to the stationary phase. The elution is initiated by increasing the ionic strength of the mobile phase. Mostly, eluents with high salt concentrations in the elution buffer are employed. Alternatively, the pH of the mobile phase can be changed. As a result, the interaction dissociates and the oligonucleotide elutes from the stationary phase and is separated from other molecules with different charge profiles.

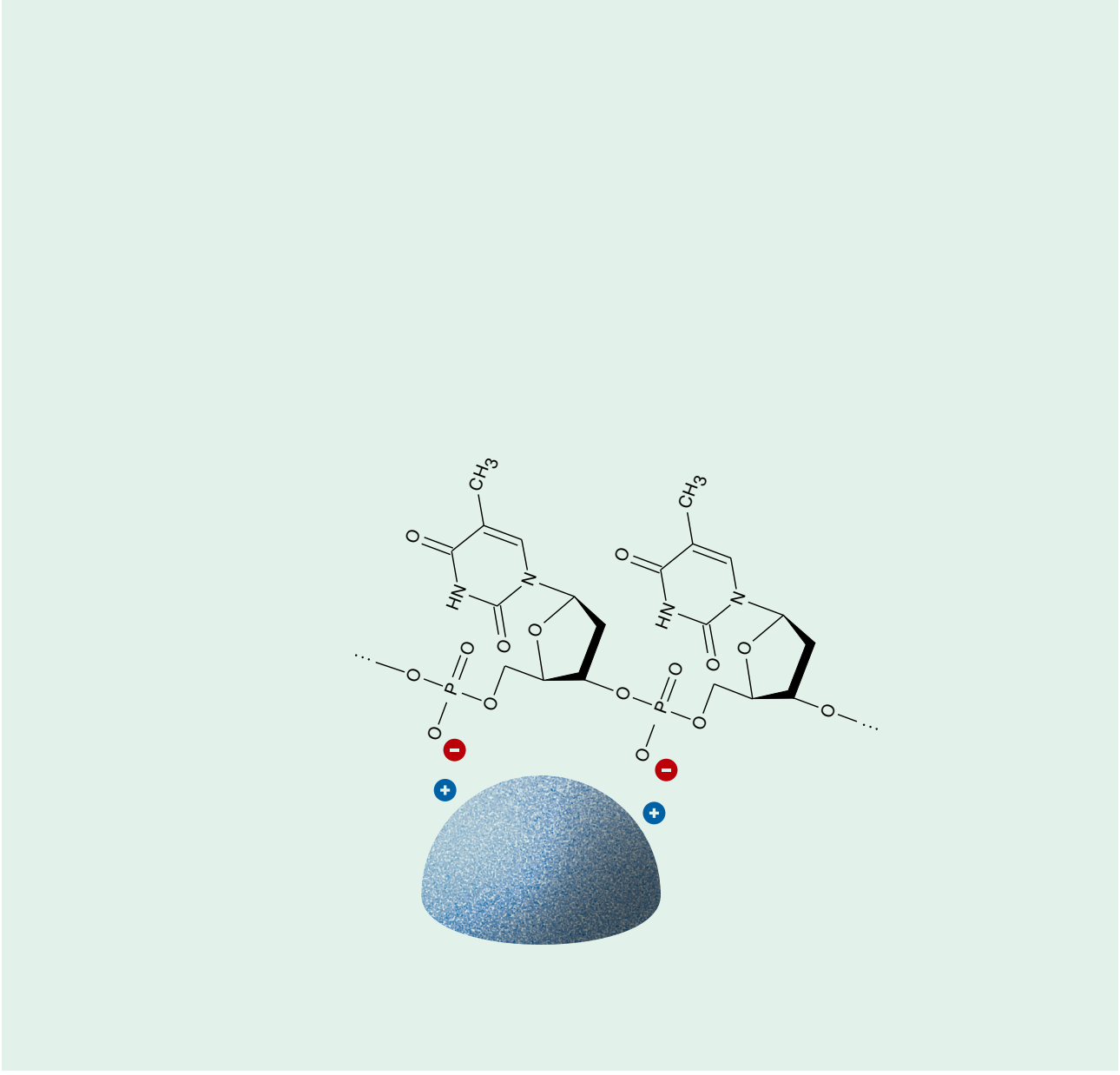


Figure 1: Separation principle of AEX.

Ion-Pairing Reversed Phase (IP-RP) Chromatography

Separation Principle

Of course, the charged oligonucleotide backbone is not able to interact with the stationary phase. Therefore ion-pair reagents with a hydrophobic group are required. They act as mediators between the charged target and the hydrophobic stationary phase. This leads to an effective retention of the oligonucleotide on the RP phase. The elution is performed by increasing the content of organic modifier of the mobile phase (including the IP-reagent), in order to elute the oligonucleotide-ion pair-complex from the stationary phase.

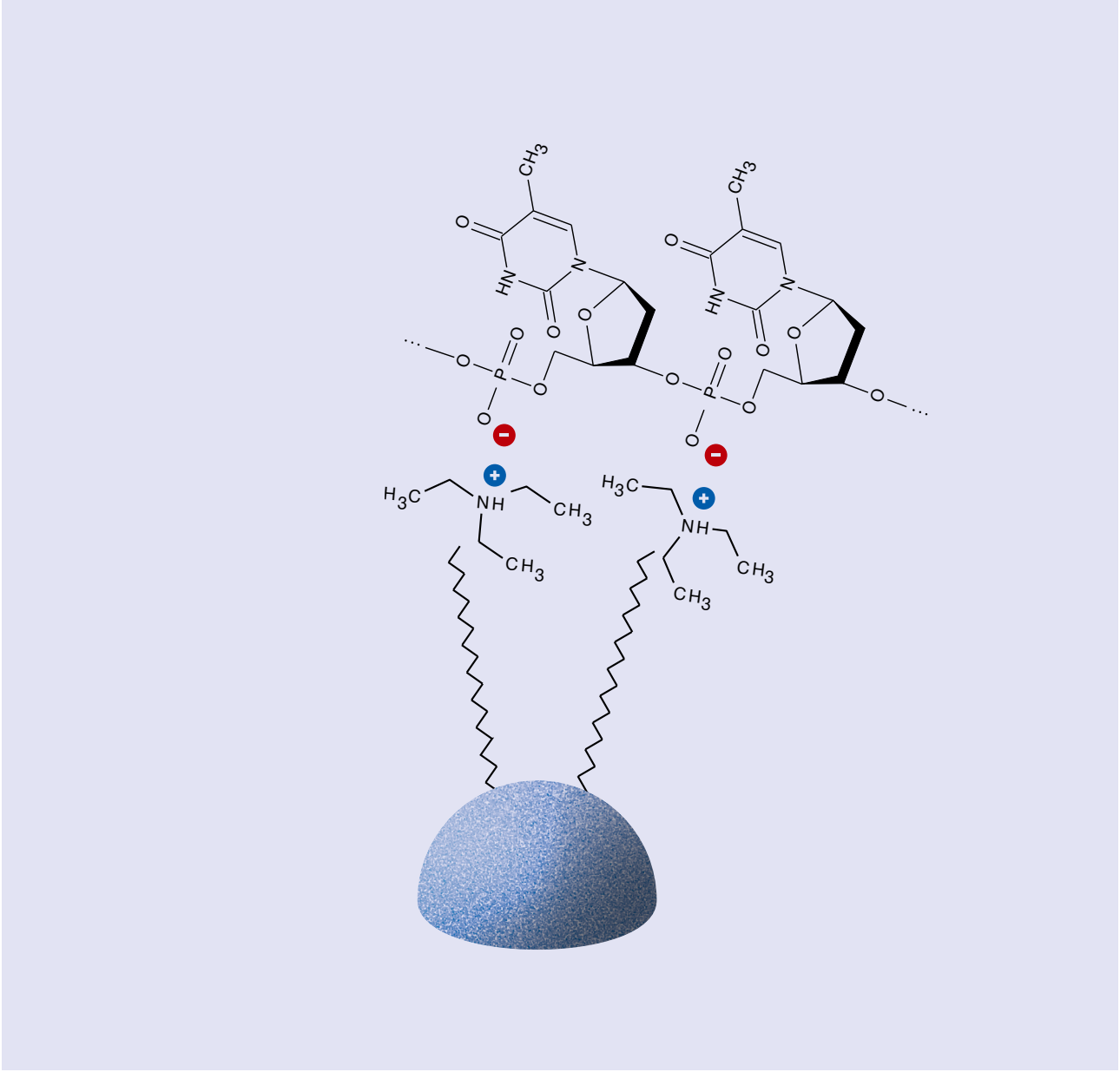


Figure 2: Separation principle of IP-RP.

1.2. Analysis and Purification of Oligonucleotides

Analysis

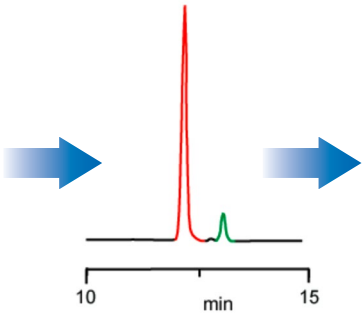
The purpose of the analysis of oligonucleotides is the quantitative and/or qualitative characterisation of the target molecules.

Aim: Obtaining Information About the Target Molecule or the Mixture

In analytical LC, the focus is on parameters that allow to gain information as fast as possible from a small amount of sample. Therefore, the resolution between the peaks of interest should be sufficient but does not need to be large. The peak shape in analytical chromatography should be as ideal as possible (asymmetry factors close to 1).

To obtain the required information from the chromatogram, the sensitivity of the system setup needs to be high because some components of the target mixture are only trace impurities that have to be detected. For high reproducibility, the method and columns have to be very robust so that the results are reproducible and reliable independent from possible experimental deviations.

Small column sizes



Analytical separation

Identification & Characterisation

- **Resolution**
- **Peak shape**
- **Sensitivity**
- **Throughput**
- **Robustness**

Important factors

As analytical LC only deals with a small amount of sample, the column dimensions are correspondingly small. The inner diameters (IDs) used for analytical methods typically range from 1.0–4.6 mm ID. Columns are packed with stationary phases with small particle sizes, sub 2 µm for UHPLC and 3 and 5 µm for HPLC. Decreasing the particle size is an effective tool to improve the resolution.

As smaller particles in smaller column dimensions provide higher efficiencies, the peaks obtained are sharper and therefore the resolution higher.

Mass spectrometry (MS) detection is often used as it provides more information on the oligonucleotide sample compared to UV. However, the MS-compatibility of the mobile phase has to be considered in order to obtain a reasonable sensitivity.

Summary of technical parameters for separations on **analytical scale:**

✔ **Column sizes:**	**small,** typical dimensions:	length: 30–250 mm ID: 1.0–4.6 mm
✔ **Particle sizes:**	**small,** typically <2–5 µm	
✔ **Phase selectivity:**	**aliphatic** modifications (IP-RP) or **cationic** functionalities (AEX)	
✔ **Mobile phase:**	**buffers/IP reagents for optimal sensitivity, MS-compatibility**	
✔ **Temperature:**	**ambient or elevated temperatures**	
✔ **Detection:**	**UV, MS**	
✔ **Sample loading:**	**low,** depending on detection limit	

Purification

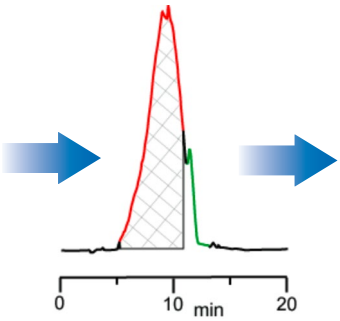
The purpose of purification processes using preparative chromatography is the isolation of a target substance from a sample.

Aim: Collection of a Target Substance with Increased Purity

In preparative LC, the focus is on the isolation of the target compound to reach a required purity. For the development of industrial scale processes, a comprehensive process development is required to identify the most productive and cost-efficient method.

In this context, the production efficiency is the determining factor in how much substance can be purified from the sample feed within a purification run. This depends on the purity level that has to be achieved.

Large column sizes



Preparative purification

Isolation & Purification

- **Purity**
- **Production efficiency**
- **Recovery**
- **Safety**
- **Costs**

Important factors

The scale of purification processes can vary greatly. This depends on the amount of target that has to be purified. If for example only a few milligrams or grams of an oligonucleotide have to be purified, the setup of the preparative system is small. The scale of the preparative column and the system increases with larger sample feed volumes.

Column IDs up to 2 m can be used for preparative LC methods, depending on the actual need for the final target compound. The particle size of preparative stationary phases is typically larger compared to analytical LC in order to reduce the backpressure at larger scales. The mobile phase needs to be safe, available in large quantities and preferably at low costs. The cost-efficiency of preparative processes strongly relies on the mobile phase used and its consumption.

For optimal productivity, the sample loading should be as high as possible with sufficient recovery levels to optimise the overall yield. The detection is only required for the collection of the target – not for its identification.

Summary of technical parameters for separations on **preparative scale:**

✔	**Column sizes:**	**larger,** especially larger IDs depends on scale
✔	**Particle sizes:**	**large,** typically 7–50 µm
✔	**Phase selectivity:**	**aliphatic** modifications (IP-RP) or **cationic** functionalities (AEX)
✔	**Mobile phase:**	**large quantities, cost-efficiency and safety required**
✔	**Temperature:**	**ambient,** high temperatures possible depending on equipment
✔	**Detection:**	**UV,** no MS-compatible buffer systems required
✔	**Sample loading:**	**as high as possible** depending on resolution

Purification aspects within this whitepaper:

This icon indicates important aspects and considerations for the purification of oligonucleotides within the whitepaper.

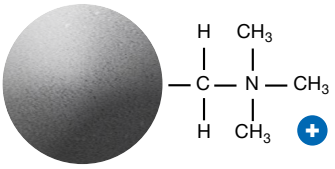
2. AEX

2.1. Stationary Phase

2.1.1. Functional Group

Quaternary amine groups, which are strong anion exchangers, are the gold standard of functional groups in the analysis and purification of oligonucleotides. Strong anion exchangers are protonated over the entire pH range allowing for the use of high pH values which are often applied in oligonucleotide separations. In contrast, weak anion exchangers are not protonated over the entire pH range.

As they lose their anion exchange functionality at high pH values, they are only suitable to a limited extend in oligonucleotide chromatography.



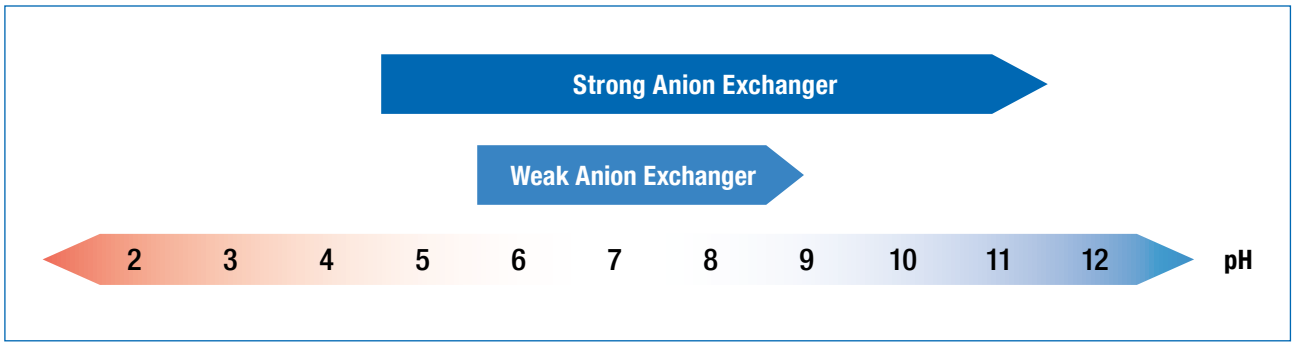
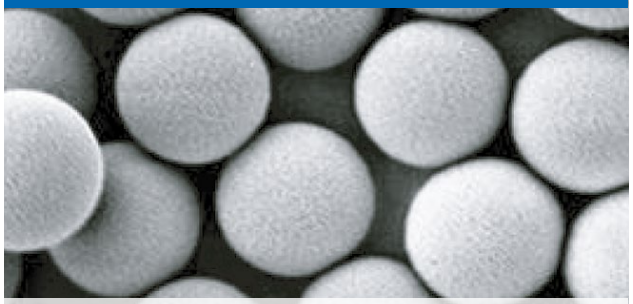


Figure 3: Protonation ranges of strong and weak anion exchangers.

2.1.2. Particle Technology

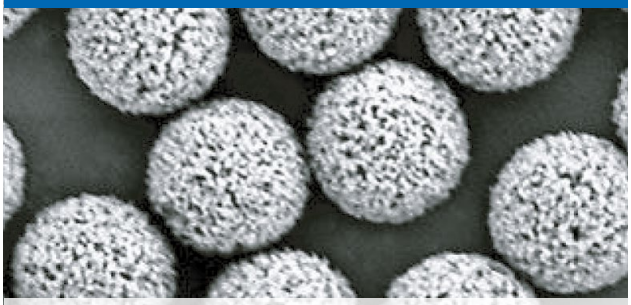
In the entire chain of oligonucleotide chromatography extreme pH values and temperatures can be required. Whilst during their analysis high temperatures are used to improve the resolution, the Cleaning-in-Place (CIP) after purification uses high pH values up to 14; both require highly robust stationary phases. Polymer-based particles provide high stability against both, high pH and temperature which is why they are often preferred over silica particles in oligonucleotide chromatography. Porous and non-porous particles are commonly available.

Analytical scale



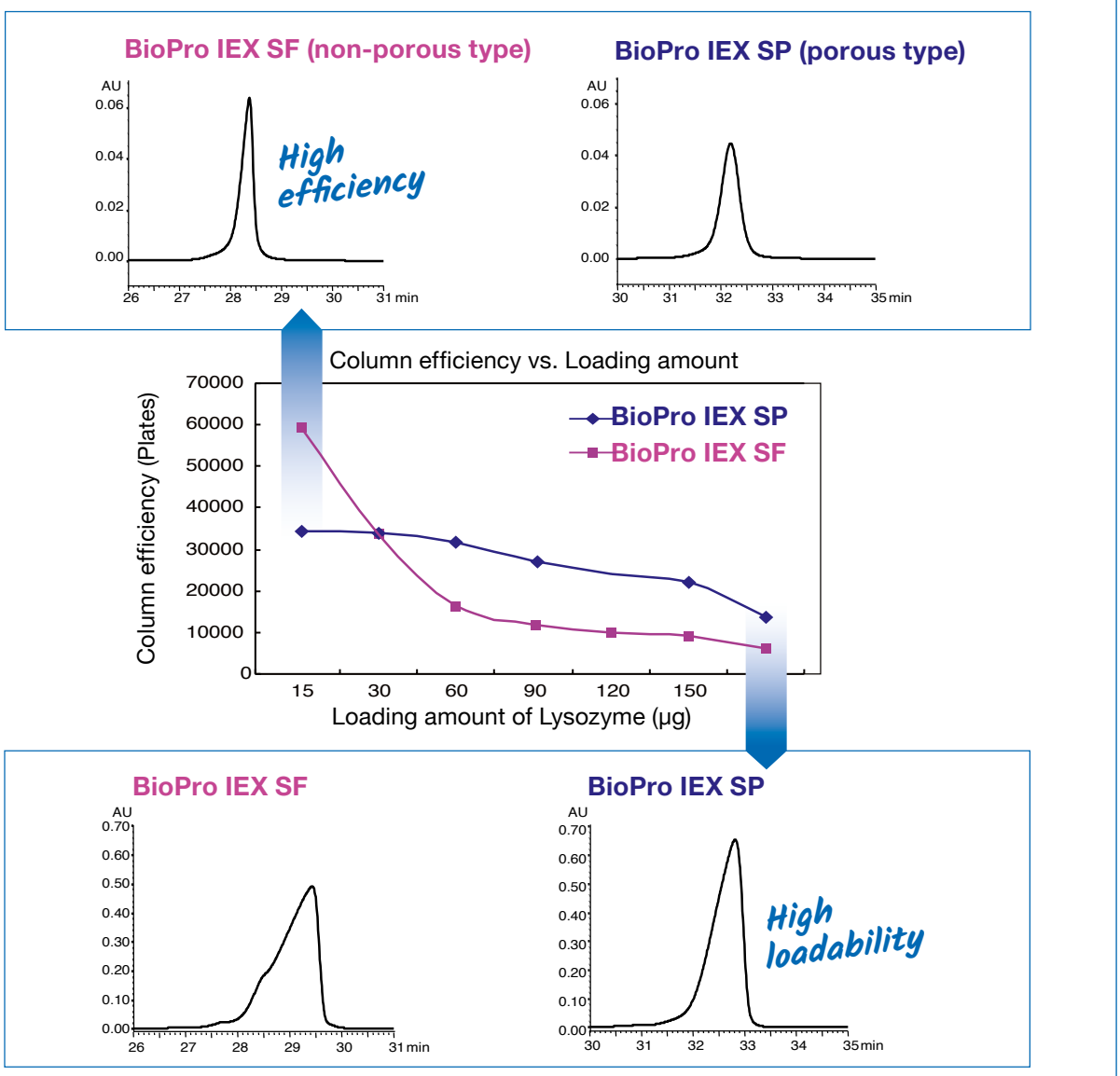
Non-porous polymer beads

(Semi) preparative scale



Porous polymer beads

Non-porous particles are usually applied on an analytical scale due to their high efficiency at low loadings. Due to the enhanced mass transfer sharp peaks can be obtained. For higher loadings required for purifications, porous particles are the better choice. Thanks to the porous structure the accessible surface area is increased allowing for a higher loadability. Figure 4 demonstrates the benefits at different loading amounts.



Column:	BioPro IEX SF/SP (5 µm) 50 x 4.6 mm ID	Flow rate:	0.5 mL/min
Part Nos.:	SF00S05-0546WP/SPA0S05-0546WP	Temperature:	25°C
Eluent:	A) 20 mM NaH_2PO_4-Na_2HPO_4 (pH 6.8)	Detection:	UV at 280 nm
	B) 20 mM NaH_2PO_4-Na_2HPO_4 (pH 6.8) containing 0.5 M NaCl	Injection:	100 µL
Gradient:	0–100%B (0–60 min)	Sample:	Lysozyme

Figure 4: Column efficiency and loadability of non-porous and porous BioPro IEX columns.

Purification of Oligonucleotides: Particle Technology

For the efficient purification of oligonucleotides, IEX resins with optimised base materials are needed. The particles need to allow high sample loading and should have good pressure-flow characteristics for high productivity. Therefore, resins with high dynamic binding capacities (DBC) and low backpressures that also enable high resolution purifications are the key to success. Hydrophilic polymer-based materials provide high chemical and mechanical stability and consequently are well-suited for oligonucleotide purification processes.

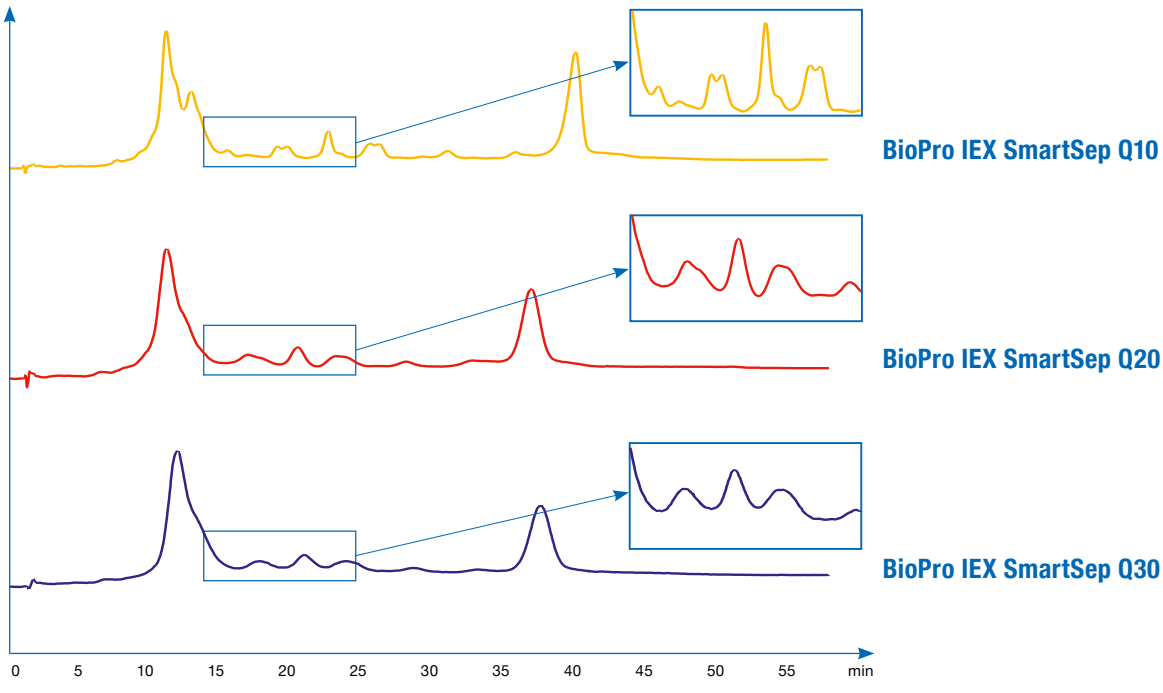
2.1.3. Particle Size

When developing a new method, chromatographers have to consider the particle size. If there is a separation task for analysis, small particle sizes such as 3 and 5 µm are typically used. For purification purposes, larger particle sizes such as 10, 20 or 30 µm can be employed. It depends on the final scale of the purification. The limiting factor is the resulting backpressure which must meet the limitations of the equipment specification. In order to perform a smooth scale-up, it is very important to make sure that various particle sizes are available. If scouting columns were packed with analytical particle size it is necessary to check that the corresponding preparative particle sizes provide the same selectivity to deliver consistent resolution.

Purification of Oligonucleotides: Selecting the Most Suitable Particle Size

The purification of oligonucleotides requires very high resolution because highly similar impurities including longmers and shortmers have to be removed. On the other hand, the process has to be productive and cost-efficient. Therefore, resolution and reasonable backpressure need to be balanced.
IEX resins like YMC's BioPro IEX SmartSep are available in different particle sizes and allow full flexibility in method development: 30, 20 as well as 10 µm.

For large scale purifications, 30 µm particle sizes are suitable. Polishing steps and high-resolution purifications are usually developed with 20 and 10 µm particle sizes. State of the art resins such as BioPro IEX SmartSep provide comparable retention profile and differ only in the resolution of the eluting peaks. As a result, the particle size can be changed during process development which allows tailored solutions.

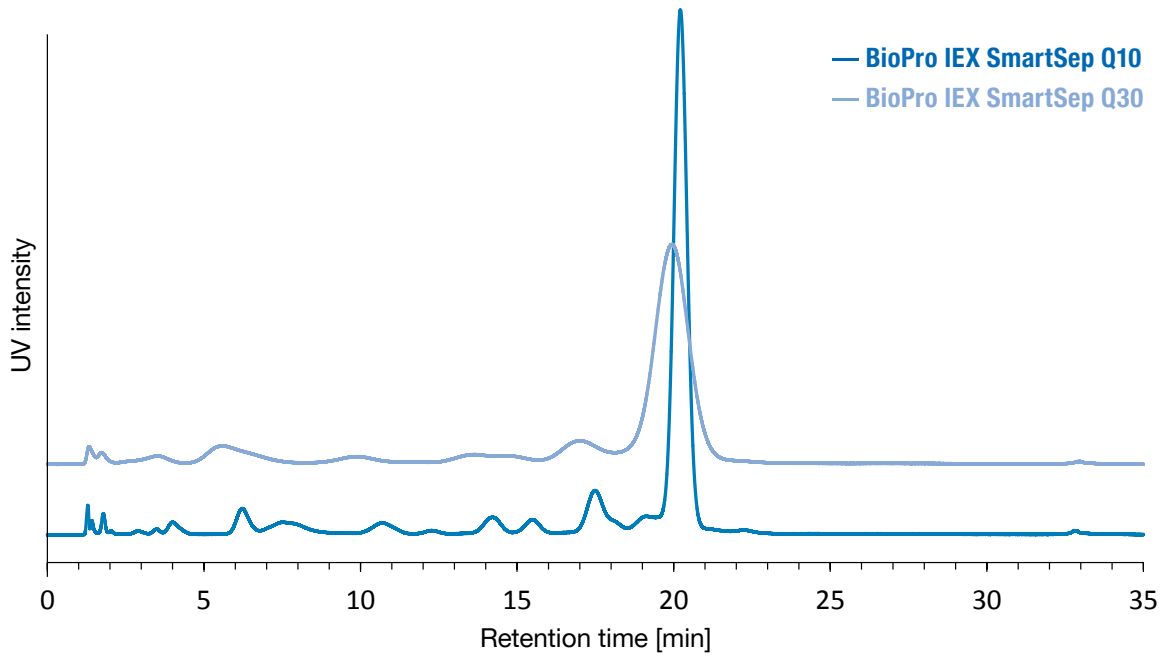


Column Dimension:	50 x 4.6 mm ID	Gradient:	0–80%B (0–60 min)
Resin Part Nos.:	BioPro IEX SmartSep Q10 (10 µm): QSA0S10	Flow rate:	180 cm/h (0.5 mL/min)
	BioPro IEX SmartSep Q20 (20 µm): QSA0S20	Temperature:	30 °C
	BioPro IEX SmartSep Q30 (30 µm): QSA0S30	Detection:	UV at 220 nm
Eluent:	A) 20 mM Tris-HCl (pH 8.1)	Sample:	Transferrin and trypsin inhibitor
	B) 20 mM Tris-HCl (pH 8.1) containing 0.5 M NaCl		

Figure 5: Scalability of BioPro IEX SmartSep.

Practical Example: Purification of Oligonucleotides with Different Particle Sizes

Figure 6 shows the separation of a DNA 20mer oligonucleotide using two different particle sizes (10 µm and 30 µm). The resolution of both traces differs significantly, whereas the retention profile of both separations remains the same. Resins that are available in different particle sizes allow more flexibility in process development because different particle sizes can be tested. If the particle sizes are scalable, which means they provide the same retention behaviour, a change of the particle size can easily be implemented without modifying the separation parameters.



Column Dimension:	100 × 4.6 mm ID
Resin Part Nos.:	BioPro IEX SmartSep Q10 (10 µm): QSA0S10
	BioPro IEX SmartSep Q30 (30 µm): QSA0S30
Eluent:	A) 10 mM NaOH
	B) 10 mM NaOH + 1 M $NaClO_4$
Gradient:	25–40%B (0–30 min), 40–100%B (30–35 min)
Flow rate:	1 mL/min
Temperature:	25 °C
Detection:	UV at 260 nm
Injection:	5 µL
Sample:	Trityl-OFF 5'-ATA CCG ATT AAG CGA AGT TT-3' (DNA 20mer)

Figure 6: Purification of 20mer DNA oligonucleotide using 10 and 30 µm BioPro IEX SmartSep.

Important Considerations for Process Scale Up

The smaller the particle size of the stationary phase, the higher is the resulting backpressure. This correlation should be considered at an early stage of process development. The systems and column hardware used for the purification of oligonucleotides determine the pressure limit. Figure 7 illustrates the pressure-flow curves of the different particle sizes of the BioPro IEX SmartSep resins. As shown in the diagram, the 10 µm particles lead to an elevated backpressure at higher linear flow rates. With adequate hardware and system, small particles are well-suited for very high-resolution purifications. This requires systems and column hardware with higher pressure ratings that can cope with the higher backpressure of the small particles. If low pressure equipment is used – especially for very large-scale applications – larger particles are the preferred option.

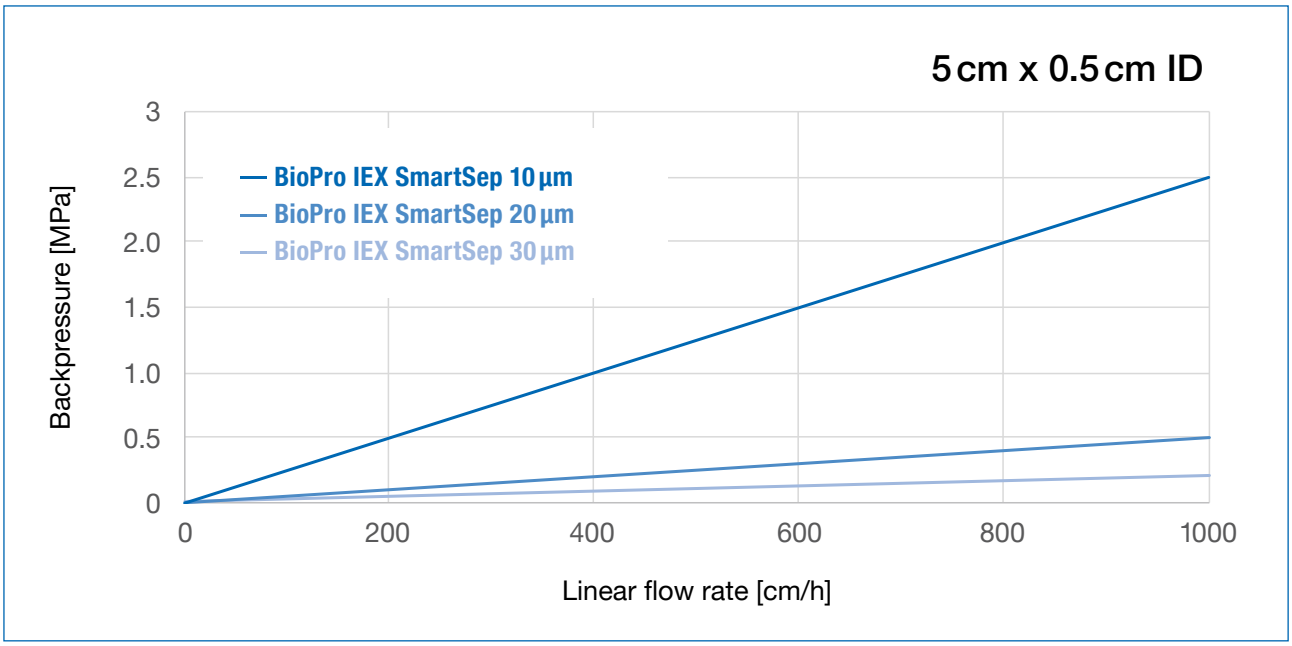


Figure 7: Pressure-flow characteristics for BioPro IEX SmartSep.

Important Check List

Before choosing a phase for a method screening in preparative LC, the following check list might be helpful:

- **1. Optimal particle technology**
- **2. Variable particle sizes available**
- **3. Reproducibility**
- **4. Scalability**
- **5. Supply guarantee**

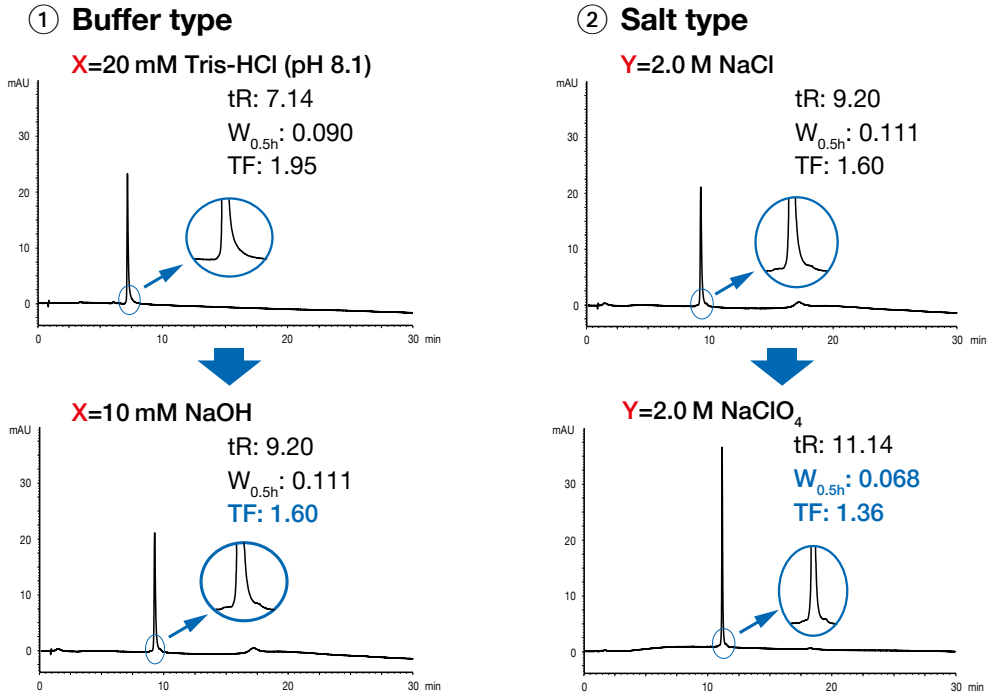
2.2. Mobile Phase

2.2.1. Choice of Buffer and Counter Ion

In addition to the stationary phase, the mobile phase has a great influence on oligonucleotide chromatography. During method development, it is worth trying different buffers and salt types as both can significantly influence peak shape and the subsequent resolution. Different buffers and salt combinations are suitable, but Tris-HCl and NaOH based buffers mixed with NaCl and $NaClO_4$ are typically used.

Figure 8 shows the comparison of Tris-HCl and NaOH buffer. The sample used is an RNA 20mer sample. By selecting the NaOH buffer, the mobile phase pH was increased from 8.1 up to 12 which was beneficial for the ionisation states of the oligonucleotide, resulting in improved tailing factors.

In a second step, the counter ion was changed in order to improve the tailing factor further. By replacing NaCl with $NaClO_4$ the tailing was further suppressed and higher sensitivities could be obtained. The gradient profile for $NaClO_4$ was adjusted because the elution strength of $NaClO_4$ is two to three times greater than that of NaCl in IEX. Especially, when high resolution is required the choice of an appropriate buffer and salt can be very beneficial.



Eluent: A) X
B) X containing 2.0 M NaCl
Gradient: 15–100%B (0–30 min)

Eluent: A) 10 mM NaOH
B) 10 mM NaOH containing 2.0 M Y
Gradient: 15–100%B (0–30 min) for NaCl
5–50%B (0-30 min) for $NaClO_4$

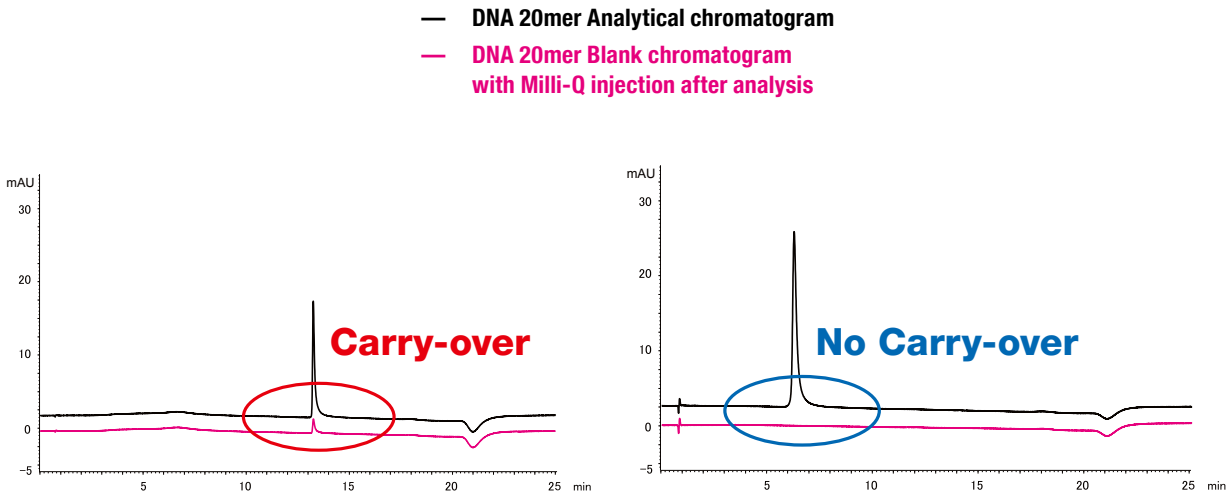
Column: BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part No.: QF00S05-1046WP
Flow rate: 1.0 mL/min
Temperatur: 25 °C
Detection: UV at 260 nm
Injection: 2 µL (10 nmol/mL)
Sample: 5'-UCAUCACACUGAAUACCAAU-3' (RNA 20mer)

Figure 8: Improved peak shapes by using different buffer and salt types.

Check the pH stability of your sample if you want to work with high pH mobile phases to prevent degradation, especially for long oligonucleotides.

2.2.2. Ionic Strength

The previous example already emphasised the importance of using of an appropriate salt type. In addition, the ionic strength plays a key role. It controls secondary interactions. Higher initial ionic strength can prevent carry-over effects. However, it must be noted that a high salt concentration can decrease the solubility of the analyte and decrease the retention time.



A) 20 mM Tris-HCl (pH 8.1)
B) 20 mM Tris-HCl (pH 8.1) containing 1.0 M NaCl
5–70%B (0–15 min), 74%B (15–18 min), 5%B (18–33 min)
Initial : 50 mM NaCl

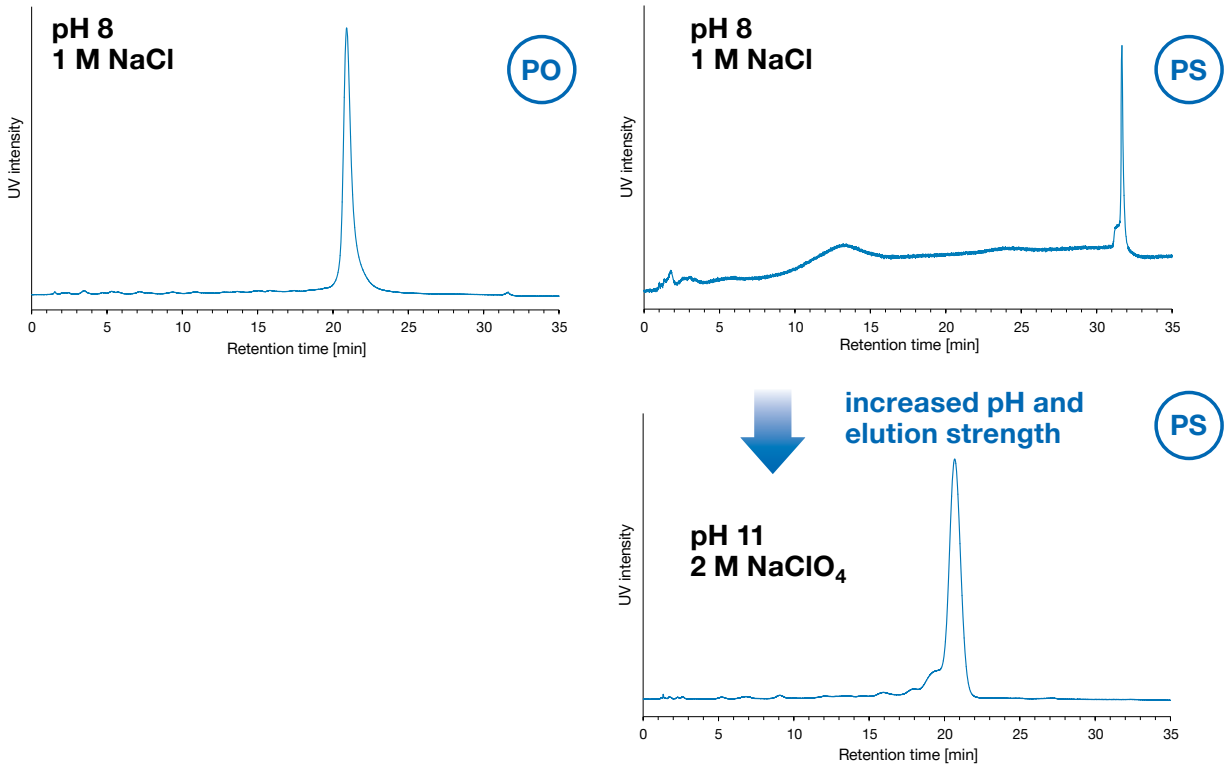
A) 20 mM Tris-HCl (pH 8.1)
B) 20 mM Tris-HCl (pH 8.1) containing 1.0 M NaCl
40–70%B (0–15 min), 74%B (15–18 min), 40%B (18–33 min)
Initial : 400 mM NaCl

Column:	BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part No.:	QF00S05-1046WP
Flow rate:	1.0 mL/min
Temperature:	25°C
Detection:	UV at 260 nm
Injection:	2 µL (10 nmol/mL)
Sample:	5'-TCATCACACTGAATACCAAT-3' (DNA 20mer)

Figure 9: Influence of initial ionic strength on carry-over behaviour.

Purification of Oligonucleotides: PS Modified Oligonucleotides Require High pH and Ionic Strength

A challenging task is the purification of phosphorothioate (PS) oligonucleotides. Their elevated negative charge leads to greater retention on the AEX resin. The following example shows the elution profile of an DNA oligonucleotide with a complete phosphodiester backbone (PO) and a phosphorothioate backbone. The PO oligonucleotide shows good elution results using a slightly alkaline buffer (pH 8.0) and 1 M NaCl for elution. In contrast, the same conditions lead to a massive increase in retention for the PS modified oligonucleotide. These conditions are not suitable for purification of the molecule. Therefore, the pH as well as the ionic strength of the mobile phase needed to be increased. The modified conditions with 10 mM NaOH and 2 M $NaClO_4$ gave to an improvement of the oligonucleotide elution.



Column Dimension:	100 × 4.6 mm ID
Resin Part No.:	BioPro IEX SmartSep Q10 (10 µm): QSA0S10
Eluent:	A) 10 mM Tris (pH 8)
	B) 10 mM Tris (pH 8) containing 1 M NaCl or 10 mM NaOH containing 2 M $NaClO_4$
Gradient:	20–70%B (0–30 min), 70–100%B (30–35 min) or
	35–70%B (0–30 min), 70–100&B (30–35 min) or
	20–40%B (0–30 min), 40–100%B (30–35 min)
Flow rate:	0.2 mL/min
Temperature:	25 °C
Detection:	UV at 260 nm
Sample:	5'-TCT CTA TCA ATA TCG TGG-3' (DNA 18mer)

Figure 10: Different requirements for the elution of PO and PS oligonucleotides.

For the purification of PS modified oligonucleotides, high pH and ionic strength are beneficial and can improve the elution of such high-binding molecules.

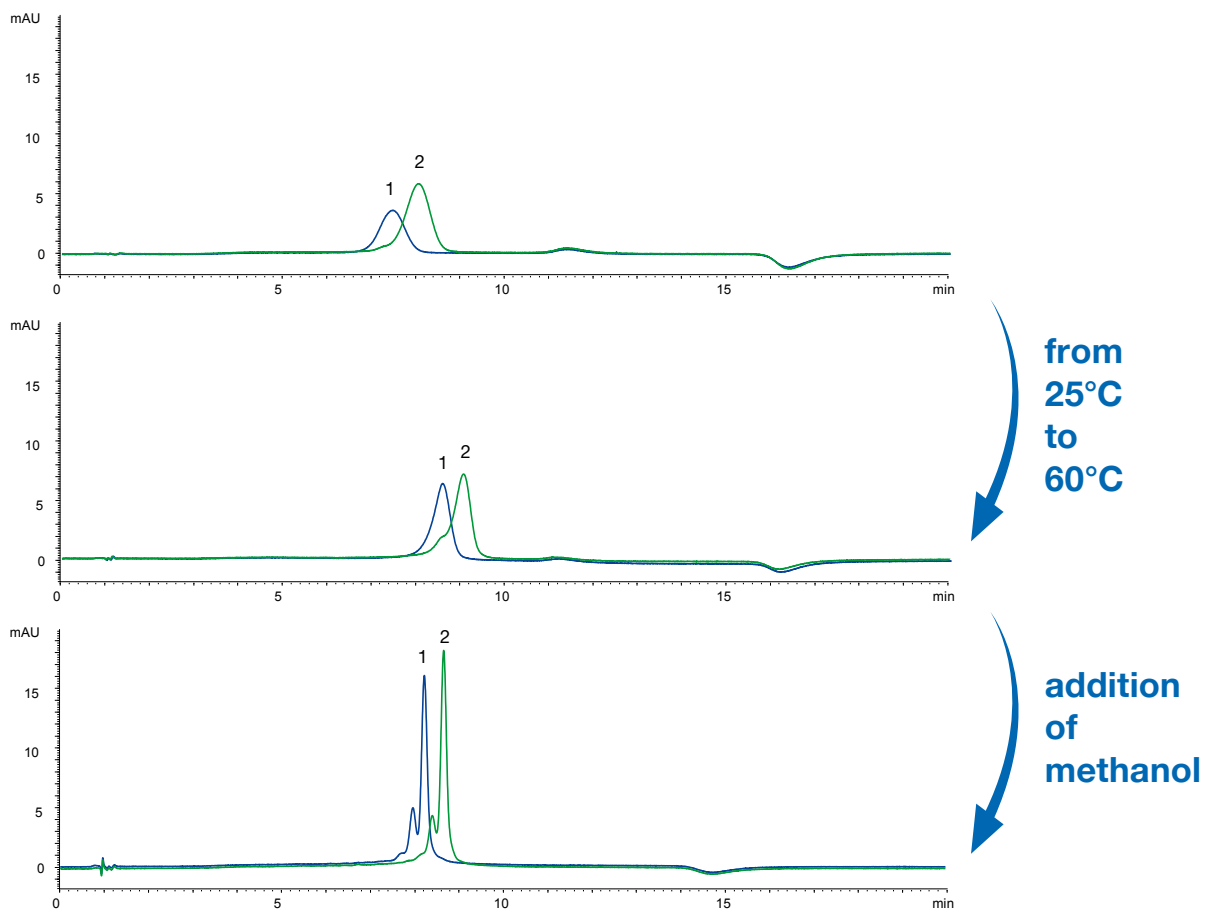
The total net charge increases with the oligonucleotide length as more negatively charged phosphates are added to the oligonucleotide backbone. This is why especially long oligonucleotides require a high ionic strength at the end of the gradient.

2.2.3. Organic Modifier and Temperature

Elevated temperature and the use of an organic modifier can influence the oligonucleotide chromatography (Figure 11). Phosphorothioated RNA samples with only a single base difference in length are ideally suited to show the benefit. By increasing the temperature from 25 to 60 °C narrower peaks and improved resolution can be obtained. The elevated temperature enhances the mass transfer and minimises the influence of the oligonucleotides' secondary structure on the retention.

In the following steps, methanol was added to the mobile phase and the gradient was adjusted.

Both the resolution and sensitivity could be improved and even narrow peaks with partly resolved variants could be detected.



Column:	BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part No.:	QF00S05-1046WP
Eluent:	*Upper and middle chromatogram:*
	A) 10 mM NaOH/methanol (100/0)
	B) 10 mM NaOH containing 1.0 M $NaClO_4$/methanol (100/0)
	Bottom chromatogram:
	A) 10 mM NaOH/methanol (70/30)
	B) 10 mM NaOH containing 1.0 M $NaClO_4$/methanol (70/30)
Gradient:	*Upper and middle chromatogram:*
	32–80%B in 8 min
	Bottom chromatogram:
	40–100%B in 6.3 min
Flow rate:	1.0 mL/min
Temperature:	*Upper and middle chromatogram:*
	25 °C
	Bottom chromatogram:
	60 °C
Detection:	UV at 260 nm
Injection:	2 µL (10 nmol/mL)
Sample:	1: RNA 20mer 5'-U~C~A~U~C~A~C~A~C~U~G~A~A~U~A~C~C~A~A~U-3'
	2: RNA 21mer 5'-G~U~C~A~U~C~A~C~A~C~U~G~A~A~U~A~C~C~A~A~U-3'
	~phosphorothioate

Figure 11: Influence of temperature increase and the addition of organic modifier.

Purification of Oligonucleotides: Elevated Temperatures

Elevated temperatures can also be beneficial for preparative processes. If the overall system allows elevated temperatures, the elution of the oligonucleotide can be improved, especially for strongly binding molecules. However, the optimal temperature depends on the oligonucleotide itself and the benefit of elevated temperatures has to be considered carefully regarding the overall cost-efficiency and the thermal stability of the target oligonucleotide.

Purification of Oligonucleotides: Organic Modifiers for Improved Elution

Generally, aqueous buffer systems are used for the purification of biomolecules via IEX but for the purification of oligonucleotides, high demands on the separation efficiency are required. The addition of an organic modifier to the mobile phase can improve the elution depending on the oligonucleotide and the critical impurities. Typically, acetonitrile or isopropanol is added and their benefit on the elution is investigated. The process environment, the preparative system as well as the column hardware and the resin itself have to be compatible with such conditions.

2.2.4. Gradient Slope

Every oligonucleotide is different and so are the requirements for the chromatographic separation. This is why the method development is more complex compared to the development of separation methods for other target molecules. In principle, fast and steep gradients provide sharp peaks but the peaks are eluted closely together.
By decreasing the slope, the resolution increases, however, the peaks become broader and elution takes longer. The trade-off between sharp peaks and high resolution has therefore to be considered thoroughly, especially when thinking of oligonucleotide purifications. High resolution means higher efficiency and any increase in resolution is beneficial. Using a shallower gradient, the resolution might be increased by 19% (see Figure 13) for DNA samples with differences in the type of base of 5' terminal base and in length (20 and 21mer).

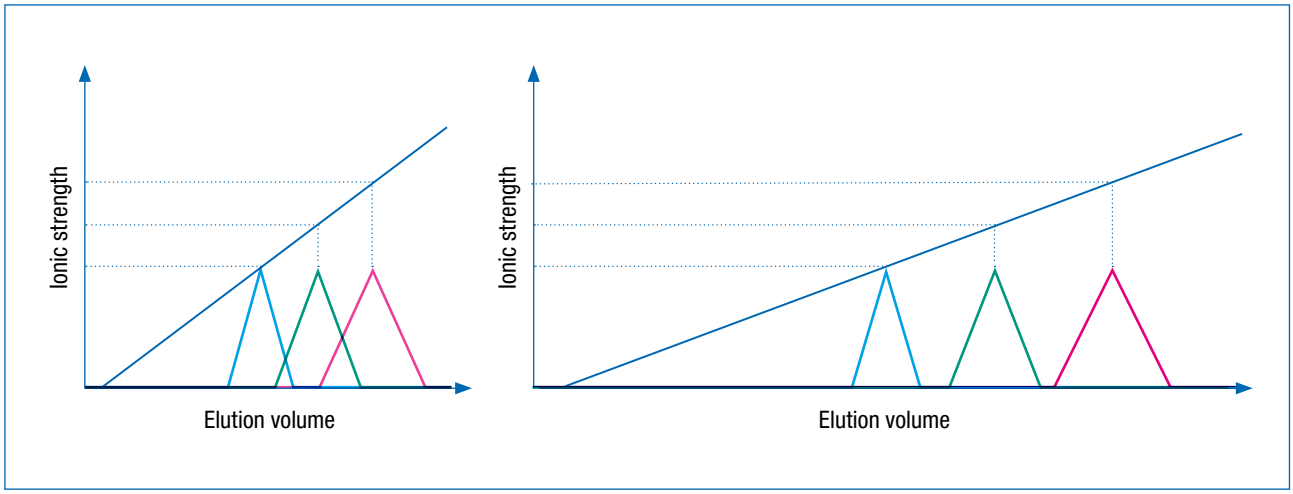
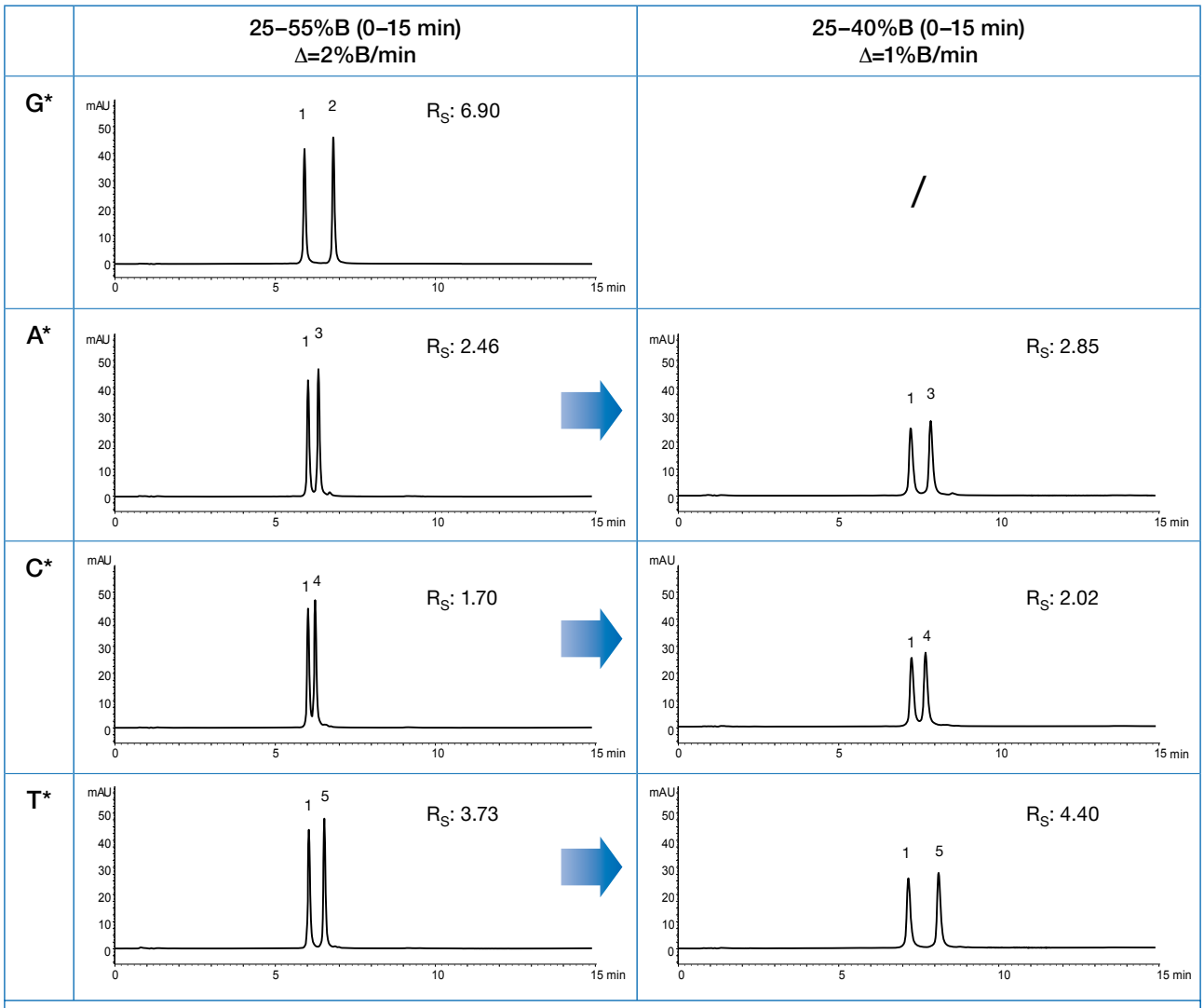


Figure 12: Influence of gradient slope on resolution and peak shape.



Column: BioPro IEX QF (5 µm) 100 x 4.6 mm ID
Part No.: QF00S05-1046WP
Eluent: A) 10 mM NaOH
B) 10 mM NaOH containing 1.0 M $NaClO_4$
Flow rate: 1.0 mL/min
Temperature: 25 °C
Detection: UV at 260 nm
Injection: 4 µL (5 nmol/mL each)
Sample: 1: 5'-TCATCACACTGAATACCAAT-3' (DNA 20mer)
2: 5'-GTCATCACACTGAATACCAAT-3' (DNA 21mer)
3: 5'-ATCATCACACTGAATACCAAT-3' (DNA 21mer)
4: 5'-CTCATCACACTGAATACCAAT-3' (DNA 21mer)
5: 5'-TTCATCACACTGAATACCAAT-3' (DNA 21mer)

*base of 5'end of DNA 21mer

Figure 13: Gradient optimisation for DNA samples with 5' terminal base differences.

Purification of Oligonucleotides: Gradient Optimisation

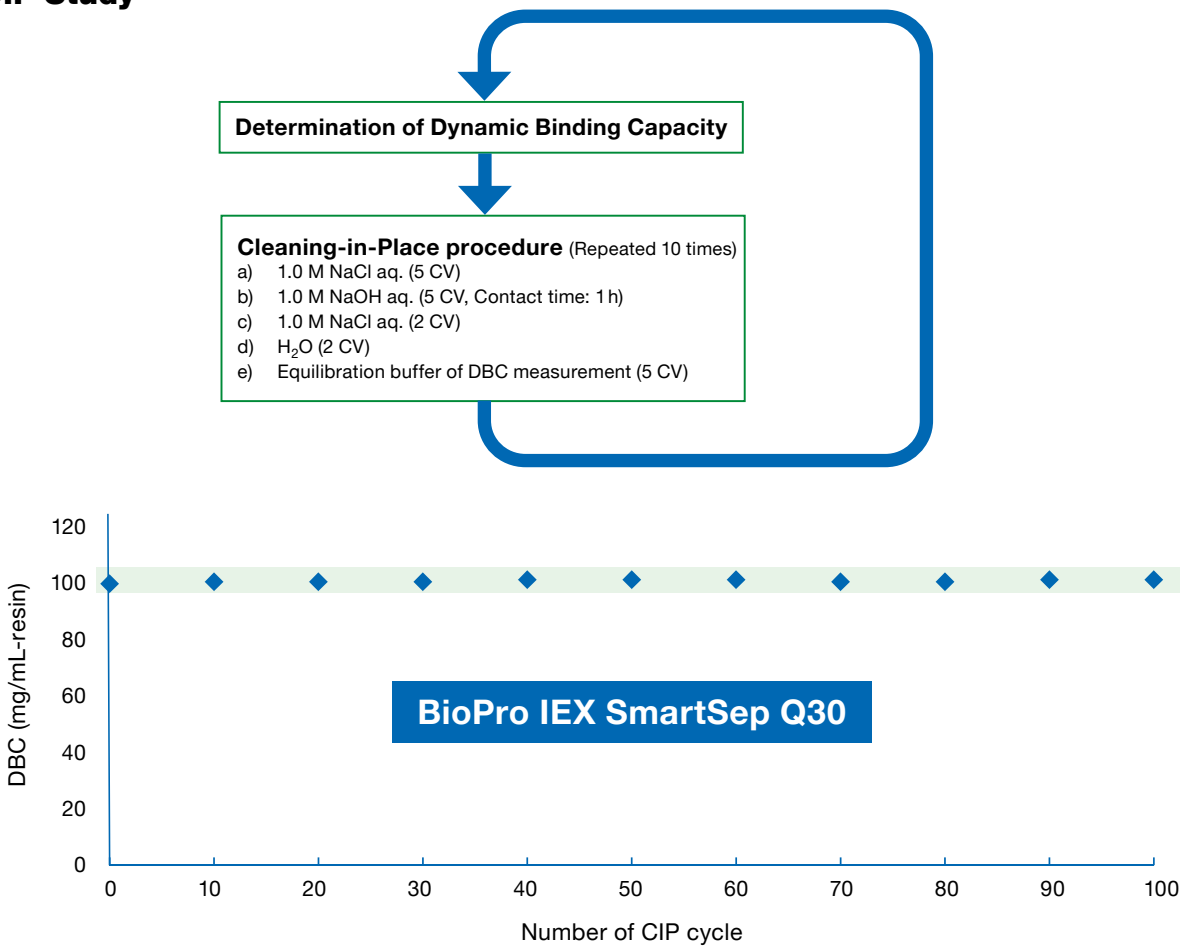
Detailed information on the optimisation of gradient elution in IEX can be found in the whitepaper about method development using IEX.

2.3. Cleaning-in-Place

Efficient Cleaning-in-Place (CIP) procedures are needed for productive and cost-efficient oligonucleotide purifications. The purification of biomolecules such as oligonucleotides leads to non-specific adsorption and precipitation on the resin over time. Aqueous native buffers add the risk of microbial growth. Cleaning procedures with alkaline solutions remove the precipitations and non-specific adsorbed substances efficiently and additionally sanitise the column. Typically, high alkaline solutions up to 1 M NaOH are used. These conditions are highly effective to increase the column lifetime. Consequently, process resins that are stable towards the continuous application of these conditions are needed.

The regularly applied flushing with alkaline solutions has an additional benefit: endotoxins, a major impurity in oligonucleotide purification are efficiently removed.

CIP Study



Column Dimension:	50 × 5.0 mm ID	Flow rate:	200 cm/h (0.66 mL/min)
Resin Part No.:	BioPro IEX SmartSep Q30 (30 µm): QSA0S30	Temperature:	25 °C
Equilibration buffer:	20 mM Tris-HCl (pH 8.6)	Detection:	UV at 280 nm
Elution buffer:	Equilibration buffer containing 0.5 M NaCl	Sample:	1.5 mg/mL BSA in equilibration buffer

CIP cycle

Column Dimension:	50 × 5.0 mm ID
Flow rates:	200 cm/h (1.0 M NaCl, H_2O, Buffer)
	30 cm/h (1.0 M NaOH)
Temperature:	25 °C

Figure 14: Stability of BioPro IEX SmartSep Q30 under alkaline CIP conditions.

2.4. YMC Stationary Phases for AEX

	BioPro IEX QF	BioPro IEX QA	BioPro IEX SmartSep Q10	BioPro IEX SmartSep Q20	BioPro IEX SmartSep Q30
Base Particle	hydrophilic polymer (polymethacrylate)				
Ion Exchange Type	strong anion exchanger				
Charged Group	quaternary amine				
Pore Size [nm]	non-porous	porous			
Particle Size [µm]	3; 5	5	10	20	30
Counter Ion	Cl^-				
pH Range	2.0–12.0				
Temperature Range	4–60 °C				
Pressure Limit	3 µm: 18–25 MPa 5 µm: 6–12 MPa	2.5–3.5 MPa	4.0 MPa	3.0 MPa	3.0 MPa
Available Formats	pre-packed columns	pre-packed columns	bulk resin + pre-packed columns		
Ion Exchange Capacity	N/A	N/A	min. 0.08 meq/mL-resin		
Dynamic Binding Capacity	N/A	N/A	> 100 mg BSA/ mL Resin		
Typical Flow Rate	5 µm: 0.2–0.8 mL/min (72–289 cm/h)* 3 µm: 0.2–0.5 mL/min (72–181 cm/h)*	0.4–0.5 mL/min (144–181 cm/h)*	200–1,000 cm/h Max. 2,000 cm/h		

*for a 100 x 4.6 mm ID column

3. IP-RP

3.1. Stationary Phase

3.1.1. Particle Technology

For IP-RP oligonucleotide separations, stationary phases with different base particles can be used. Fully porous or core-shell based silica and hybrid silica particles are the most typical phases used. Hybrid silica particles are the preferred choice, due to their higher mechanical and chemical stability. High pH mobile phase conditions or performing alkaline cleaning studies oblige to use hybrid silica resins.

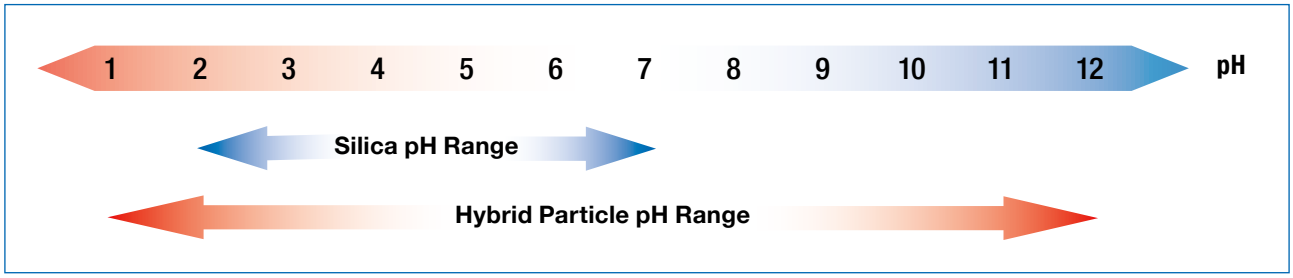
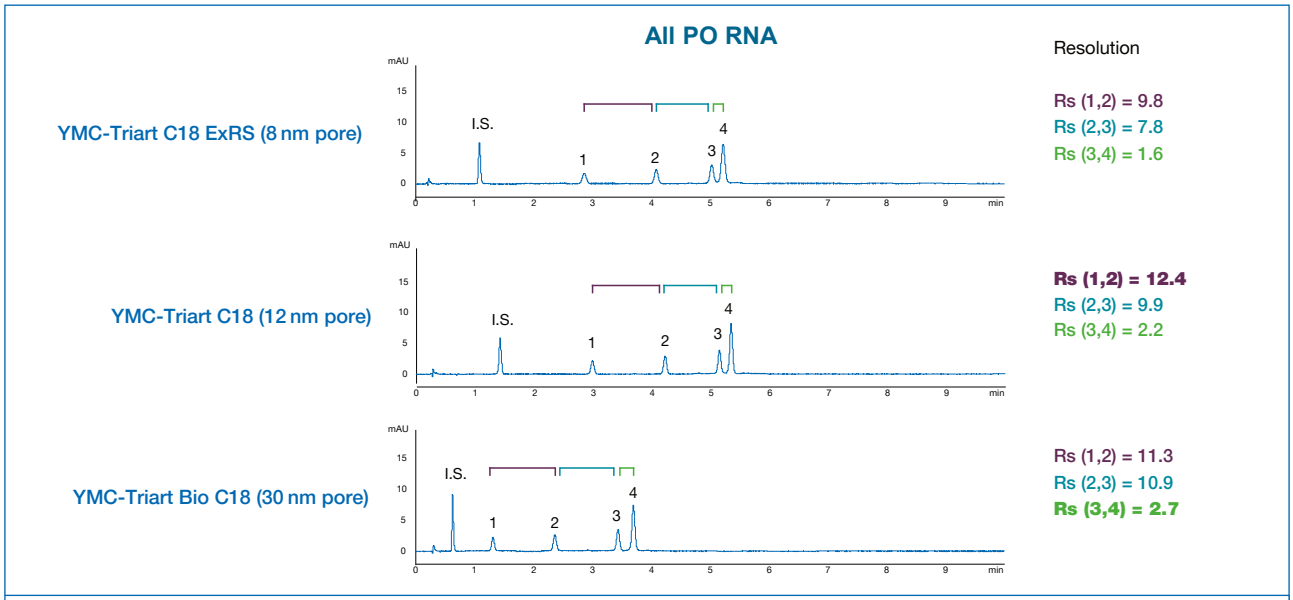


Figure 15: Applicable pH range for silica and hybrid silica particles.

3.1.2. Modifications and Selectivity

The selectivity is the most powerful tool to increase the resolution. In IP-RP oligonucleotide separations, alkyl phases are most commonly used, especially C18 or C8 modified phases but also C4 phases may be required sometimes.

Also, different C18 columns provide varying results as shown for all PO RNAs in Figure 16. Pore size and hydrophobicity play an important role, which is further discussed in section 3.1.3.

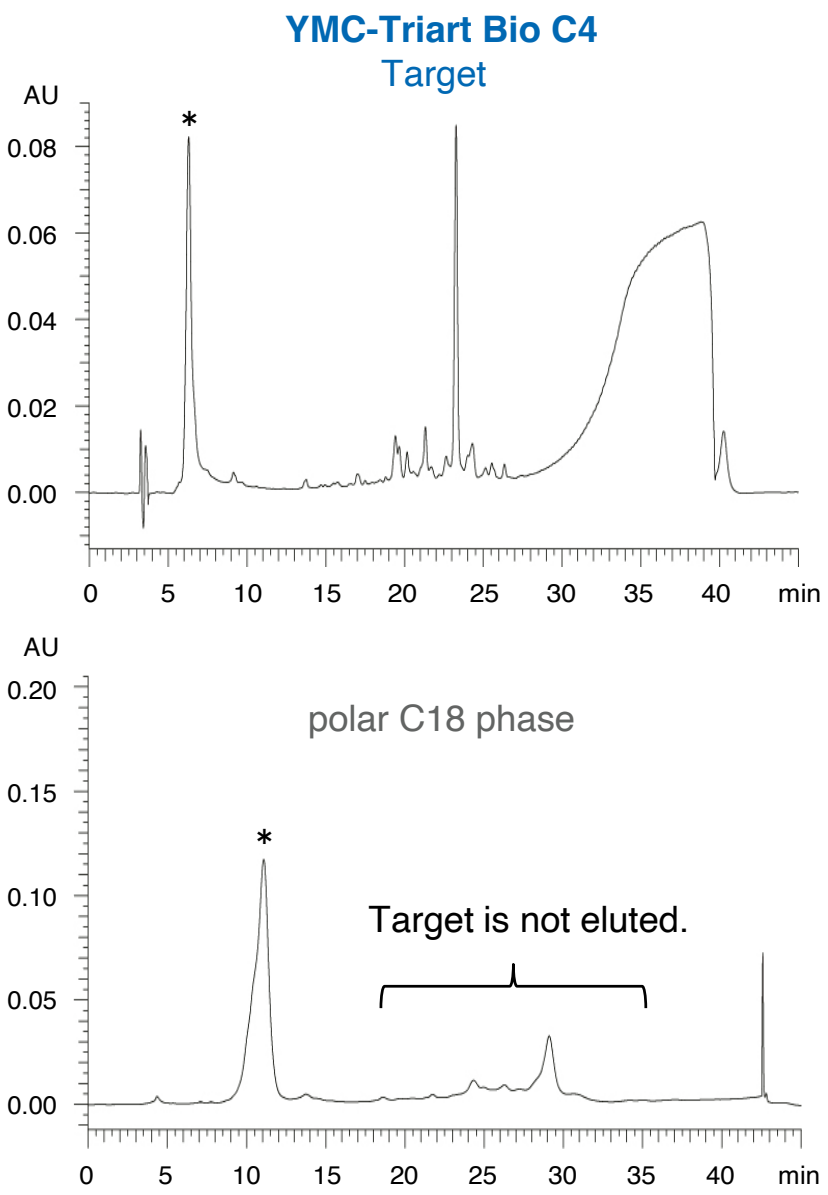


Columns:	YMC-Triart C18 ExRS (3 µm, 8 nm) 50 x 2.1 mm ID YMC-Triart C18 (3 µm, 12 nm) 50 x 2.1 mm ID YMC-Triart Bio C18 (3 µm, 30 nm) 50 x 2.1 mm ID	Flow rate:	0.42 mL/min
Part Nos.:	TAR08S03-05Q1PTH TA12S03-05Q1PTH TA30S03-05Q1PTH	Temperature:	65°C
		Detection:	UV at 260 nm
		Injection:	1 µL
Eluent:	A) 8 mM TEA, 200 mM HFIP B) methanol	Sample:	1. 5'-CACUGAAUACCAAU-3' (14mer) 2. 5'-UCACACUGAAUACCAAU-3' (17mer) 3. 5'-UCAUCACACUGAAUACCAAU-3' (20mer) 4. 5'-GUCAUCACACUGAAUACCAAU-3' (21mer) I.S. caffeine
Gradient:	10–20%B (0–10 min)		

Figure 16: Influence of C18 modification of all PO RNA analysis.

Even though C18 columns are most commonly used, considering other selectivities can be helpful when facing challenging analytes. In Figure 17 the separation of disulfide modified oligonucleotides is shown using both a polar C18 and a C4 stationary phase.

When using the C18 column, the target could not be eluted as the hydrophobic character of the disulfide units require a less hydrophobic stationary phase. Good peak shapes could be obtained using a widepore C4 phase.



Column:	5 µm, 250 x 4.6 mm ID
Product code:	TB30S05-2546PTH HS12S05-2546WT
Eluent:	A) 50 mM TEAA* (pH 7.0)/acetonitrile (95/5) B) acetonitrile
Gradient:	5–95%B (0-30 min), 95%B (30–35 min), 95–5%B (35–35.1 min), 5%B (35.1–45 min)
Flow rate:	1 mL/min
Temperature:	50 °C
Detection:	UV at 260 nm
Sample:	Crude reaction mixture

*Triethylammonium acetate

By courtesy of Saki Kawaguchi, Chemistry Department, Nagoya University, Japan

Figure 17: Separation of oligonucleotides modified with disulfides using C18 and C4 modified stationary phases.

3.1.3. Pore Size

The stationary phase pore size has a big influence on the chromatographic results in RP processes, as it determines the accessibility of the specific surface area and influences the hydrophobicity of the stationary phase. On the one hand, the pore has to be large enough so that the target oligonucleotide can enter the pores. On the other hand, it needs to be small enough in order to allow sharp peaks and a good resolution.

As oligonucleotides can vary in their length from just a few up to multiple hundred nucleotides, different pore sizes need to be evaluated. In general, the pore size is recommended to be as the smallest possible and the largest necessary. Generally, for short single-stranded oligonucleotides without any bulky modifications, a smaller pore size is recommended. For larger oligonucleotides up to multiple hundreds of nucleotides in length, larger pore sizes are required for an efficient separation. The pore size selection tool (Figure 18) gives a general overview on the recommended pore size based on the length of single-stranded oligonucleotides.

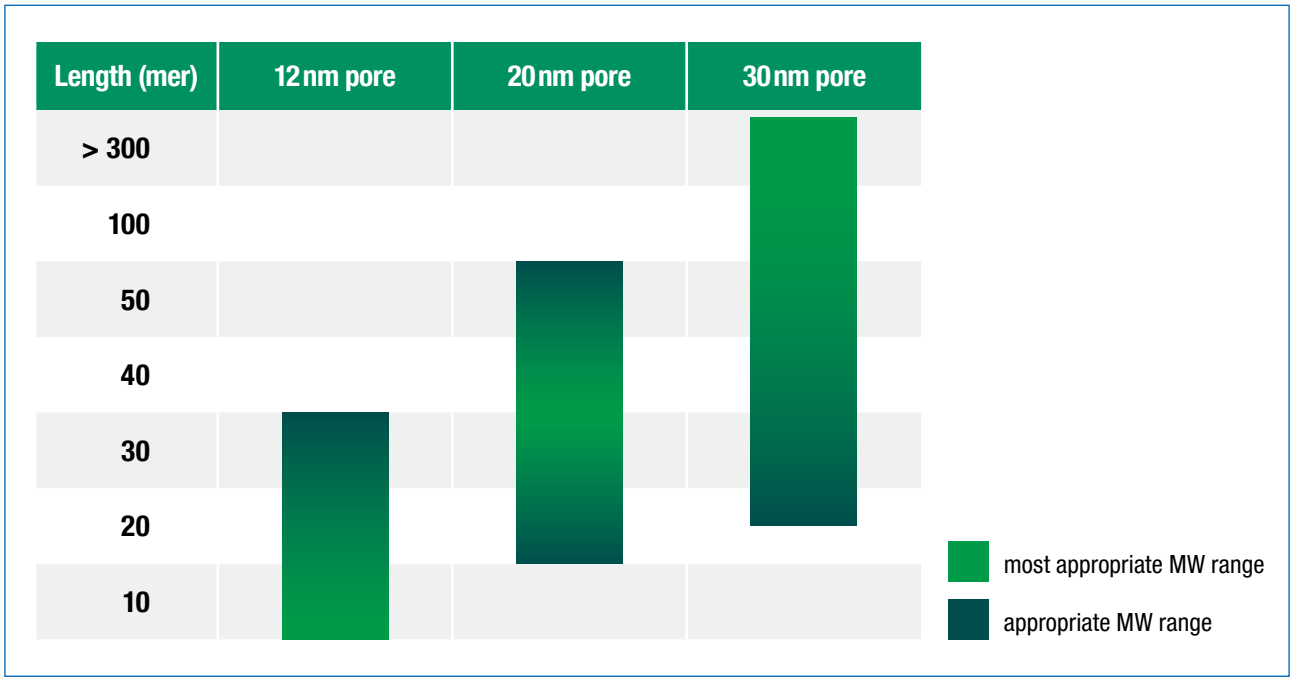
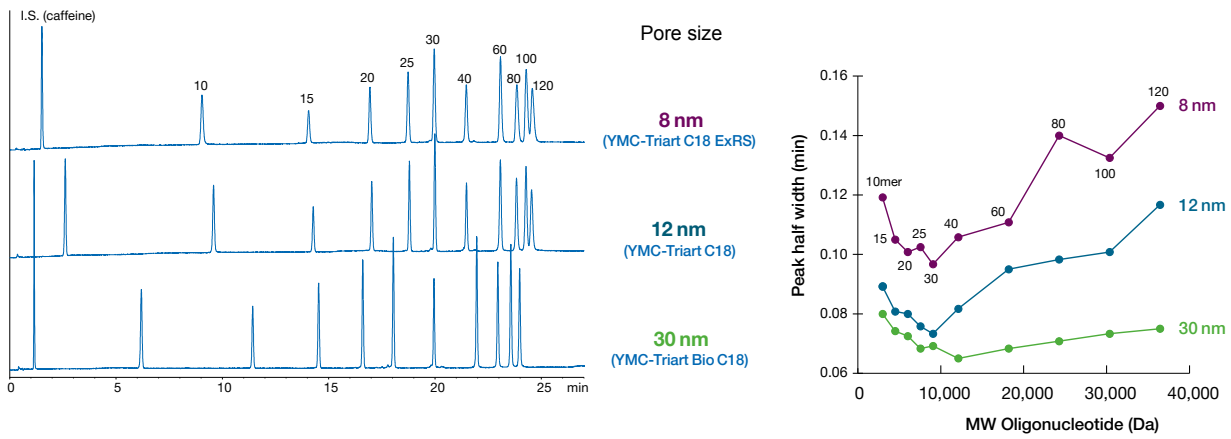


Figure 18: Pore size selection tool.

These values are only recommendations and the limits are only approximate, as potential structures or modifications can lead to different requirements. Therefore, the testing of different pore sizes can be useful.

In this example, several pore sizes have been tested for 10–120mer poly dT DNA strands. It showed that the 8 nm pore was inappropriate for oligonucleotide separation at all, as it showed an increased peak half width for the entire oligonucleotide lengths. This may be caused by the non-accessibility due to the small pore size. The 12 nm pore size showed comparable behaviour to 30 nm for short oligonucleotides. For 30mer or more, the 30 nm pore was the best choice as the oligonucleotides could fully permeate the pore and interact with the bonded C18 phase.



Columns:	YMC-Triart C18 ExRS (3 µm, 8 nm) 50 x 2.1 mm ID
	YMC-Triart C18 (3 µm, 12 nm) 50 x 2.1 mm ID
	YMC-Triart Bio C18 (3 µm, 30 nm) 50 x 2.1 mm ID
Part Nos.:	TAR08S03-05Q1PTH
	TA12S03-05Q1PTH
	TA30S03-05Q1PTH
Eluent:	A) 4 mM TEA, 100 mM HFIP
	B) methanol
Gradient:	5–20%B (0–30 min)
Flow rate:	0.42 mL/min
Temperature:	65 °C
Detection:	UV at 260 nm
Injection:	1 µL (0.2–2.0 µM)
Sample:	Poly dT (10–120mer)

Figure 19: Influence of the stationary phase pore size on peak width.

Remember that the molecular weight of double-stranded oligonucleotides can be nearly double the size of a single-stranded oligonucleotide with the same length. Modifications increase the molecular weight further. Potential secondary structures of the molecule can have an additional influence on the pore accessibility.

3.1.4. Particle Size

It is well known that by decreasing the particle size, the resolution increases, but with an increase in the backpressure. In analytical scale, 3 and 5 µm particle sizes are typically used for HPLC, whereas sub 2 µm particles are used for highly sensitive UHPLC analyses.

Purification of Oligonucleotides: Selection of the Appropriate Particle Size

In preparative chromatography, particle sizes such as 7, 10 or 20 µm are commonly used in RP separations. Particle sizes such as 7 or 10 µm provide higher resolution, but with higher backpressures (see chapter 2.1.3.). Therefore, the selection of the optimal particle size depends on the required resolution as well as on the preparative system that is used for purification. A stationary phase available with different particle sizes provides an increased flexibility in IP-RP processes development. YMC-Triart (Prep) for example is available with completely scalable particle sizes – from analytical to preparative scale.

Important Check List

Before choosing a stationary phase for analytical methods or purification processes, the following check list might be helpful:

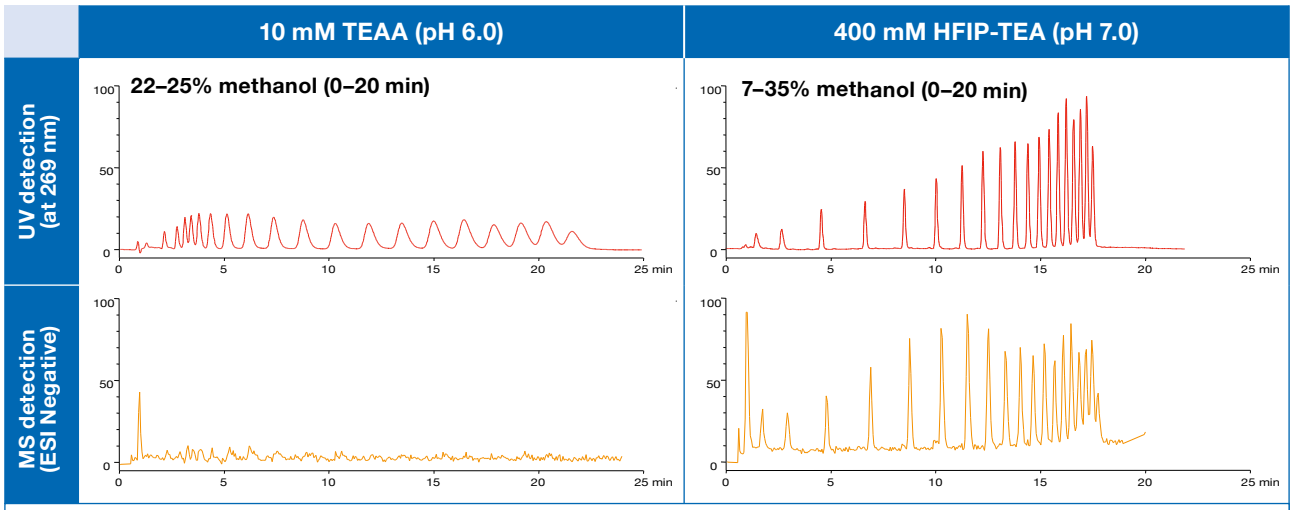
1. **Optimal particle technology**
2. **Variable particle sizes available**
3. **Reproducibility**
4. **Scalability**
5. **Supply guarantee**

3.2. Mobile Phase

3.2.1. Choice of Buffer System

Sensitivity and resolution strongly depend on the mobile phase composition. Therefore, not every buffer system is appropriate for use in oligonucleotide chromatography. On an analytical scale, the use of fluoroalcohols with alkyl amines such as HFIP-TEA (1,1,1,3,3,3-hexafluoro-2-propanol-triethylamine) is the gold standard as it provides a good sensitivity and reproducible chromatographic results in combination with UV detection as well as with MS detection. Figure 20 shows the UV (red) and MS (orange) traces of separations of oligodeoxythymidylic acid d(pT)2–20 using different buffer systems. HFIP-TEA provides much higher sensitivity and better resolution compared to triethylammonium acetate (TEAA). However, it is immiscible with acetonitrile.

Alternative alkyl amine acetates such as DBAA (dibutylammonium acetate) or TEAA buffers can also be used, but higher buffer concentrations may provide increased resolution and stronger retention (see Figure 21). On the contrary, lower concentrations reduce system contamination in (U)HPLC-MS analyses.



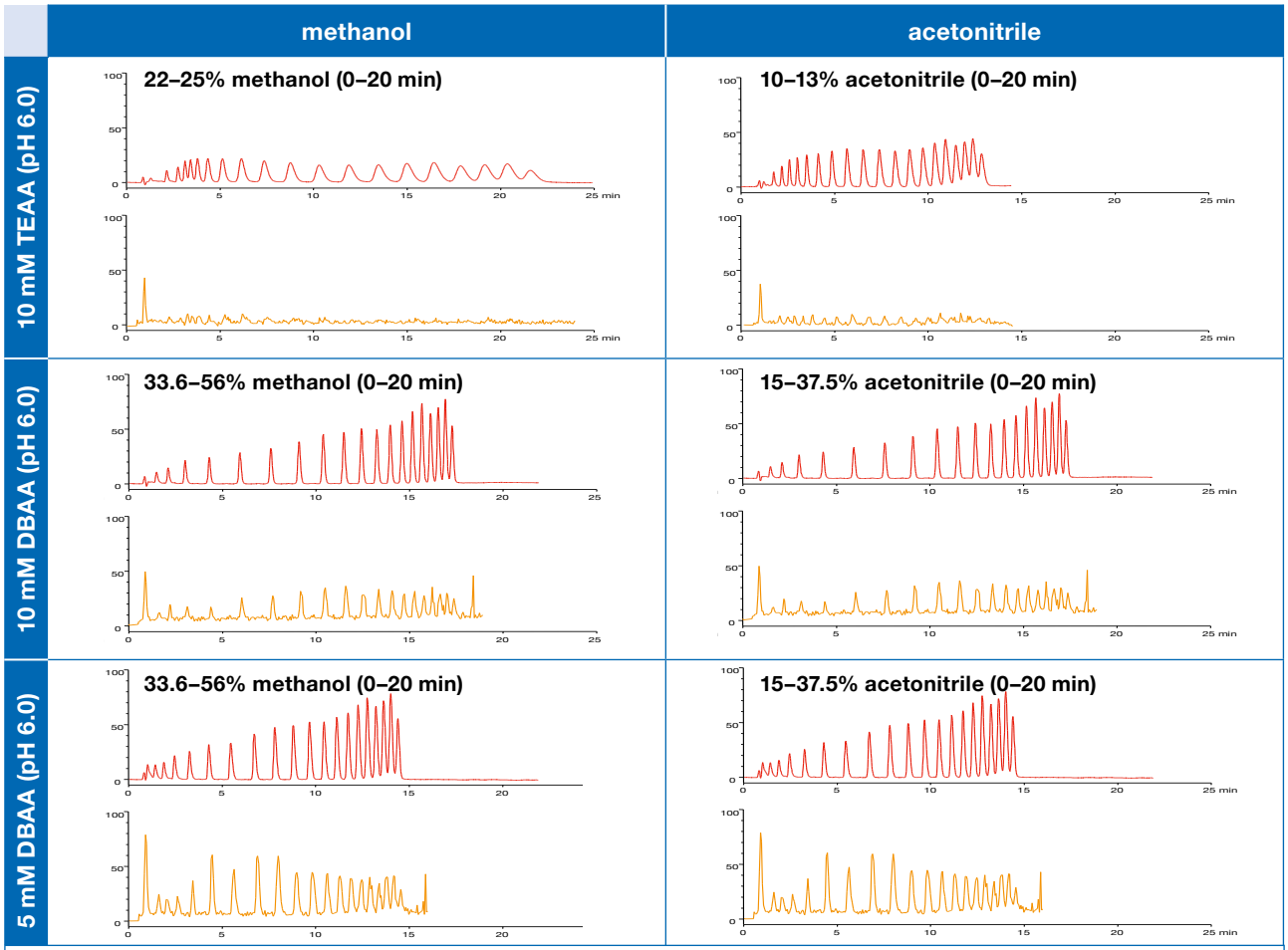
Column:	Hydrosphere C18 (3 µm, 12 nm) 50 x 2.0 mm ID
Part. No.:	HS12S03-0502WT
Flow rate:	0.2 mL/min
Detection:	UV at 269 nm (red), ESI negative mode (orange)
Temperature:	35°C
Injection	5 µL (25 pmol/mL)
Sample:	Oligo(deoxythymidylic acid) [$d(pT)_{2-20}$]

Figure 20: Signal intensity in UV and MS detection using different buffer systems.

In order to prevent contamination and to ensure reproducible results, buffers should be prepared daily.

Purification of Oligonucleotides: Cost-Efficient Buffer Systems and IP Reagents

For preparative applications, economic buffer systems are required, such as DBAA or TEAA. Generally, the cost-efficiency and safety are the major factors that influence the selection of suitable buffer systems and IP-reagents.



Column: Hydrosphere C18 (3 µm, 12 nm) 50 x 2.0 mm ID
Part. No.: HS12S03-0502WT
Flow rate: 0.2 mL/min
Detection: UV at 269 nm (red), ESI negative mode (orange)

Temperature: 35°C
Injection 5 µL (25 pmol/mL)
Sample: Oligo(deoxythymidylic acid) [$d(pT)_{2\text{-}20}$]

Figure 21: Signal intensity in UV and MS detection using different mobile phases.

Purification of Oligonucleotides: Efficient Desalting to Remove Buffer Salts Using RP

The purification of oligonucleotides requires the use of (buffer-) salts and IP reagents. If these salts need to be removed from the final product, the use of a RP column is a convenient strategy. Using the same column for both the separation and desalting saves investment costs and lowers the footprint of the process.

A big advantage can be a stationary phase which retains the target molecule under preferably 100% aqueous conditions such as YMC-Triart Prep C18-S and Phenyl-S. This will allow a cost-efficient desalting process as post-treatment after the purification run to be applied. The general principle is shown in Figure 22.

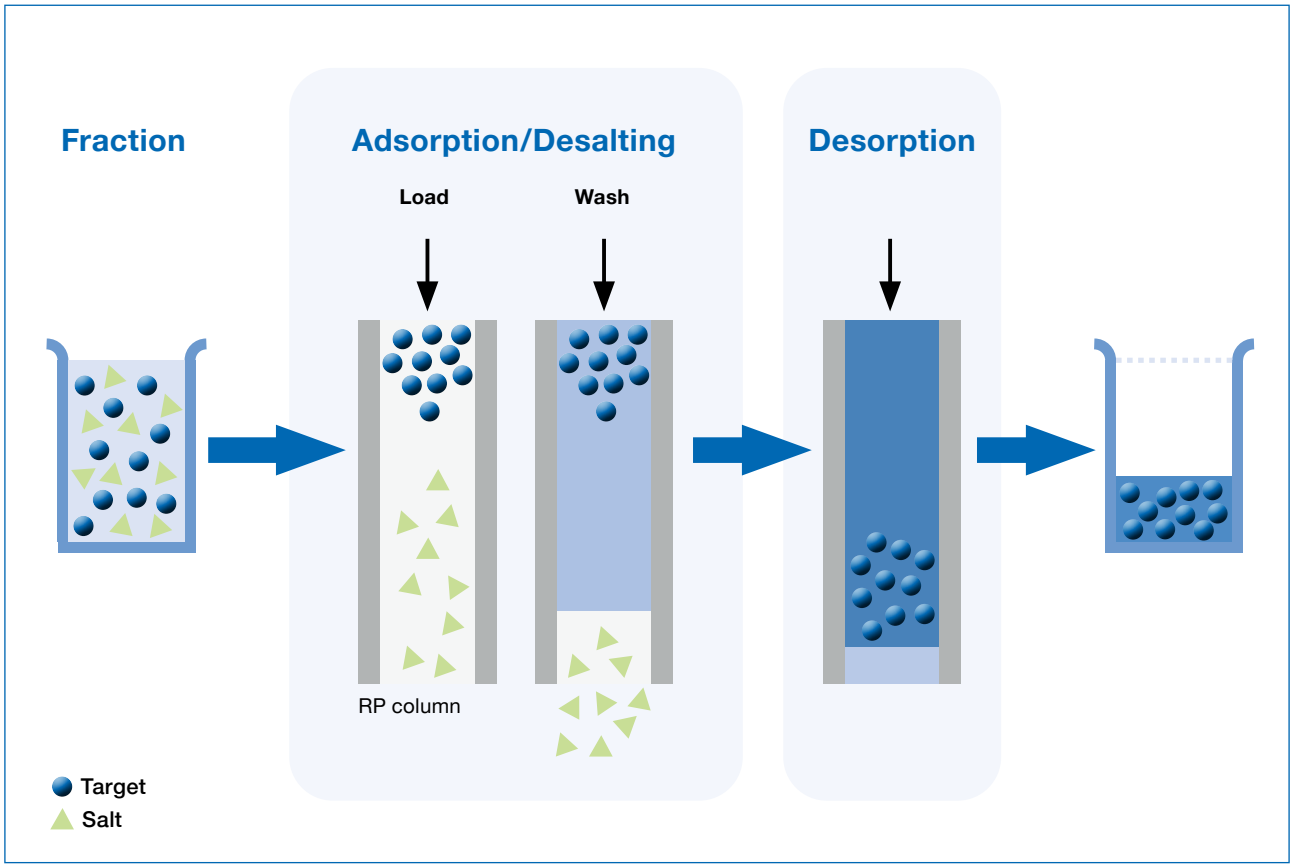


Figure 22: General desalting principle using an RP column.

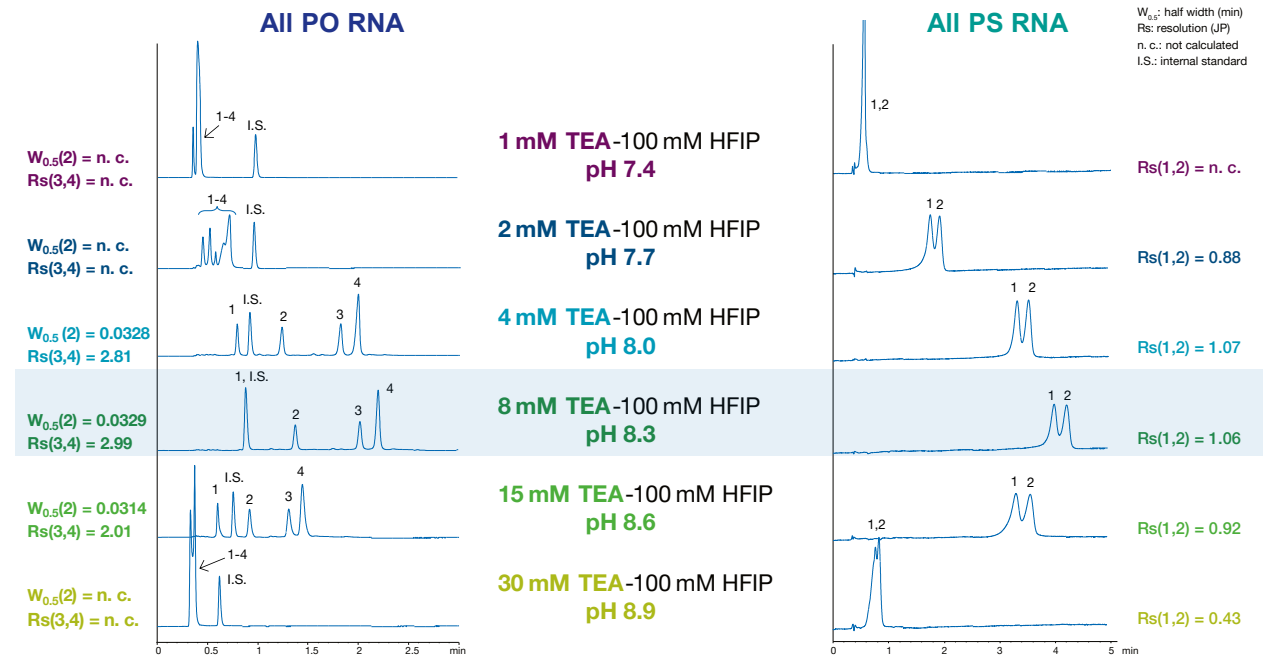
Desalting / Resalting

The detailed procedure of a desalting process using RP can be found in the technical note: Desalting/Resalting using a RP column.

3.2.2. High pH Stable Resins

In addition to the buffer composition and the selection of the most appropriate ion-pair reagent, the pH of the mobile phase is an important factor to influence the elution of the target compound. Depending on the target oligonucleotide, mobile phases with high pH can improve the elution. However, alkaline conditions are not possible for conventional silica-based RP stationary phases. Therefore, hybrid silica-based RP phases with an extended pH range are a great tool for efficient oligonucleotide separations – for analysis and preparative purifications.

The example below shows a screening of various concentrations of TEA and HFIP for all PO and PS RNA (Figure 24). In a first step, the concentration of HFIP was held constant at 100 mM, whilst the concentration of TEA was varied between 1 and 30 mM resulting in pH values between 7.4 and 8.9. Between 1 and 8 mM TEA, retention increased for both all PO and PS RNA with increasing pH. Using higher concentrations of 15 or 30 mM TEA, retention decreased again and resolution decreased which is a commonly observed for PO/PS oligonucleotides.



Column:	YMC-Triart Bio C18 (1.9 µm, 30 nm), 50 x 2.1 mm ID
Part No.:	TA30SP9-05Q1PT
Eluent:	A) buffer
	B) methanol
Gradient:	Initial: 8%B, gradient slope: 1%B/min
Flow rate:	0.42 mL/min
Temperature:	65 °C
Detection:	UV at 260 nm
Injection:	1 µL

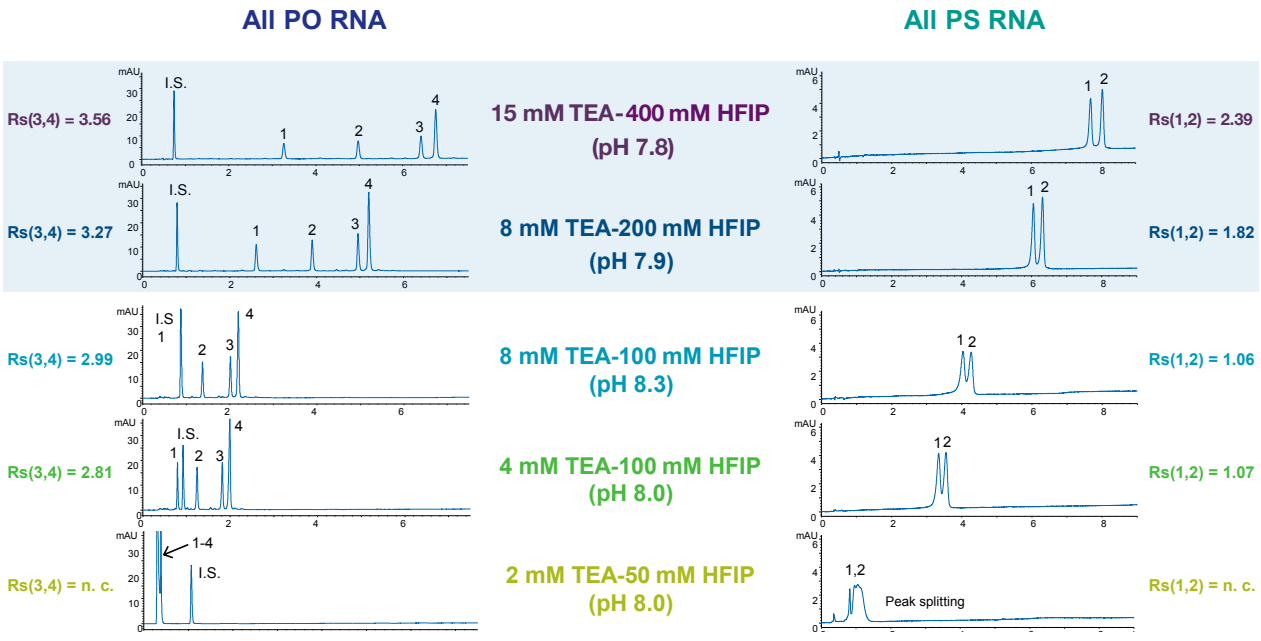
Sample:
all PO RNA:
1. 5'-CACUGAAUACCAAU-3' (14mer), 1.8 nmol/mL
2. 5'-UCACACUGAAUACCAAU-3' (17mer), 1.8 nmol/mL
3. 5'-UCAUCACACUGAAUACCAAU-3' (20mer), 1.8 nmol/mL
4. 5'-GUCAUCACACUGAAUACCAAU-3' (21mer), 3.6 nmol/mL
I.S. = caffeine

all PS RNA:
1. 5'-U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3' (20mer), 1.0 nmol/mL
2. 5'-G*U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3' (21mer), 1.0 nmol/mL
*=Phosphorothioated

Figure 23: Influence of the IP concentration on All-PO and All-PS RNA separations.

As TEA has a pKa value of 10.7, the number of ionised TEA and therefore the ion pairing functionality decreases at higher pH. Therefore, the optimal pH for analysing oligonucleotides using a TEA-HFIP system is pH=8.

In a second step, separations with varying TEA and HFIP concentrations at a pH around 8 were performed. This showed that higher TEA-HFIP concentrations provide better resolution. Whilst high TEA concentrations improve the retention, high HFIP concentrations are required to keep the pH value as low as possible. Especially for all PS RNA, peak shape got better and baseline resolution was achieved with 8 mM TEA – 200 mM HFIP or higher concentrations. Please note that the results are based on UV detection. Lower concentrations may be more favourable for MS detection due to ion suppression.



Column:	YMC-Triart Bio C18 (1.9 µm, 30 nm), 50 x 2.1 mm ID
Part No.:	TA30SP9-05Q1PT
Eluent:	A) buffer
	B) methanol
Gradient:	Initial: 8%B, gradient slope: 1%B/min
Flow rate:	0.42 mL/min
Temperature:	65 °C
Detection:	UV at 260 nm
Injection:	1 µL

Sample:
all PO RNA:
1. 5'-CACUGAAUACCAAU-3' (14mer), 1.8 nmol/mL
2. 5'-UCACACUGAAUACCAAU-3' (17mer), 1.8 nmol/mL
3. 5'-UCAUCACACUGAAUACCAAU-3' (20mer), 1.8 nmol/mL
4. 5'-GUCAUCACACUGAAUACCAAU-3' (21mer), 3.6 nmol/mL
I.S. = caffeine

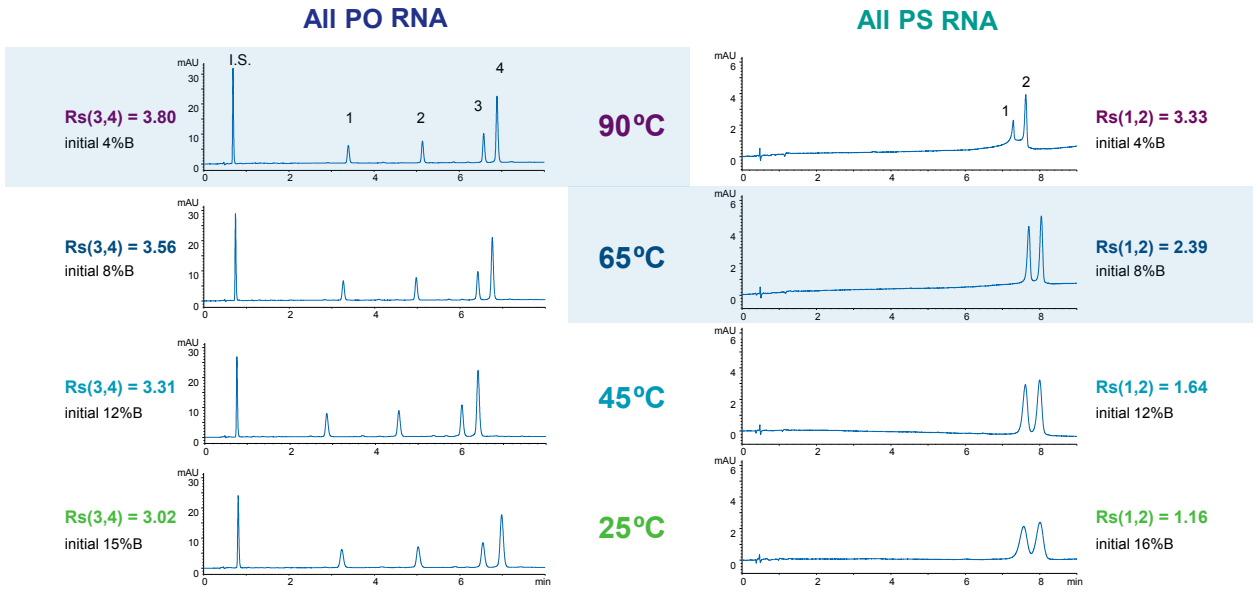
all PS RNA:
1. 5'-U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3' (20mer), 1.0 nmol/mL
2. 5'-G*U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3' (21mer), 1.0 nmol/mL
*=Phosphorothioated

Figure 24: Influence of different TEA-HFIP concentrations.

3.3. Temperature

The use of elevated temperature is a common tool to improve peak shape and resolution. Increased temperature influences the secondary structure and provides enhanced dispersion and distribution velocity of an oligonucleotide.

Figure 25 shows the separations of all PO and PS RNA using 25°C, 45°C, 65°C and 90°C. For all PO RNA, good separation is achieved even at lower temperature. However, separation was improved at higher temperature. For all PS RNA, elevated temperatures were essential to obtain sharp peaks and good resolution. At 45°C, acceptable results were obtained, and 65°C were the best conditions. At higher temperatures, such as 90°C peak deterioration was observed, which probably occurred due to a structural change of the RNA sample.



Column:	YMC-Triart Bio C18 (1.9 µm, 30 nm), 50 x 2.1 mm ID
Part No.:	TA30SP9-05Q1PT
Eluent:	A) buffer
	B) methanol
Gradient:	Initial: 8%B, gradient slope: 1%B/min
Flow rate:	0.42 mL/min
Temperature:	65 °C
Detection:	UV at 260 nm
Injection:	1 µL

Sample:
all PO RNA:
1. 5'-CACUGAAUACCAAU-3' (14mer), 1.8 nmol/mL
2. 5'-UCACACUGAAUACCAAU-3' (17mer), 1.8 nmol/mL
3. 5'-UCAUCACACUGAAUACCAAU-3' (20mer), 1.8 nmol/mL
4. 5'-GUCAUCACACUGAAUACCAAU-3' (21mer), 3.6 nmol/mL
I.S. = caffeine

all PS RNA:
1. 5'-U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3' (20mer), 1.0 nmol/mL
2. 5'-G*U*C*A*U*C*A*C*A*C*U*G*A*A*U*A*C*C*A*A*U-3' (21mer), 1.0 nmol/mL
*=Phosphorothioated

Figure 25: Temperature influence on All-PO and All-PS RNA separations.

Purification of Oligonucleotides: High Temperature Purifications

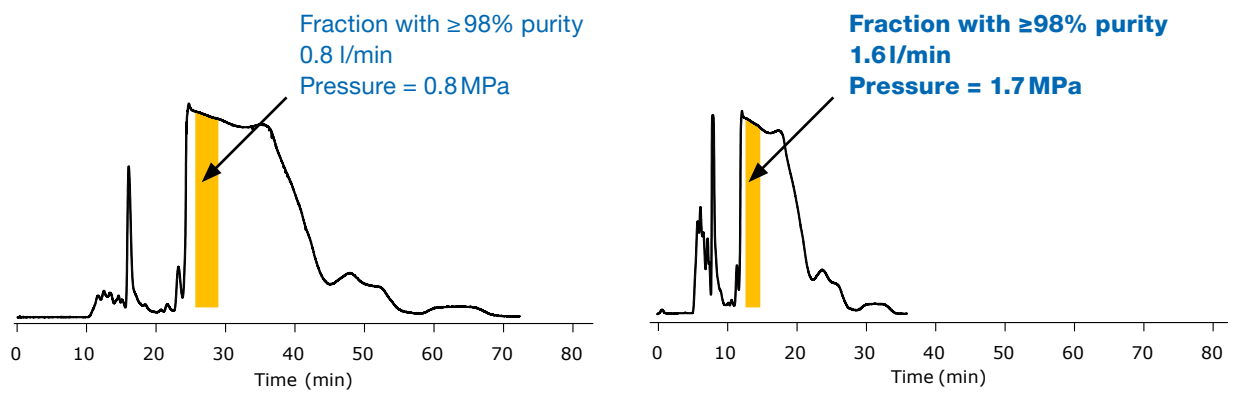
The benefit of increased temperatures on the separation efficiencies of oligonucleotides becomes more important for preparative applications. Elevated temperatures can improve the separation efficiency and therefore the productivity of oligonucleotide purifications but require special equipment. For purification processes larger column volumes and mobile phase quantities have to be heated so that the benefit of higher temperatures on the overall productivity and the economics of the process have to be considered. In the end, the cost-efficiency is the determining factor and this depends on the overall purification process and the value of the target substance.

3.4. Flow Rate

The adaption of the flow rate is a powerful tool to adjust the throughput of a method. At analytical scale, a higher flow rate decreases the cycle time and consequently allows more analytical runs per day because analysis time is shortened. At preparative scale, the higher throughput improves the productivity because more substance can be purified in the same time. The maximum flow rate is generally limited by the resulting resolution and backpressure. Therefore, the optimal flow rate of a method has to be evaluated.

Purification Aspects: Higher Flow Rates for Higher Productivity

The following example shows two purification runs of the same sample at two different flow rates. The collected fractions with the required purity of ≥98% are marked in yellow. With the elevated flow rate, the backpressure increases from 0.8 MPa (8 bar) to 1.7 MPa (17 bar). Although the yield in case of higher flow rate was lower, the overall productivity could be significantly increased as shown in the table below.



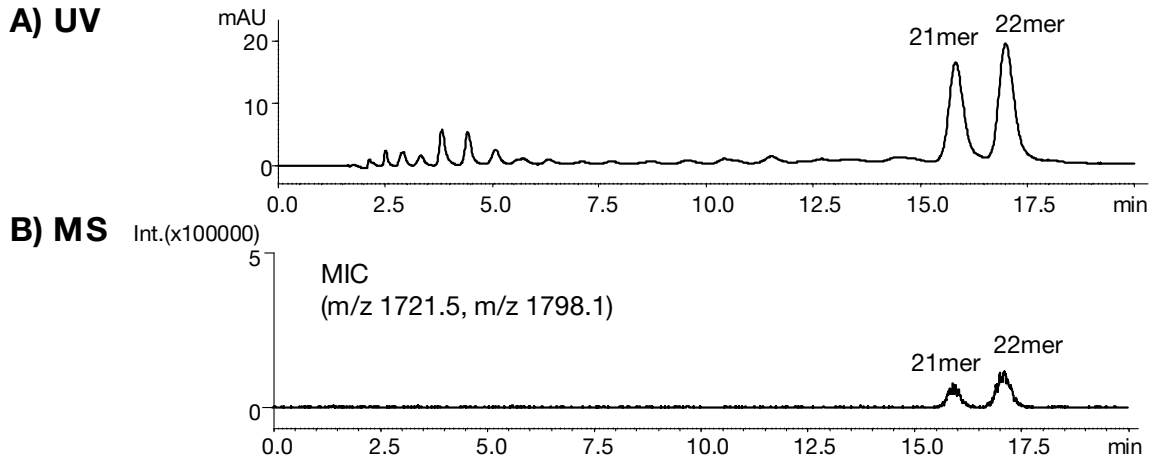
0.8 mL/min	Flow Rate	1.6 mL/min
60 min	Cycle time	30 min
1 run	Run/Hour	2 runs
30.1 mg/g gel	Loading	30.1 mg/g gel
71.8%	Theoretical Yield	64.7%
17.1 mg/g gel	Productivity/Hour	30.8 mg/g gel

Figure 26: Higher flow rates influence the productivity.

This example illustrates the influence of the flow rate on the overall productivity. To identify the optimal flow rate, a screening of different flow rate conditions is required.

3.5. Detection

The liquid chromatography of oligonucleotides on analytical scale is typically coupled to mass spectrometry due to its benefits over other detection techniques. MS provides high accuracy and sensitivity for high throughput analyses. LC-MS analyses are applied over the entire chain, during the oligonucleotide development up to quality control. Determining the oligonucleotide structure is required over the entire process to ensure the patient's safety. In contrast to AEX, RP is easier to couple with MS due to the absence of non-volatile salts in high amounts. In addition to MS, UV detection is often used which is also the state of the art detection for preparative chromatography.



Courtesy of M. Yamada, SHIMADZU CORPORATION

5'-pUGG AGU GUG ACA AUG GUG UUG-3' (21mer, MW 6,890.1)
5'-pUGG AGU GUG ACA AUG GUG UUG U-3' (22mer, MW 7,196.3)

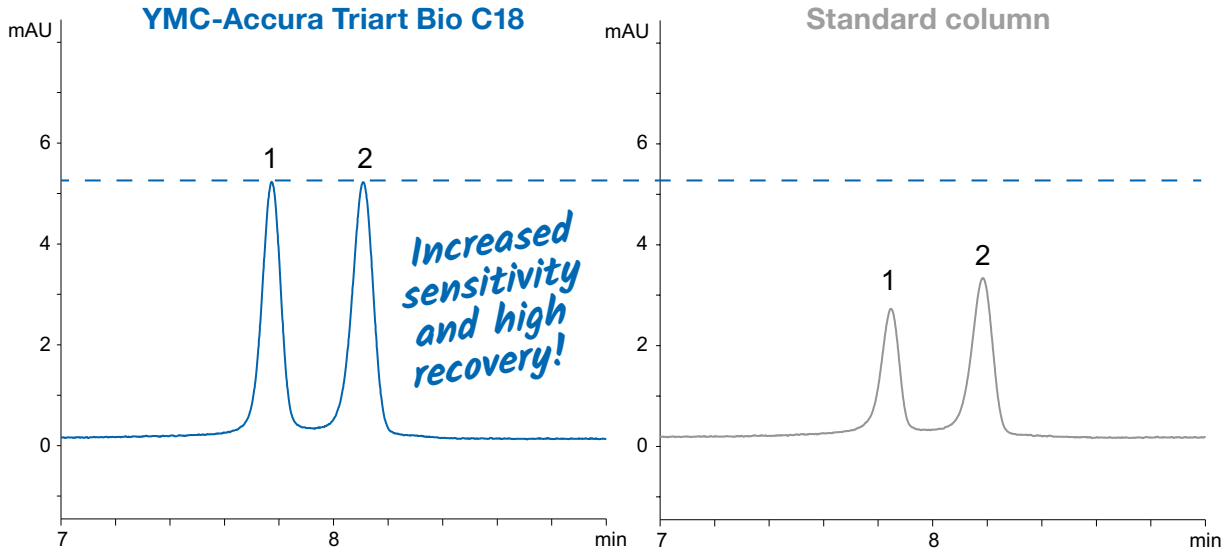
Column:	YMC-Triart C18 (3 µm, 12 nm) 150 x 2.0 mm ID
Part No.:	TA12S03-1502WT
Eluent:	A) 10 mM di-n-butylamine-acetic acid (pH 7.5)
	B) 10 mM di-n-butylamine-acetic acid (pH 7.5)/acetonitrile (50/50)
Gradient:	62–72%B (0–20 min)
Flow rate:	0.2 mL/min
Temperature:	30 °C
Detection:	A) UV at 260 nm
	B) ESI-negative mode
Injection:	4 µL (5 nmol/mL)
System:	LC) Shimadzu Prominence
	MS) Shimadzu LCMS2020

Figure 27: UV and MS detection of miRNA analysis.

3.6. Bioinert Column Hardware

Due to the negatively charged phosphate groups in the backbone of the oligonucleotides, they are able to interact with positively charged metal ions of the conventionally used stainless steel column hardware. The coordination with metals can lead to a poor peak shape and decreased recovery. Both can be improved by the use of a special column hardware which minimises these interactions. The most common columns for metal coordination analytes either have a bioinert coating or a PEEK-lining inside the stainless steel body such as YMC-Accura Triart and YMC-Triart metal-free, respectively. Both column types provide an increased sensitivity and high recovery without any preconditioning necessary.

! Optimum results are achieved by using both a bioinert column and system. However, as the column accounts for 70% of the metallic surface of the flow path its change has the biggest influence on the chromatographic results.



Column:	YMC-Accura Triart Bio C18 (1.9 µm, 30 nm) 50 x 2.1 mm ID
Part No.:	TA30SP9-05Q1PTC
Eluent:	A) 15 mM triethylamine - 400 mM HFIP*
	B) methanol
Gradient:	8–18%B (0–10 min)
Flow rate:	0.42 mL/min
Temperature:	65°C
Detection:	UV at 260 nm
Injection:	1 µL
Sample:	All PS RNA 20mer (1) (5'-U^C^A^U^C^A^C^A^C^U^G^A^A^U^A^C^C^A^A^U-3')
	All PS RNA 21mer (2) (5'-G^U^C^A^U^C^A^C^A^C^U^G^A^A^U^A^C^C^A^A^U-3')
	^=Phosphorothioate

*1,1,1,3,3,3-hexafluoro-2-propanol

Figure 28: Increased sensitivity and high recovery using bioinert column hardware.

3.7. Cleaning-in-Place

Cleaning-in-Place procedures can massively improve the column lifetimes in preparative RP-processes. Problems and solutions are similar as for the purification of biomolecules via IEX (see chapter 2.3.). The washing of the RP column with alkaline solutions (e.g. 0.1 M NaOH) restores the column performance. This is only possible with stationary phases that provide a high pH stability.

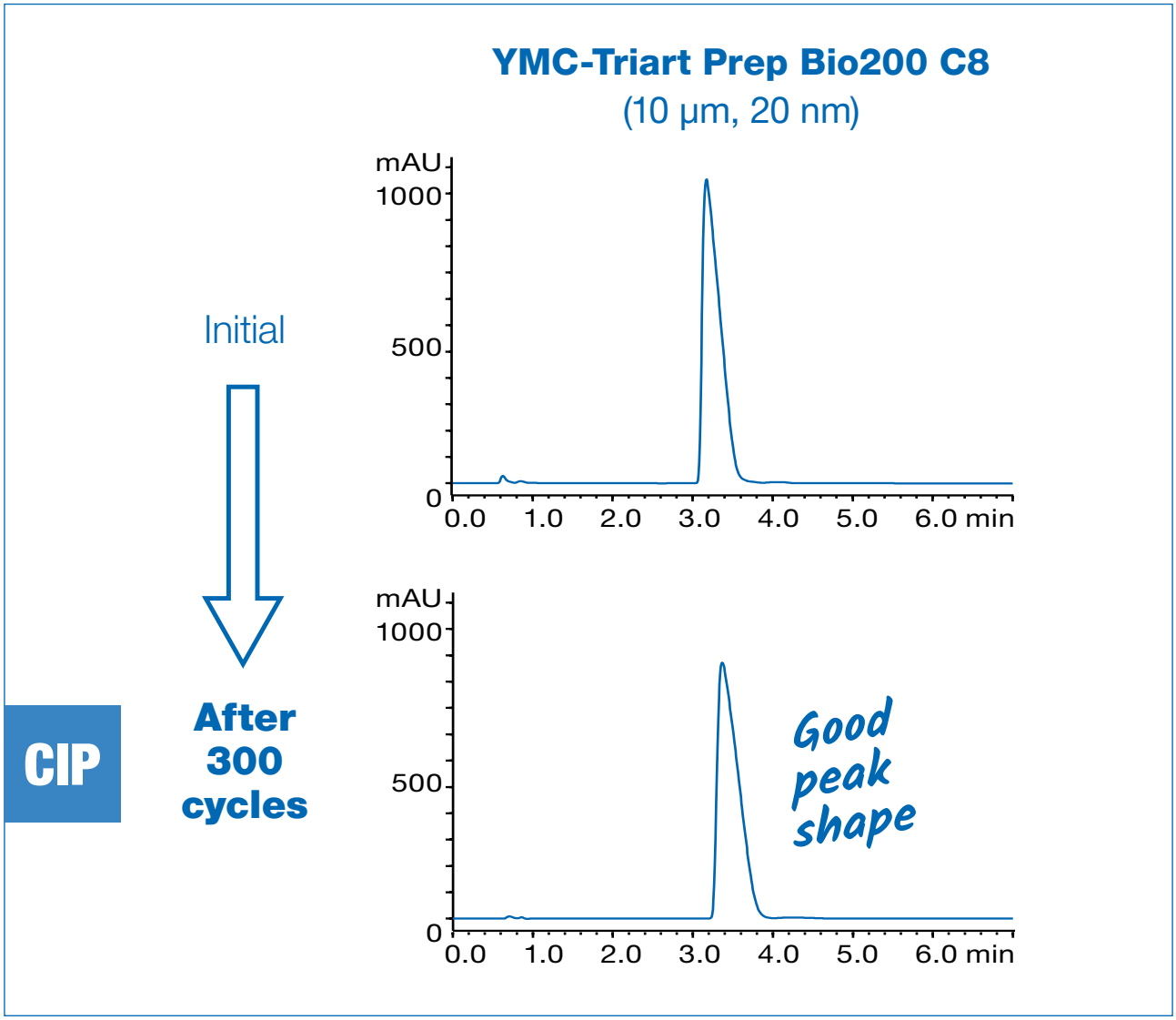


Figure 29: Cleaning-in-Place stability after 300 cycles.

Purification of Oligonucleotides: YMC Triart-Prep

Further information on CIP-procedure with pH stable RP stationary phases can be found in the Application Note about the CIP-Stability of YMC-Triart Prep.

3.8. YMC Stationary Phases for IP-RP

Analysis of Oligonucleotides: Stationary Phases for Analytical Separations

	YMC-Triart C18	YMC-Triart Bio C18	Hydrosphere C18	YMC-Triart C8	YMC-Triart Bio C4
Base Material	inorganic/ organic hybrid silica	inorganic/ organic hybrid silica	silica	inorganic/ organic hybrid silica	inorganic/ organic hybrid silica
Modification	C18 (USP L1)	C18 (USP L1)	C18 (USP L1)	C8 (USP L7)	C4 (USP L26)
Particle Size [µm]	1.9, 3, 5	1.9, 3, 5	2, 3, 5	1.9, 3, 5	1.9, 3, 5
Pore Size [nm]	12	30	12	12	30
Spec. Surface Area [m^2/g]	360	–	330	360	–
pH Range	1.0–12.0	1.0–12.0	2.0–8.0	1.0–12.0	1.0–10.0
Temperature Range	pH < 7: 90°C pH > 7: 50°C	pH < 9: 90°C pH > 9: 50°C	50 °C	pH < 7: 90°C pH > 7: 50°C	pH < 7: 90°C pH > 7: 50°C
Available Formats*	pre-packed columns: SST, bioinert coated SST or PEEK-lined SST	pre-packed columns: SST, bioinert coated SST or PEEK-lined SST	pre-packed columns: SST	pre-packed columns: SST, bioinert coated SST or PEEK-lined SST	pre-packed columns: SST, bioinert coated SST or PEEK-lined SST

**SST: stainless steel*

Purification of Oligonucleotides: Bulk Stationary Phases for Preparative Scale

	YMC-Triart Prep C18-S	YMC-Triart Prep C8-S	YMC-Triart Prep Bio200 C8	YMC-Triart Prep C4-S	YMC-Triart Prep Phenyl-S
Base Material	inorganic / organic hybrid silica				
Modification	C18	C8	C8	C4	Phenyl
Particle Size [µm]	7, 10, 15, 20	10, 15, 20	10	10	10
Pore Size [nm]	12	12	20	12	12
Spec. Surface Area [m^2/g]	360	360	proprietary	360	360
pH Range	2.0–10 CIP: pH 12.0				
Available Formats	bulk resin + pre-packed columns				

4. Loadability and Scale-up

4.1. Loadability Studies at Analytical Scale

After the successful method development at small scale, loadability studies at analytical scale are the next step towards a productive oligonucleotide purification. During this step, the sample load is increased to identify the maximum amount of sample that can be purified in each cycle. The loadability can be increased in different ways to achieve an overloading effect that leads to efficient recovery results.

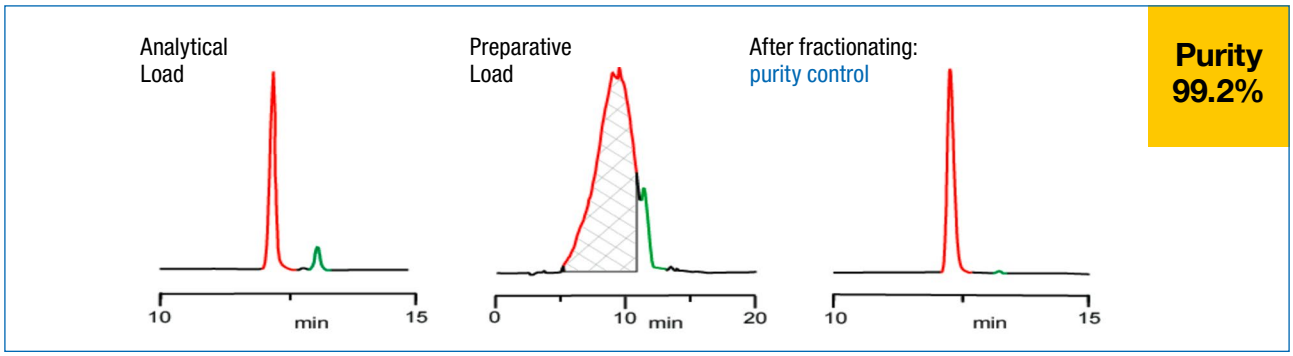


Figure 30: Purity control after performing analytical loading and loadability studies.

4.2. Scale-Up to Preparative Scale

If the method is completely developed including the identification of the optimal loading conditions, the final setup can easily be transferred to the final process scale. This is done by applying the linear scale up. Within these procedures, all non-scalable parameters, including the particle size of the stationary phase, the separation mode and the column length, remain the same. To increase the final scale, the diameter of the preparative column is increased and the flow rate is adjusted according to the scaling factor (Figure 31).

Purification of Oligonucleotides: Linear Scale-Up

A detailed guideline can be found in the Technical Note about linear scale-up in preparative LC method development.

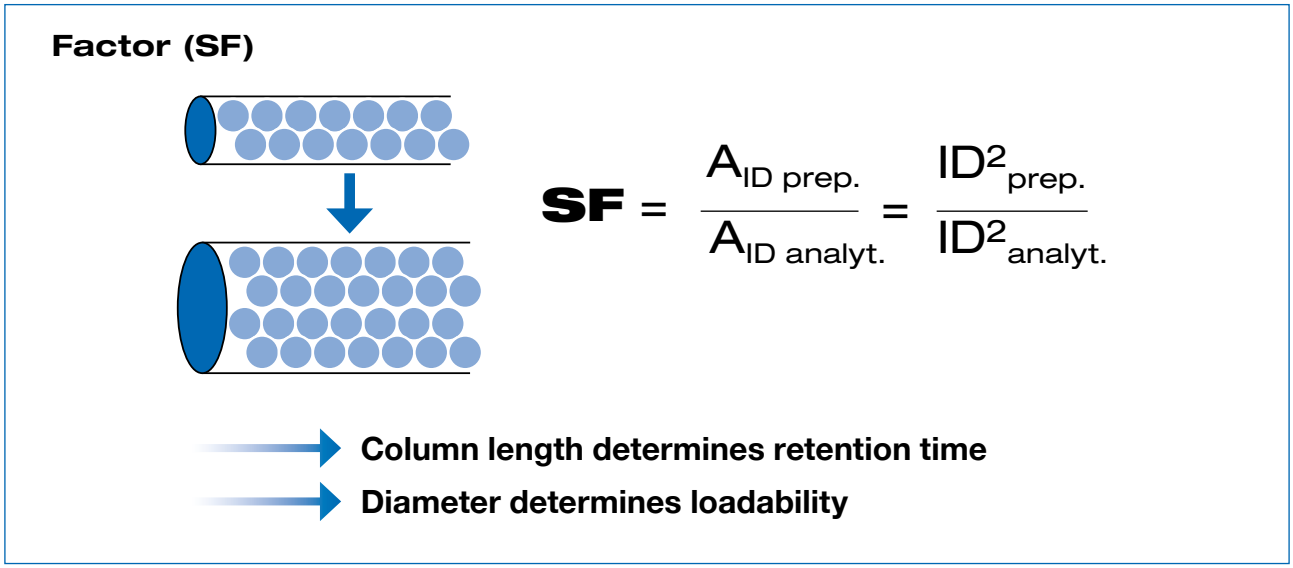
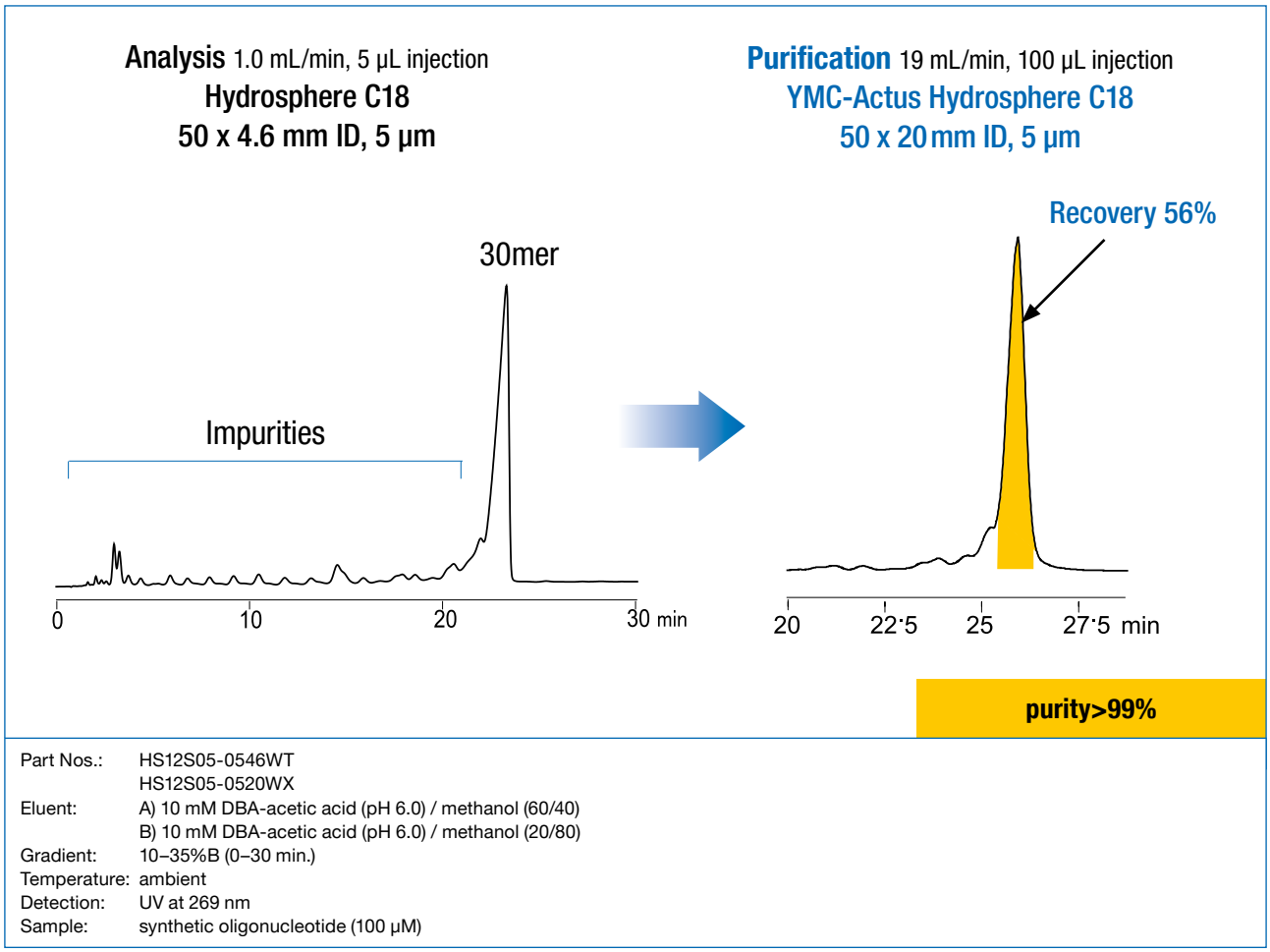


Figure 31: Scale-Up Factor calculation.

4.2.1. Practical Example: Scale-Up of an Oligonucleotide Purification Process

This practical example shows the purification of a synthetic 30mer oligonucleotide using a C18 modified stationary phase. The analytical run is performed using a 50 x 4.6 mm ID column and the resulting chromatogram shows the main peak and the impurity peaks. The flow rate at this scale was set to 1.0 mL/min. For the preparative purification, the ID of the column was increased to 20 mm and the column length remained the same. To follow the rules of the linear scale-up, the flow rate was adjusted to 19 mL/min according to the scaling factor of approx. 19. The loading was increased at the same scale. The resulting chromatogram is completely comparable to the analytical one: the peak shape and the retention time of the target peak are nearly the same.



Part Nos.:	HS12S05-0546WT
	HS12S05-0520WX
Eluent:	A) 10 mM DBA-acetic acid (pH 6.0) / methanol (60/40)
	B) 10 mM DBA-acetic acid (pH 6.0) / methanol (20/80)
Gradient:	10–35%B (0–30 min.)
Temperature:	ambient
Detection:	UV at 269 nm
Sample:	synthetic oligonucleotide (100 µM)

Figure 32: Easy purification with YMC-Actus semi prep columns.

5. Conclusion

Therapeutic oligonucleotides are synthetic DNA or RNA oligomers that can be quite diverse, not only in their function but also in their structure. Different separation modes can be applied for oligonucleotide LC, but the current gold standards these days are anion exchange and ion-pairing reversed phase chromatography. Additional modes such as HILIC (hydrophilic interaction chromatography) or SEC (size exclusion chromatography) are also gaining more interest as alternative approaches.

Even though the goals of oligonucleotide analyses and purifications are quite different, it is important to consider them together in order to design an efficient process. Whereas small column dimensions and particle sizes are used to identify and characterise oligonucleotides in analytical LC, large column IDs and particles are used for their isolation and purification. This leads to different important factors which play a role:

Analytical LC	Preparative LC
Resolution	Purity
Peak shape	Production efficiency
Sensitivity	Recovery
Sample throughput	Safety
Method robustness	Process costs

Similar to the different aspects to be considered in analytical and preparative scale, selected parameters have a significant influence in AEX and IP-RP mode:

	AEX	IP-RP
Functional Group	Strong or weak exchanger	C18, C8, C4, Phenyl
Particle Technology	Porous or non-porous polymer	(Hybrid) silica
Particle Size	3-5 µm (analytical) 10-30 µm (preparative)	<2–5 µm (analytical) 7–50 µm (preparative)
Mobile Phase	Tris-HCl or NaOH + NaCl or $NaClO_4$ MeOH as organic modifier	HFIP-TEA (analytical) DBAA or TEAA (analytical, preparative)
Temperature	25–60°C	25–90°C
Hardware	Bioinert or stainless steel	
Column Cleaning	CIP using NaOH	

Despite the different aims in analytical and preparative LC of oligonucleotides, often analytical methods can be scaled up for preparative purposes or are the starting point for a preparative application. This scale-up is a straight-forward approach as long as a linear scale-up is applicable, therefore the same selectivity is provided by a stationary phase independent from its particle size. As a result, the loading study can be performed in analytical scale followed by a scale-up to the final preparative dimensions and method parameters. YMC offers robust AEX and IP-RP stationary phases to analyse and purify oligonucleotides. YMC is your preferred supplier addressing all required chromatographic parameters for analytical and preparative chromatography of oligonucleotides!

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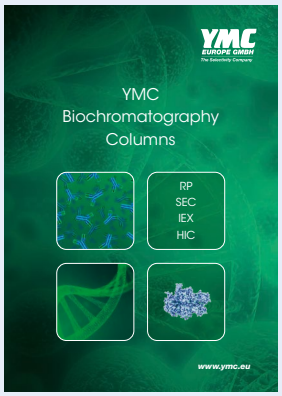
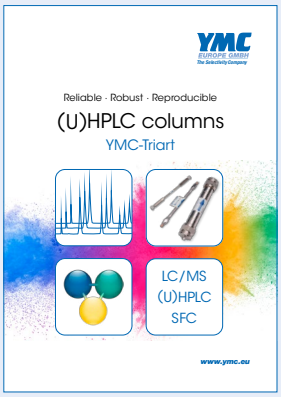
FURTHER INFORMATION

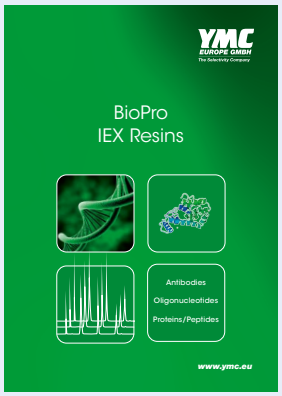
YMC BROCHURES



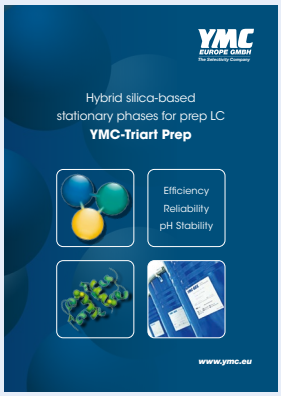
YMC Biochromatography Columns



YMC-Triart

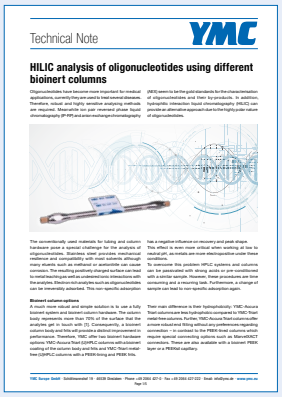


YMC BioPro IEX Resins



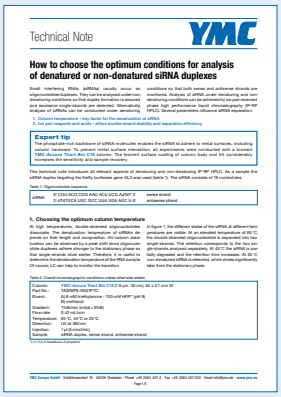
YMC-Triart Prep

TECH NOTES


Technical Note

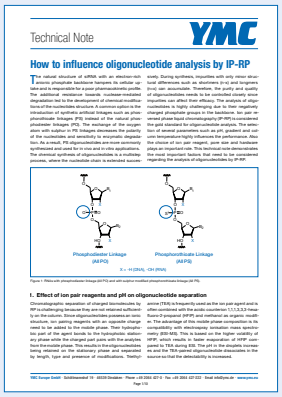
HILIC analysis of oligonucleotides using different bioinert columns

HILIC analysis of oligonucleotides using different bioinert columns


Technical Note

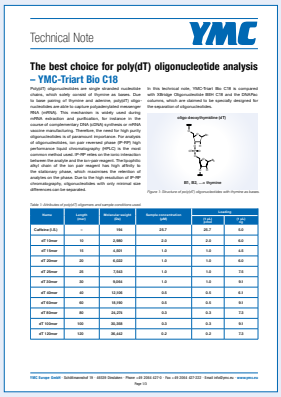
How to choose the optimum conditions for analysis of denatured or non-denatured siRNA duplexes

How to choose the optimum conditions for analysis of denatured or non-denatured siRNA duplexes


Technical Note

How to influence oligonucleotide analysis by IP-RP

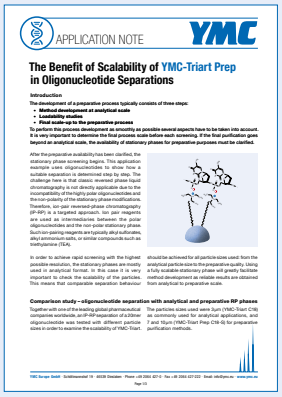
How to influence oligonucleotide analysis by IP-RP


Technical Note

The best choice for poly(dT) oligonucleotide analysis – YMC-Triart Bio C18

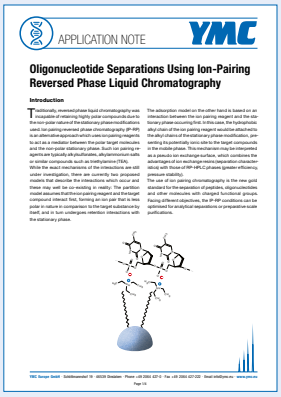
The best choice for poly(dT) oligonucleotide analysis - YMC-Triart Bio C18

APP NOTES


APPLICATION NOTE

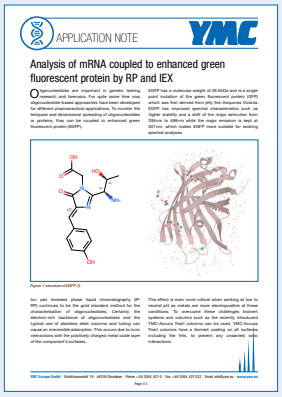
The Benefit of Scalability of YMC-Triart Prep in Oligonucleotide Separations

The Benefit of Scalability of YMC-Triart Prep in Oligo-nucleotide Separations


APPLICATION NOTE

Oligonucleotide Separations Using Ion-Pairing Reversed Phase Liquid Chromatography

Oligonucleotide Separations Using Ion-Pairing Reversed Phase Liquid Chromatography


APPLICATION NOTE

Analysis of mRNA coupled to enhanced green fluorescent protein by RP and IEX

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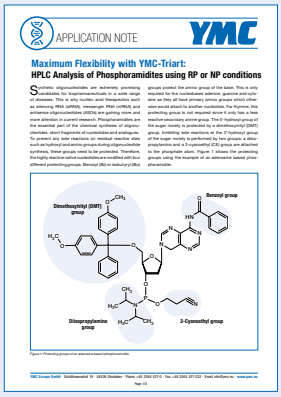
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Maximum Flexibility with YMC-Triart: HPLC Analysis of Phosphoramidites using RP or NP conditions

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