Oligonucleotide Purification Solutions
Agilent PLRP-S and PL-SAX HPLC columns
It’s All About Scalability

Whether you’re working on the bench or in manufacturing, Agilent PLRP-S and PL-SAX columns deliver robustness and reproducibility. What’s more, they are available in pore sizes that are selective and appropriate for your unique oligonucleotide purification requirements.

A diverse class of molecules

Research has shown that synthetic oligonucleotides and nucleic acids are promising therapeutic agents as well as an essential part of next generation sequencing (NGS) for research and diagnostics. These oligonucleotides can range in size, sequence complexity, and overall modifications. Small oligonucleotides can span a range of sizes from a few nucleotides to 40 or 50 bases, with varying DNA or RNA modifications. Beyond their primary structure, these molecules can be single or double stranded, and modified with molecular or biological tags. Larger oligonucleotides—including guide RNA or NGS primers and probes—can span from 50 to 200 bases, with varying modifications and tags for their particular use. RNA therapeutics can have sequences that are thousands of bases in length, synthesized through in-vitro production, requiring unique purification considerations. Sequence-derived structure formations and impurities resemble the full-length product, so optimizing purification and analysis is essential when developing a final pure molecule. However, oligonucleotide diversity demands chemistries that can meet the challenges of purification and analysis through a range of particles and pore sizes.

Polymeric particles, such as PLRP-S and PL-SAX, are ideal for analyzing and purifying oligonucleotides. Because these materials are thermally and chemically stable, they enable diverse techniques:

– Working at elevated temperature enhances mass transfer, increasing resolution for longer oligonucleotides.
– Denaturing conditions, such as high pH, can be used to avoid aggregation of modified, self-complementary, and GC-rich oligonucleotides during analysis and purification.
– Performing anion exchange at high pH can suppress ionization of individual bases, enabling resolution of phosphodiester and phosphorothioate forms of the oligonucleotide.
Purification your way: siRNA to mRNA

Agilent recognizes the diversity of the oligonucleotide landscape, offering the optimal chemistry and pore size no matter your molecule size or purification scale.

Choosing the right pore size is an important factor for large molecules like oligonucleotides. Agilent offers a range of pore sizes to find the balance between resolution and binding capacity.

<table>
<thead>
<tr>
<th>Pore sizes</th>
<th>siRNA/ASO</th>
<th>Guide RNA</th>
<th>mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 Å</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>300 Å</td>
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<td></td>
<td></td>
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<tr>
<td>1000 Å</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4000 Å</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Agilent PLRP-S columns:**
  - Ideal for your small-scale analysis, and scales as your production needs grow
  - Ion pair reversed-phase chemistry
  - Five pore sizes: 100 Å, 200 Å, 300 Å, 1000 Å, and 4000 Å
  - Particles from 3 to 50 µm

- **Agilent PL-SAX columns:**
  - The purity you need whether you are working with guide RNA or large nucleic acid molecules
  - Best-in-class polymeric anion exchange media
  - Two pore sizes: 1000 Å and 4000 Å
  - Particles from 5 to 30 µm

Having the same quality stationary phase available in the scale needed is critical—from analytical dimensions, semi-prep and prep, to bulk material—Agilent PLRP-S and PL-SAX columns allow you to utilize the same optimized chemistry regardless of the scale of production.
The Agilent 6545XT
AdvanceBio LC/Q-TOF system:
Designed to handle multiple workflows
Whether you need to perform oligonucleotide analysis at the intact level, sequence and modification confirmation through fragment identification, or identify and quantify impurities, the 6545XT delivers. It’s the analytical cornerstone in a comprehensive set of tools designed for biopharma characterization.

Withstands high temperatures and pH
Agilent PLRP-S columns are available in a range of pore and particle sizes, all with identical chemistries and fundamental adsorptive characteristics. The particles are inherently hydrophobic, so there is no bonded phase required for reversed-phase separations. As such, PLRP-S particles can withstand extreme temperature and pH ranges that are incompatible with silica-based columns. That means you get the broadest allowable functionality for method development with particles as small as 3 µm.

PLRP-S has excellent binding capacity across five pore sizes: 100 Å, 200 Å, 300 Å, 1000 Å, and 4000 Å. This allows you to capture a wide range of oligonucleotides, whether you are looking at small antisense oligonucleotides or large mRNA.

In this example, we used an Agilent AdvanceBio 6545XT LC/Q-TOF to analyze poly-A tail sequences formed by e. coli poly-a polymerase (PAP). PAP is commonly used for post in-vitro transcription addition of polyA tails to mRNA, an essential component for translation.

**Analysis of poly-A sequences extended by PAP on synthetic RNA Primer**

**Figure 1.** UV absorbance at 260 nm (A: reference = 360 nm) and total ion chromatogram (B) of RNA primers extended with PAP in the presence of ATP only. Separation was carried out on a PLRP-S column and shows bimodal distribution of two distinct populations.

### Conditions

- **Instrument:** Agilent 1290 Infinity II LC system
- **Column:** Agilent PLRP-S, 5 µm, 2.1 × 50 mm, 1,000 Å
- **Solvent A:** 15 mM dibutylamine + 25 mM HFIP in DI water
- **Solvent B:** 15 mM dibutylamine + 25 mM HFIP in methanol
- **Gradient:** 15% B 0–1 min, 45% B 1.1–10.5 min, 90% B 10.6–11.5 min
- **Flow rate:** 0.4 mL/min
- **Temperature:** 80 °C
- **Injection volume:** 10–20 µL
Analytical separation of crude 25, 50, 75, and 100 mer using PL-SAX 1000 Å, 5 μm, 2.1 x 50 mm column

Delivering excellent resolution

Ion exchange analysis is often used for release testing of oligonucleotides, and offers robustness with common buffers and reagents found in QC labs.

Whether you are performing analytical characterization of oligonucleotide impurities or scaling up for future purification, PL-SAX delivers the flexibility you need. PL-SAX columns are composed of a strong anion exchange functionality covalently linked to a fully porous chemically stable polymer, extending its operating pH range and stability. In addition, the anion-exchange capacity is independent of pH, enabling separations that use denaturing conditions of temperature, organic solvent, and high pH for synthetic oligonucleotides.

The 5 μm media delivers high-resolution separations, while the 30 μm media is used for medium-pressure liquid chromatography. With pore sizes of 1000 and 4000 Å, PL-SAX offers high binding capacity and superior flow dynamics for oligonucleotides ranging from a few bases to thousands of bases in length.

**Conditions**

Solvent A: 10 mM Tris, pH 8.0 in water/ACN 10:90 (v:v)  
Solvent B: 2 M NaCl in solvent A  
Column temp: 30 °C unless otherwise noted  
Flow rate: 0.5 mL/min  
Gradient: 20–40% B in 10 min, except for 25 mer which was conducted at 10–30% B  
Sample: 1 mg/mL samples  
Injection: 2 μL except for 25 mer which was 1 μL
PLRP-S: Providing the Scale and Pore Size for Your Purification Goals

PLRP-S polymeric materials are an ideal choice for ion pair reversed-phase oligonucleotide purification. They are physically and chemically robust and therefore well suited to the conditions required. Choosing the correct pore size for the length of oligonucleotide is important to optimize the binding capacity and overall oligonucleotide purification. Available in a range of pore sizes (100 Å, 200 Å, 300 Å, 1000 Å, and 4000 Å), accessibility of very large molecules may be hindered in smaller pore sizes commonly seen across the market. Even though smaller pore size particles have higher internal surface area, if the molecule is too large to fit into the pores, the capacity will be compromised.

Purification of Single-Stranded RNA Oligonucleotides using the Agilent 1290 Infinity II Preparative LC system

By coupling PLRP-S and the Agilent 1290 Infinity II Preparative system, you can achieve ideal, efficient separations using ion pair reversed-phase methods. As you can see below in Figure 3, PLRP-S allows high sample loading, and the combined autosampler/fraction collector is ideal for large scale injections and flexible fraction collection.

![Chromatogram and overlay](image)

Figure 3. A chromatogram and overlay (UV 260nm) of an injection, fraction collection, and reanalysis of 11, 9 second fractions collected over the FLP.

**Conditions for chromatographic parameters for purification**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mobile phase A: 0.1 M Hexylammonium acetate in water, pH = 7.0</th>
<th>Mobile phase B: Acetonitrile</th>
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<tr>
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<tr>
<td>Gradient</td>
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</tr>
<tr>
<td></td>
<td>0 32</td>
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<td></td>
<td>12.07 100</td>
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<td></td>
<td>13.06 32</td>
<td>Stop time: 11 min</td>
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<tr>
<td>Injection volume</td>
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<td>Fraction collection</td>
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<td>UV downslope: 1 mAU/s</td>
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**Conditions for chromatographic parameters for fraction reanalysis**

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<tr>
<td>Gradient</td>
<td>Time (min) % B</td>
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<tr>
<td>UV detection</td>
<td>260/4 nm Reference 360/100 nm 5 Hz data rate</td>
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Choosing the right pore size

As the pore size of the particle increases, the corresponding surface area decreases. As such, the smaller 100 Å pore has the highest binding capacity for the smaller oligonucleotides (25, 50 mer), while larger pores provide increased binding capacity for larger oligonucleotides (75 mer) through increased permeability and flow dynamics. Therefore, it is important to balance pore size against target to maximize your purification (Figure 4).

Dynamic binding capacity provides a useful indication of how a stationary phase may behave for oligonucleotide purification. It can help determine which material may give the best results for purifying a particular oligonucleotide, and give an indication to the column size that may be required.

Estimated loading capability by column internal diameter

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<tr>
<th>ID (mm)</th>
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<td>318</td>
<td>404</td>
<td>36</td>
<td>38</td>
<td>50</td>
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</tbody>
</table>

Figure 5. Estimate of the amount of oligonucleotide (mg) that might be purified as an initial injection quantity from which optimization and validation is performed. Quantities were calculated using 5% of dynamic binding capacity determined from analytical runs.
Prep-to-process purification with PL-SAX columns

Exceptional pH and thermal stability

Polymeric PL-SAX is chemically and thermally stable under diverse HPLC conditions. These robust ion-exchange columns can be used over a wide range of linear velocities, with fast loading of dilute solutions and wash cycles.

In addition, the increased binding capacity of PL-SAX makes it an attractive choice when scaling up and transitioning from small-scale ion pair reversed-phase purifications to more traditional buffering systems. Their high binding capacity helps you meet your yield requirements using aqueous buffer/non-volatile conditions.

You can also count on:
- Excellent reproducibility and long column life.
- Purifications over a wider pH range. Strong, hydrophilic ion exchange functionalities are covalently linked to a chemically stable polymeric particle.
- Rapid HPLC flow rates and equilibration for reduced purification cycle times.
- Faster sanitation and clean-up through increased column stability.
- High flexibility. The 1000 Å pore size is ideal for high-capacity purifications, while the 4000 Å gigaporous particles with improved mass transfer are best for large biomolecules such as mRNA.

### Conditions

- **Column:** PL-SAX 1000 Å 8 µm, 4.6 x 50 mm
- **Eluent A:** 93% 0.1 M TEAA, pH 8.5/7% ACN
- **Eluent B:** 93% 0.1 M TEAA, 1 M ammonium chloride, pH 8.5/7% ACN
- **Gradient:** 0–40% B in 10 min, 40–70% B in 14 min, 70–100% B in 25 min
- **Flow rate:** 1.5 mL/min
- **Temperature:** 60 °C

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![Physical Stability](image)

**Figure 6.** Pressure vs linear velocity. PL-SAX particles are physically robust and stable up to 6000 psi.

![Thermal Stability](image)

**Figure 7.** Separation of a poly T-standard at 60 °C. Thermal stability lets you use denaturing conditions and stabilizing/solubilizing agents to purify target synthetic oligonucleotides with self-complementary sequences.
When scaling oligonucleotide purification, it is critical to select a particle and column dimension that will meet yield and throughput goals. Determination of dynamic binding capacity is a useful tool for determining the expected quantity of oligonucleotide that can be purified under a given set of conditions.

Following accurate DBC determination, injection of a low percent of the total DBC (typically 1–5%) can allow estimation of the total injection quantity per column volume that can be expected while scaling to prep columns. The percent of the DBC that can be used for purification injections is highly dependent on the impurity profile of the oligonucleotide, though 5% of the DBC is a good initial starting point by which optimization can be performed. No matter the quantity of oligonucleotide being purified, from µg to kg, PL-SAX is offered in a range of column dimensions and bulk media for your preparative needs.

<table>
<thead>
<tr>
<th>Representative loading quantity by column diameter for PL-SAX</th>
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</thead>
<tbody>
<tr>
<td><strong>Column id (mm) x 50 mm</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>2.1</td>
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<tr>
<td>4.6</td>
</tr>
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<td>7.5</td>
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<tr>
<td>25</td>
</tr>
<tr>
<td>50</td>
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</table>

Figure 8. Illustrative dynamic binding capacity of PL-SAX 1000 Å, 8 µm for 100, 50, and 25 mer ssDNA. Binding capacity was measured at 25% of breakthrough curve by overloading the oligonucleotide of interest.

Figure 9. Representative starting load quantities of 25, 50, and 100 mer oligonucleotide for AEX purification utilizing 5% of the dynamic binding capacity of PL-SAX. Loading quantities should be tested for each oligonucleotide and may allow for loading more or less depending on purification goals and impurity profile.

Representative Dynamic Binding Capacity experiment using 100 mer DNA on PL-SAX

**Conditions**

- DNA concentration: 100 mer–1189 ng/µL
- Column: PL-SAX 1000 Å 8 µm 2.1 x 50 mm
- Flow rate: 0.5 mL/min

Figure 10. PL-SAX large pore size and high binding capacity ensures high purity and yield.
Separating guide RNA and modified oligonucleotides with PL-SAX Anion Exchange columns

With their 1000 Å and gigaporous totally porous particles, PL-SAX columns are uniquely suited for the purification of therapeutic oligonucleotides, utilizing common buffers like Tris-HCl with organic modifiers for gRNA separations, or strong basic conditions for separating oxidation impurities of thiolated oligonucleotides.

Unlike traditional silica and small-pore chemistries, PL-SAX has the pH and temperature stability—paired with optimal pore size—required to separate various oligonucleotide sizes and modifications.

Figure 11. Using PL-SAX columns, a high-pH eluent can be used to separate a fully thiolated oligonucleotide from an impurity where thiolation is incomplete. The strong anion exchange functionality on a chemically inert polymeric matrix provides charge differentiation even in 1 M NaOH.

Figure 12. HPLC separation of sgRNA (105 bases) using PL-SAX 1000 Å, 5 µm 2.1 x 50 mm (p/n PL1951-1502). Figure A demonstrates separation of sgRNA utilizing standard 10 mM Tris, pH 8 + 10% ACN at elevated temperature. Figure B utilizes decrease in temperature and high pH to achieve increased resolution with a pH 10 ethylenediamine hydrochloride buffer EDA·HCl.
The benchmark in purification

The 1290 Infinity II Preparative LC system embodies the next generation of instrumentation for preparative LC, delivering the maximum in purification, instrument, and laboratory efficiency. Set the benchmark in your laboratory and boost your daily throughput.

Automated calculation of focused gradient profiles for each target compound helps you to achieve highest purity and recovery.

Our wide range of detectors for fraction triggering—including mass-based detection—ensure that you achieve maximum purity and recovery.

The 1290 Infinity II Preparative Open-Bed Sampler/Collector optimizes bench space. Benefit from the built-in fraction delay calibration of all InfinityLab fraction collectors.
Agilent Load & Lock columns

Ideal for routine, large-quantity purification, Agilent Load & Lock columns exhibit excellent packed bed stability and enhanced flow distribution. Combined with Agilent purification systems, these columns offer maximum efficiency at higher flow rates and delivery pressures, meeting the high-throughput demands of pilot-scale purification.

Easy to set up, easy to use

Within a few minutes, you can pack or unpack your column with any commercially available material—even in hazardous environments. Even better, the column and packing station are combined into a convenient stand, making them easy to move.

High performance on a large scale

Agilent Load & Lock columns are available with inside diameters from 1 to 3 inches (27 to 75 mm). They also offer both dynamic axial compression (DAC) and static axial compression (SAC).

Axial compression is used in the column packing process to compress the sorbent particles into a tightly packed, void-free bed for high-performance purifications. DAC constantly compresses the packed bed during use. With SAC, the column is first compressed by a plunger, which is held in position by a locking mechanism.

Pack multiple columns into one mobile station, and use the unlocking feature to deploy the packed columns anywhere in your lab.
## Ordering Information

### Agilent PL-SAX analytical columns

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<tr>
<th>Dimensions (mm)</th>
<th>Particle Size (µm)</th>
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<th>PL-SAX 4000 Å</th>
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### Custom columns and bulk media

If you do not see the combination of pore/particle size and column dimensions or the bulk media quantity you are looking for in these tables, please contact your local sales office, who will assist you with our custom ordering process.

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