



Advanced Ion Exchange Chromatography for Antibody-Oligonucleotide Conjugate (AOC) Characterization-Oligonucleotide-to-Antibody Ratio Analysis

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Sepax Proteomix® IEX Phase



Antibody-Oligonucleotide Conjugate

Oligonucleotide-to-Antibody Ratio (OAR) Analysis by Sepax Proteomix® IEX

Key Features

- Reliable and accurate Oligonucleotide-to-Antibody Ratio (OAR) analysis enabled by robust, high-resolution separation of unconjugated antibody, AOC conjugates, and free oligonucleotide by Ion Exchange Chromatography (IEX).
- In general, each oligonucleotide attachment increases the overall negative charge of the antibody, thus conjugates with higher OAR bind more strongly to the positively charged stationary phase and elute later under a salt or pH gradient.
- Tris and Phosphate buffer systems being discussed in this application for the development of method flexibility and compatibility, with high resolution AOC separation achievable under diverse mobile-phase conditions for method optimization.
- Innovative SCX + SAX tandem universe platform method for complex bioconjugation workflows without harsh high-pH conditions that risk degrading oligonucleotide payloads.

Antibody-oligonucleotide conjugates (AOCs) are an emerging class of hybrid biomolecules that combine the high specificity and favorable pharmacokinetics of antibodies with the versatile structural and functional properties of oligonucleotides. This unique design enables broad applications, especially in innovative antibody delivery strategies, thereby expanding their therapeutic, diagnostic, and other emerging applications.

Various synthetic strategies have been developed for AOC preparation, ranging from less-controlled, stochastic chemical conjugation (which links randomly across the antibody) to more precise site-specific linkage through engineered reactive sites. Ongoing research continues to focus on improving synthetic precision and accurately controlling the Degree of Conjugation (DoC), or the Oligonucleotide-to-Antibody Ratio (OAR), as this control is critical for ensuring product consistency, maximizing biological activity, and enabling reliable analytical reproducibility.

Ion-exchange high-performance liquid chromatography (IEX-HPLC) provides a robust, charge-based analytical approach for characterizing AOCs. Each oligonucleotide attachment increases the overall negative charge of the conjugates, enabling IEX-HPLC to separate and quantify species with different degrees of conjugation. Among IEX modes, strong anion-exchange (SAX) chromatography is particularly effective, as it exploits these charge differences to distinguish variants: conjugates with higher OAR bind more strongly to the positively

charged stationary phase and elute later under a salt or pH gradient. This charge-dependent separation mechanism makes SAX ideally suited for profiling AOC conjugation variants, providing both qualitative and quantitative insight into conjugation efficiency, product heterogeneity, and purification optimization.

Technical Specifications

Resin Matrix	Spherical, hydrophilic layer coated on PS/DVB
Pore Size	Non-porous
Particle Size	1.7, 3, 5, 10 µm
pH Stability	2-12
Operating Temperature	Up to 80 °C
Operating Pressure Limit	4,000 psi for 10 µm resins; 6,000 psi for 5 µm resins; 8,000 psi for 3 µm resins; 12,000 psi for 1.7 µm resins

Characteristics

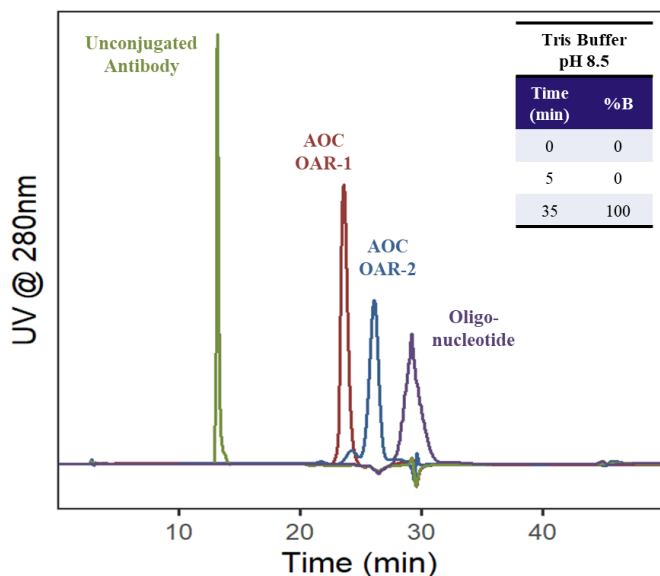
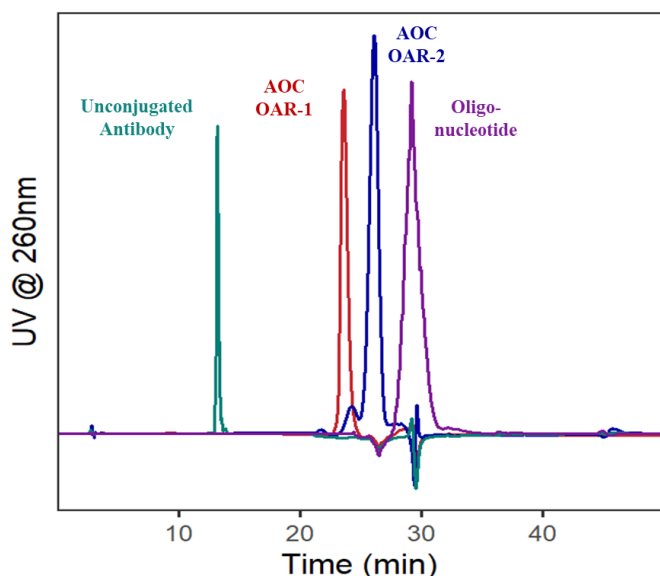
- Uniform polymer beads as the packing support
- Proprietary surface chemistry specially designed for the elimination of non-specific bindings
- Tunable separation capacity with combination of the pore structure and the controllable surface chemistry
- Unprecedented separation efficiency, selectivity and resolving power
- Complete selection for analytical, semi-preparative and preparative separations
- Wide particle size selection
- High stability
- High recovery
- High lot-to-lot reproducibility
- Well suited for UPLC system

Method Details

Mobile Phase I	A: 20 mM Tris Buffer, pH 8.5		
	B: 20 mM Tris Buffer + 1.2 M NaCl, pH 8.5		
Mobile Phase II	A: 20 mM Phosphate Buffer pH 8.5		
	B: 20 mM Phosphate Buffer + 1.2 M NaCl, pH 8.5		
Mobile Phase III	A: 20 mM Phosphate Buffer, pH 7.0		
	B: 20 mM Phosphate + 1.0 M NaCl, pH 7.0		
Flow Rate	0.5 mL/min	Temperature	25°C
Detection	UV 280 nm and 260 nm	Instrument	HPLC Agilent
Gradient	0-100% MPB in 30 min		
Column Info	Proteomix SAX-NP5P (5 μm, 4.6 × 250 mm, PEEK)		
	Proteomix SCX-NP5P (5 μm, 4.6 × 50 mm, PEEK)		

Results and Conclusion

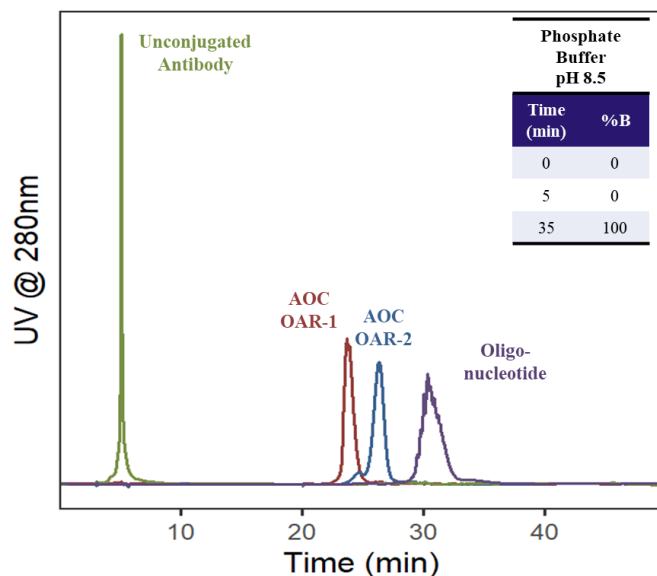
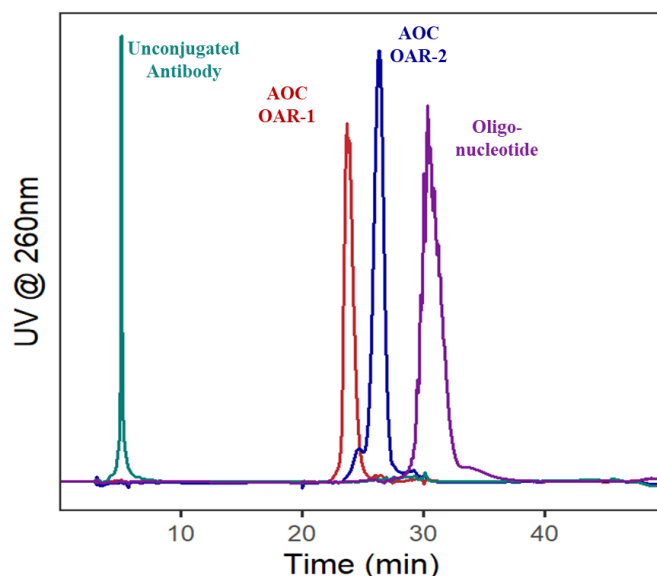
Figure 1. UV chromatogram overlay showing distinct peaks for the unconjugated antibody (green), free oligonucleotide (purple), AOC OAR-1 (red), and AOC OAR-2 (navy) obtained using the Sepax Proteomix SAX column under Tris buffer (pH 8.5).



Method Development and Buffer Selection

Anion-exchange method separates AOC species based on differences in overall surface charge, as each attached oligonucleotide increases the conjugates' net negative charge. This application note demonstrates the capability of the Sepax Proteomix SAX for monitoring the degree of conjugation during AOC production. As shown in Figure 1, the UV chromatogram overlay demonstrates distinct peak separation for AOC profiling under Tris buffer (pH 8.5) conditions. The unconjugated antibody elutes first, at approximately 13 minutes, followed by peaks corresponding to progressively higher degrees of conjugation, with less-conjugated species eluting earlier. The free oligonucleotide elutes last and exhibits well-resolved charge-variant peaks, further illustrating the method's strong resolving capability for both conjugated and free oligonucleotide species.

Figure 2. UV chromatogram overlay showing distinct peaks for the unconjugated antibody (green), free oligonucleotide (purple), AOC OAR-1 (red), and AOC OAR-2 (navy) obtained using the Sepax Proteomix SAX column under Phosphate buffer (pH 8.5).



Unlike the Tris buffer condition, under which the unconjugated antibody, AOCs (OAR-1 and OAR-2), and free oligonucleotide were successfully separated with clear retention using a simple two-phase linear gradient, the free antibody was not retained under the phosphate buffer system (pH 8.5), as shown in Figure 2. This result highlights the strong impact of buffer selectivity on AOC profiling and purification assessment.

The choice of buffer has a significant impact on AOC separation in ion-exchange HPLC, even when pH and ionic strength are held constant. Phosphate and Tris differ markedly in charge properties and interaction behavior with both the stationary phase and analyte. Phosphate, a multivalent anion, strongly interacts with positively charged resin sites and enhances electrostatic retention through its kosmotropic, structure-stabilizing nature, often producing sharper peaks and later elution. In contrast, Tris is a weakly cationic, more chaotropic buffer that interacts less strongly and can partially shield charged sites, resulting in weaker retention and broader peaks. These ion-specific effects, combined with differences in buffering range and microenvironmental pH near the resin surface, lead to notable changes in selectivity and resolution.

As a result, the effective surface charge density and competition between buffer ions and analyte binding are not equivalent, even when the nominal ionic strength (e.g., 20 mM) is identical. Moreover, although the bulk pH (e.g., 8.5) may match, phosphate ($pK_{a2} \approx 7.2$) and Tris ($pK_a \approx 8.1$) differ in buffering capacity, creating distinct micro-pH environments around charged surfaces. Consequently, phosphate and Tris buffers can yield substantially different elution profiles due to their unique chemical characteristics and interaction mechanisms within the IEX system.

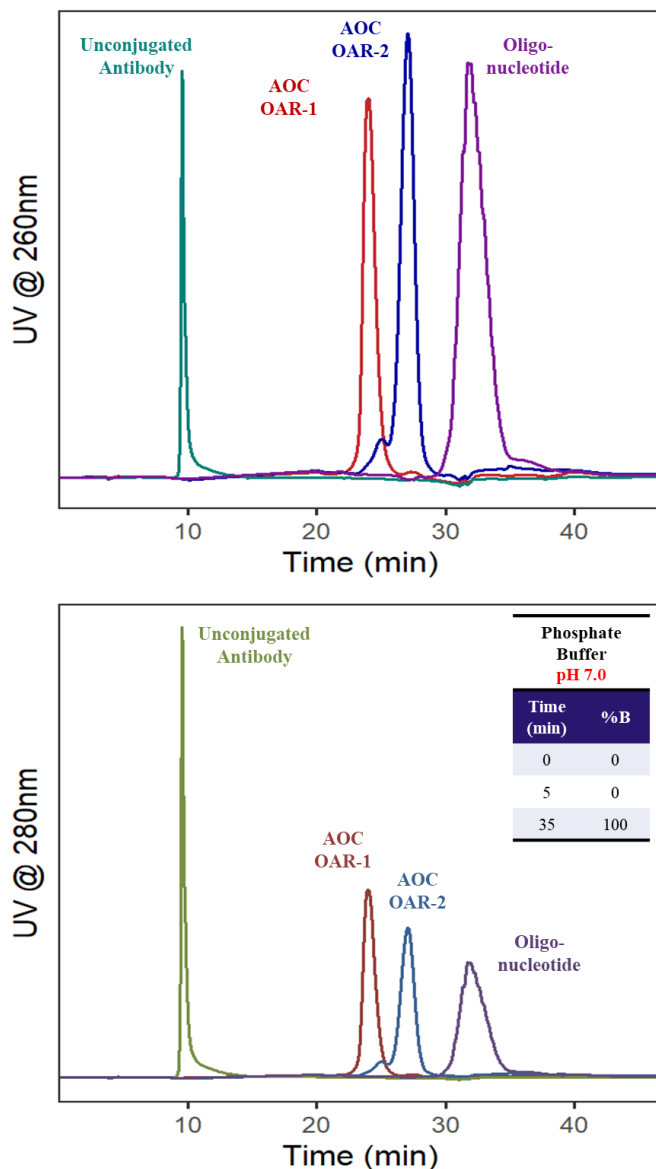
► Platform Method: SCX + SAX in Tandem

The pH stability of AOCs reflects the combined behavior of the antibody, the oligonucleotide payload, and the linker chemistry. Antibodies are generally most stable near neutral pH, while extreme acidic or alkaline conditions can trigger unfolding or aggregation. Oligonucleotides, especially DNA or chemically modified backbones, tolerate wider pH ranges, though RNA-based payloads remain sensitive to alkaline hydrolysis.

Linker stability often dictates overall pH tolerance, as certain linkages may hydrolyze or rearrange under non-optimal conditions. For analytical methods such as IEX-HPLC, AOCs are typically most stable in near-neutral buffers (pH 6.5 – 7.5), with only brief exposure to ex-

treme pH recommended. As pH sensitivity varies with molecular design, each AOC should be evaluated individually to determine appropriate handling and storage conditions.

Figure 3. UV chromatogram overlay showing distinct peaks for the unconjugated antibody (green), free oligonucleotide (purple), AOC OAR-1 (red), and AOC OAR-2 (navy) obtained using a tandem SCX+SAX method under Phosphate buffer (pH 7.0).



The tandem SCX + SAX configuration provides two orthogonal charge-based interactions in a single chromatographic run and balances charge interactions across both stationary phases. This dual-mode environment enables effective resolution of conjugation variants under milder, neutral-pH conditions while a highly basic mobile phase can risk sample degradation and reduced column stability, as shown in Figure 3.

Neutral pH normally reduces charge differences among biomolecules, but the SCX + SAX tandem setup restores effective discrimination by leveraging the distinct balance of positive and negative charge domains in each

conjugate class. Antibodies, even those with relatively high pI value, retain localized positive patches that interact sufficiently with the SCX segment. In contrast, AOCs and mRNA conjugates carry incrementally stronger negative charge from their oligonucleotide or mRNA payloads and therefore exhibit greater retention on the downstream SAX segment. By combining these complementary retention mechanisms, the SCX + SAX configuration enhances charge resolution at pH 7–8 and enables clean separation of species with different conjugation levels or payload types without requiring the highly basic conditions normally needed for SAX-only methods, preserving molecular integrity while maintaining effective chromatographic discrimination.

Mild organic modifiers such as ethanol or acetonitrile also can improve AOC separations in IEX-HPLC, particularly when hydrophobic or structural effects interfere under purely aqueous conditions. AOCs with higher hydrophobicity, arising from the antibody, linker, or increased OAR, often display non-specific interactions that result in peak tailing or reduced resolution. Low levels of organic solvent (5 – 20%) help suppress these interactions and restore a more charge-dominant separation. Organic modifiers also enhance solubility and reduce self-association, improving retention consistency for aggregation-prone samples. For AOCs carrying long, GC-rich, or structurally stable double-stranded payloads such as siRNA, mild organic content, optionally combined with moderate temperatures (30 – 40°C), can soften secondary structures that otherwise cause shoul-

der peaks or limited resolution. Maintaining organic levels within 5 – 10% preserves non-denaturing conditions and supports duplex integrity. Overall, organic modifiers are most useful for AOCs with high hydrophobicity, limited solubility, or pronounced oligonucleotide secondary structure.

Summary

The Sepax Proteomix SAX-NP5 column demonstrated excellent capability for resolving AOC conjugation variants, delivering clear separation of the unconjugated antibody, AOCs at different OAR levels, and free oligonucleotide under pH 8.5 conditions. Its strong retention selectivity, sharp peak shapes, and stable baselines enable accurate OAR determination. Buffer selection proved critical, as the antibody was not retained under phosphate buffer at the same pH, underscoring the importance of optimizing buffer chemistry for effective AOC profiling. In addition, the tandem SCX + SAX configuration further enhances charge discrimination at neutral pH by leveraging complementary positive and negative charge domain interactions, enabling robust separation of conjugation variants without the need for harsh, high-pH mobile phases that may compromise AOC or oligonucleotide integrity. Collectively, these findings highlight the SAX-NP5 column, and its use within SCX + SAX workflows, as a reliable, non-denaturing, and highly effective platform method for AOC characterizations and purification process development.

Order Information

Proteomix® IEX Phases

Part Number	Column	ID and Length Available
401NP2, NP3, NP5, NP10	Sepax Proteomix SCX NP2, NP3, NP5 and NP10	2.1, 4.6, 7.8, 10, 21.2, 30 and 50 mm I.D. 5, 10, 15 and 25 cm length <i>Available in PEEK and Stainless steel</i>
402NP2, NP3, NP5, NP10	Sepax Proteomix WCX NP2, NP3, NP5 and NP10	
403NP2, NP3, NP5, NP10	Sepax Proteomix SAX NP2, NP3, NP5 and NP10	
404NP2, NP3, NP5, NP10	Sepax Proteomix WAX NP2, NP3, NP5 and NP10	

**More column dimensions will be available on the Sepax website.*