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Consumables Workflow Ordering Guide

Oligonucleotide Purification using Anion Exchange Liquid Chromatography

Synthetic Oligonucleotides (ONs) are a class of compounds that have gained increasing interest over the last few years because of their use in biochemical research and as pharmaceuticals. The process of synthesizing ONs has become much more efficient and can often reach 99% coupling efficiency. However, a 25 mer ON synthesis will yield less than 80% of the desired product, with decreased yield as a function of length.

Separation of the final oligonucleotide product from its closest impurity is challenging, as these impurities are highly related to the full-length product. This challenge is exacerbated as a function of length, with longer oligonucleotides having more complex impurity profiles. Beyond n-1,2,3,...x impurities, synthesis-related base loss, incomplete thiolation of the backbone and others must also be considered.

Factors to consider

Selecting the right column chemistry

Deciding what columns chemistry to use depends on purity requirements, buffer options, and scale of purification. Ion pair reversed-phase and anion exchange are the most common tools for purification of oligonucleotides ranging from a few bases, to thousands of bases like those found in mRNA.

Anion exchange chromatography of oligonucleotides is a widely deployed separation technique for UV analysis and large-scale purification.[1-2] Anion exchange, which uses common mobile phases like tris or phosphate buffers and salt (NaCl), is a cost effective and reproducible purification technique for separating oligonucleotides from their impurities. Unlike ion pair reversed phase, anion exchange is a UV technique that is not typically paired with mass spectrometry (MS) due to the high salt concentrations used.

Agilent PL-SAX chemistry offer scalable purification solutions

- Analytical and preparative prepacked columns along with bulk media for large-scale production
- Polymeric PS-DVB base particles that are stable at high temperatures and pH
- Large pore 1000 and 4000 Å options that ensure optimal resolution of oligonucleotides ranging from small oligos with 10s of bases all the way to 1000s of bases mRNA. For most oligonucleotides, the 1000 Å pore provides the highest binding capacity and delivers excellent resolution between the full-length product and associated impurities. For large molecules, such as mRNA, 4000 Å pores provide greater permeability.

Ion pair reversed-phase (IP-RP) chromatography[3-4] is a common technique used for ON analysis and small-scale purification. It is often selected for its resolving power using alkyl amine acetate salts as ion pair reagents using UV detection. Substituting the acetate with MS compatible hexafluoroisopropanol (HFIP), enables MS analysis and is often used to elucidate and identify impurities with similar masses such as base loss, oxidation impurities in thiolated oligos, and adducts. To learn more, refer to the PLRP-S workflow ordering guide, 5994-4636EN.

Selecting the right pore and particle size

Oligonucleotides and nucleic acids come in a range of sizes and structures, from a few bases to thousands of bases. Depending on the oligonucleotide of interest and separation goals, the choice of pore size is critical to ensure effective mass transfer of the oligonucleotide into the pore structure. PL-SAX 1000 Å is preferred when working with oligos ranging from a few bases to 200 bases. This includes small therapeutic oligonucleotides such as siRNA used for RNA interference to gRNA used for gene editing. For larger oligonucleotides such as large mRNA, PL-SAX 4000 Å with a larger pore size is recommended to ensure effective mass transfer and limit particle shearing of the full-length product.

When scaling up purification methods, the size of the particle may also need to be increased to ensure that the pressure remains in the operating range of your instrument and equipment. This may mean moving to larger 10 or 30 µm particles and is especially true if working with medium or low-pressure systems.

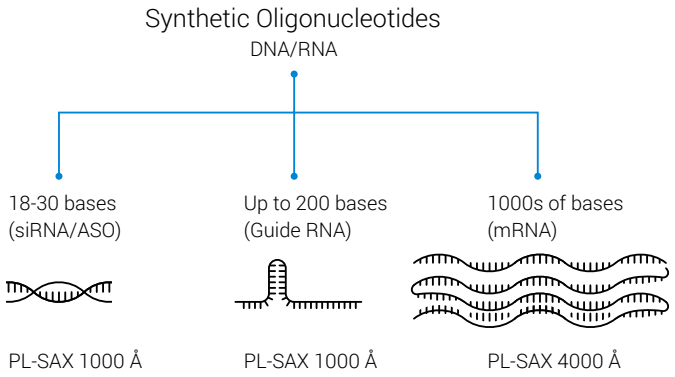

Figure 1. Types of oligonucleotides and recommended pore sizes.

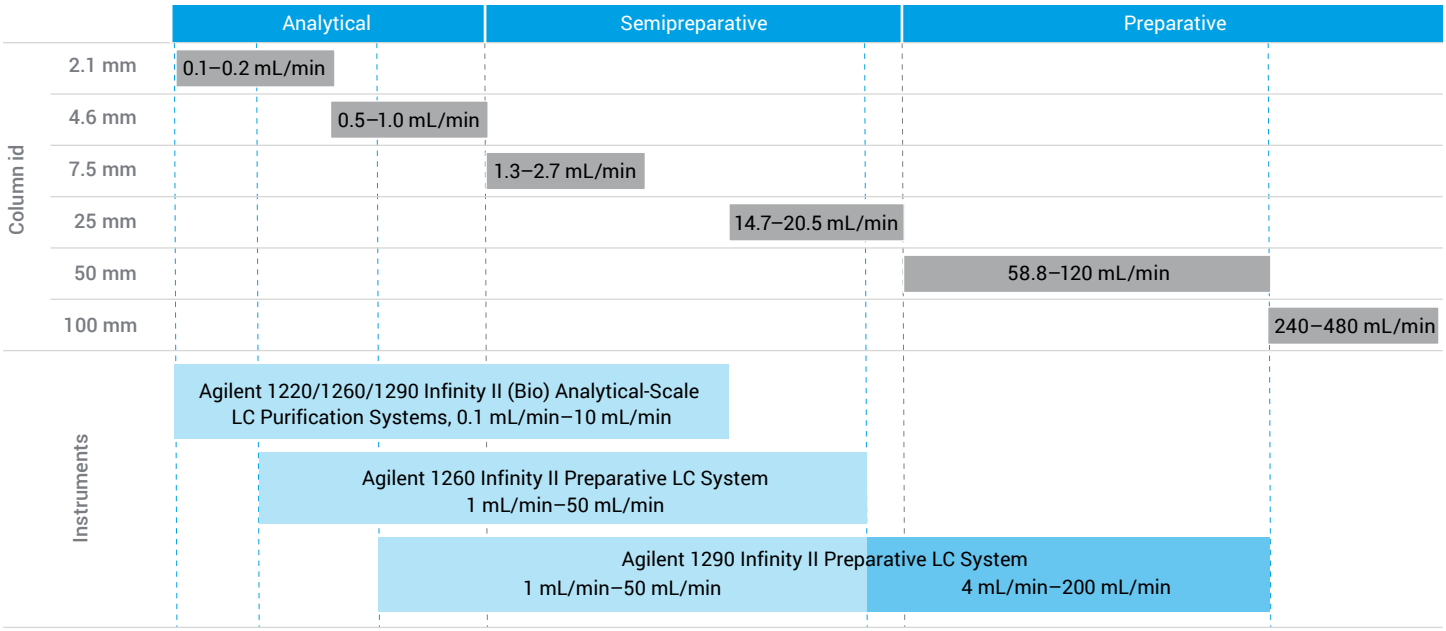

Figure 2. Range of analytical to preparative Agilent instruments and column dimensions for oligonucleotide purification. Recommended flow rates and instrumentation is outlined for each column dimension.

Determining optimal conditions for the separation

Limit oligoncleotide secondary interactions when performing anion exchange. This can be achieved by using:

1) **Increased pH:** NaOH at pH 11 or 12 can help break up secondary interactions and sharpen peaks. Using high pH, further separation of oxidation impurities in fully thiolated oligonucleotides can be seen[5]. When purifying molecules such as RNA-based oligos, high pH paired with high temperature can result in the formation of purification related impurities that should be tested and monitored.

   As the oligonucleotide length increases, the net negative charge increases. This may require higher salt concentrations or even higher pH to effectively elute long mRNA. Conditions should be tested to optimize yield.

2) **Increased temperature**[5-6]: Temperature is a common parameter to investigate whether performing ion pair reverse phase or anion exchange purifications. Instruments equipped with a column heater can be used to increase temperature, up to ~80 ˚C. This sharpens peaks by breaking up secondary interactions. Though useful, modulation of temperature may be difficult when moving up to large-scale columns.

3) **Organic additives**: Organic modifiers is a commonly used alternative when temperature modulation is not an option. Care should be taken to ensure that the concentration of organic modifier does not cause the eluting salt to precipitate. Acetonitrile (ACN), the most common organic modifier, is typically used in the binding and eluting buffers at a concentration of 10 to 15%.

Figure 3 demonstrates how to optimize separation conditions by identifying the best pH, temperature, and organic additive combination to use when purifying a sgRNA. When optimizing method conditions, it is recommended that the input quantity and method conditions are optimized on an analytical column with the same length as the column that will be used for scale-up. After optimization, scale-up column input quantity and flow rate can be calculated.

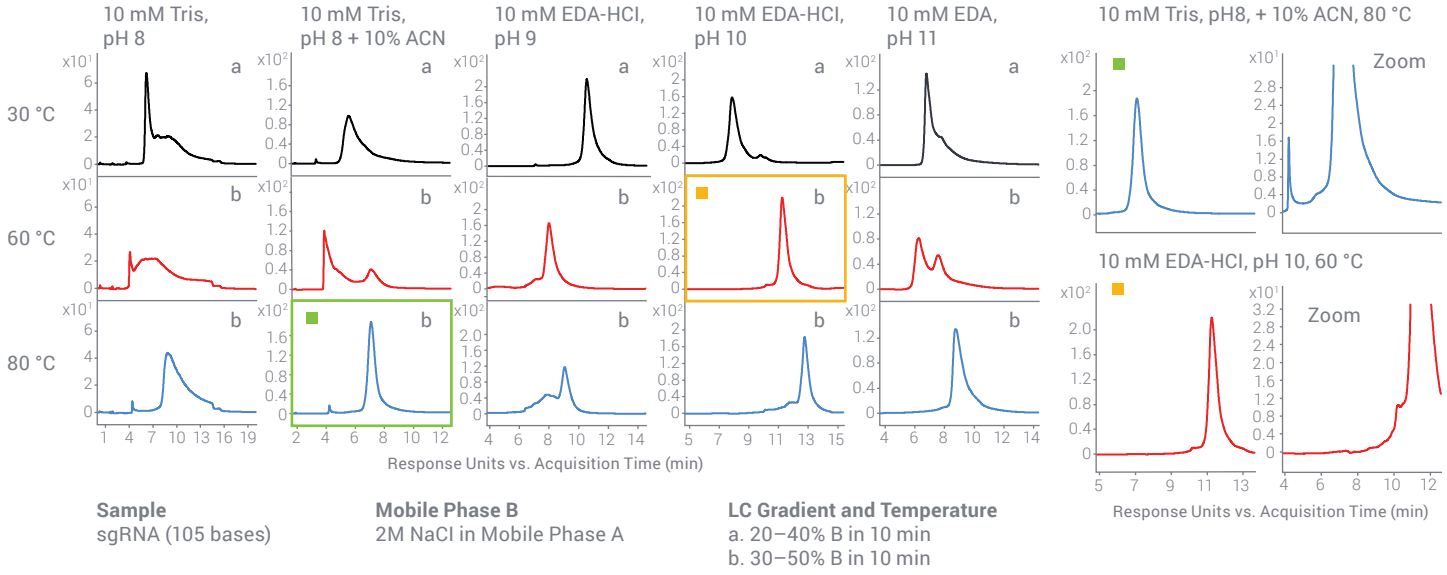

Figure 3. Method optimization for the purification of sgRNA using the Agilent PL-SAX 1000 Å column. Method mobile phase, temperature, and gradients were scouted to determine the optimal separation conditions.

Scale

Scale is one of the many factors to consider when preparing for ON purification. The quantity of oligonucleotide that needs to be purified will determine the size of the column and the instrument configuration required.

When performing analytical scale-up, it is important to determine the appropriate flow rate to apply when moving to a semiprep or prep column. For PL-SAX columns, the recommended linear velocity is between 180 and 360 cm/hr. Therefore, an analytical injection optimized with a volumetric flow rate of 0.8 mL/min on a 4.6 mm id column scales to a flow of 24 mL/min on a semiprep 25 mm id column using the flow equation:

$$V = \frac{L}{60} * \frac{\pi * d^2}{4}$$

V = volumetric flow rate (mL/min)
d = column inner diameter (cm)
L = l inear flow rate (cm/hr)

This equation can be simplified once the volumetric flow of the analytical dimension is determined, assuming particle size remains constant:

$$V_p = V_a * \left(\frac{D_p{}^2}{D_a{}^2} \right)$$

V_p = volumetric flow rate prep (mL/min)
V_a = volumetric flow rate analytical (mL/min)
D_p = diameter prep (mm)
D_a = diameter analytical (mm)

Scaling the particle size from small analytical particles (3 µm) to prep particle sizes (10 to 50 µm) may also be required to stay within the operating pressure range of your preparative instrument. A change in particle size can affect the overall resolution and mean retention time of the primary oligonucleotide product. Maintaining resolution may require increasing the column length to increase the plate count (N) so it is equivalent to the analytical column particle. Calculate theoretical plates using the following equation:

$$N_a = \frac{L_a}{Dp_a}$$

N_a= analytical column theoretical plates
L_a= column length analytical (mm)
Dp_a= particle diameter analytical (mm)

Example: If moving from a 5 µm analytical column with dimensions of 2.1 x 150 mm to a 10 µm column with a 25 mm id, consider moving to a column length of 300 mm to maintain equivalent theoretical plates.

$$N_a * Dp_p = L_p$$

N_a = Theoretical plates analytical run
Dp_p = prep particle diameter (mm)
L_p = suggested prep column length (mm)

Bulk media PL-SAX

Anion exchange with PL-SAX is often preferred for large process-scale purifications due to the lower cost of buffers used and because large volatile-buffer quantities are not needed.

When moving to bulk media for large-scale production, Agilent offers a range of Load & Lock columns[7] for use with InfinityLab Purification LC solutions to achieve maximum purity and yield. Load & Lock columns are available in 1, 2, and 3 inch columns, offering high performance and high throughput. PL-SAX media is available in 10g, 100 g or 1 kg, depending on scale and throughput requirements.

Best practices and helpful tips

Condition your PL-SAX column before use

New PL-SAX columns contain shipping solution (0.1 M Na_2SO_4 and 0.02% NaN_3) on arrival and require conditioning with an appropriate mobile phase prior before use. To condition a column:

1) Elute with five column volumes of a low ionic strength mobile phase (buffer A).
2) Exchange buffer A with a high ionic strength mobile phase (buffer B). Continue with this eluent with a minimum of five column volumes or until a stable baseline is achieved at the required sensitivity.
3) Equilibrate with buffer A for a minimum of five column volumes before use.

Recommended PL-SAX column conditions and operating ranges

Column Specifications	Particle Size	Pressure Limit	Linear Velocity	pH Range	Max Temperature
PL-SAX (1000 Å, 4000 Å)	5 µm, 8 µm, 10 µm	207 bar (20.7 MPa)	180 – 360 cm/hr	1 to 14	80 °C
	30 µm	103 bar (10.3 MPa)			
Shipping Solvent	0.1 M Na_2SO_4 and 0.02% sodium azide	Compatibility	Compatible with all commonly used ion exchange eluents, buffers, and salt as well as nonionic and zwitterionic detergents. NOT compatible with anionic detergents		

Operating tips

- Reverse flow will not usually harm the column but should be avoided except when removing a clogged frit (see "column care").
- Start the flow rate at a reduced rate and gently increase it to the desired operating flow rate.
- Always use high purity reagents and chromatography grade solvent to prepare your mobile phase. Degas and filter all mobile phase before use.
- An inline filter may be used to protect your column and increase its lifetime.
- Operating at the maximum temperature for prolonged periods will reduce column lifetime.

Cleaning and storing your PL-SAX column to extend column lifetime

An increase in column backpressure is likely to occur over time. Absorption of oligonucleotide/nucleic acid to the packing material or on the inlet frit will cause an increase in pressure and will decrease column performance. Cleaning the column can decrease the backpressure and improve performance.

Detailed operating tips, cleaning, and storage recommendations can be found in the Agilent PL-SAX user guide.

Selecting the right instrument[8, 9]

Agilent 1290 Infinity II Preparative LC Systems

Dynamic flow range up to 200 mL/min.

Seamless method transfer from rapid analytical scouting runs to scale up to gram-level purification of compounds on a single system.

Purification up to 50 mm id columns.

Agilent 1260 Infinity II Bio Analytical-Scale LC Purification System

Biocompatible solvent and sample flow path ensure integrity of biomolecules.

Binary or quaternary gradient pump delivers flow rates up to 5 mL/min.

Purification up to 10.0 mm id columns.

Agilent 1220/1260/1290 Infinity II Analytical-Scale LC Purification Systems

Ideal for purification of multi-milligram quantities of materials.

Flow rates between 0.1 and 10 mL/min.

Works with 2.1 and 10.0 mm id analytical column.

References

1. High Resolution Separations of Oligonucleotides using PL-SAX Strong Anion-Exchange HPLC Columns 5990-8297EN
2. Agilent PL-SAX Anion-Exchange Media for Nucleotide and Oligonucleotide Analysis 5990-8779EN
3. Purification of Single-Stranded RNA Oligonucleotides Using High-Performance Liquid Chromatography 5994-3514EN
4. Direct Analysis of In-Process Oligonucleotides Without Manual Purification 5991-9490EN
5. Improved Column Lifetime with Thermally Stable Polymer Columns for Oligonucleotide Ion-Pair RP HPLC 5990-7764EN
6. Use Temperature to Enhance Oligonucleotide Mass Transfer and Improve Resolution in Ion-Pair RP HPLC 5990-7765EN
7. Purify Your Way, Agilent Lock & Load Columns 5994-3907EN
8. Agilent InfinityLab LC Purification Solutions 5991-9153EN
9. Purify Your Samples with Maximum Flexibility 5991-9154EN

Easy selection and ordering information

This guide lists all the columns and supplies you will need for oligonucleotide analysis using PL-SAX and appropriately setup system. To order items listed in the tables below from the Agilent online store, add items to your Favorite Products list by clicking on the MyList # header links. You can then enter the quantities for the products you need, add the products to your Cart and proceed to checkout. Your list will remain under Favorite Products for your use with future orders.

If this is your first time using Favorite Products, you will be asked to enter your email address for account verification. If you have an existing Agilent account, you will be able to log in. However, if you don't have a registered Agilent account, you will need to register for one. This feature is valid only in regions that are e-commerce enabled. All items can also be ordered through your regular sales and distributor channels.

MyList 1: Oligonucleotide standards

Description	Part No.
Standards	
DNA ladder standard, oligos at 15, 20, 25, 30, 35, 40 mer, 1 mL	5190-9029
RNA resolution standard, oligos at 14, 17, 20, and 21 mer, 1 mL	5190-9028

MyList 2: Analytical scale PL-SAX columns

Agilent PL-SAX analytical columns		Part No.	Part No.
Dimensions (mm)	Particle Size (µm)	PL-SAX 1000 Å	PL-SAX 4000 Å
2.1 x 50	5	PL1951-1502	PL1951-1503
4.6 x 50		PL1551-1502	PL1551-1503
2.1 x 50	8	PL1951-1802	PL1951-1803
2.1 x 150		PL1951-3802	PL1951-3803
4.6 x 50		PL1551-1802	PL1551-1803
4.6 x 150		PL1551-3802	PL1551-3803
4.6 x 150	10	PL1551-3102	PL1551-3103
4.6 x 250		PL1551-5102	PL1551-5103
4.6 x 150	30	PL1551-3702	PL1551-3703
4.6 x 250		PL1551-5702	PL1551-5703

MyList 3: Analytical scale supplies

Description	Part No.
Solvent and sample preparation	
AdvanceBio Spin columns for desalting or buffer exchange, <100 µL samples, 25/pk, collection tubes included	1980-1103
AdvanceBio Spin 96-sample plate for desalting or buffer exchange, 10 to 50 µL samples, 1/pk	1980-1104
96-well plate, polypropylene, 0.33 mL, 14 mm, round wells, V shape, 25/pk Recommended for final collection step with p/n 1980-1104	5043-9312
Captiva disposable syringe, 5 mL, 100/pk	9301-6476
Captiva premium syringe filter, PES, 4 mm, 0.2 µm, 100/pk (sample volume <1 mL)	5190-5094
Captiva premium syringe filter, PES, 15 mm, 0.2 µm, 100/pk (sample volume 1–15 mL)	5190-5096
InfinityLab Ultrapure LC/MS water, 1 L	5191-4498
InfinityLab Ultrapure LC/MS acetonitrile, 1 L	5191-4496
InfinityLab Quick Change inline filter assembly, for HPLC	5067-1602
InfinityLab Quick Change inline filter assembly, for UHPLC	5067-1603
Column fittings and connectors	
Agilent InfinityLab Quick Connect fitting (for connection on column inlet)	5067-5965
Agilent InfinityLab Quick Connect capillary MP35N 0.12 x 105 mm (for Quick Connect fitting)	5500-1578
Agilent InfinityLab Quick Turn fitting (for connection on column outlet)	5067-5966
Quick Turn capillary MP35N 0.12 x 280 mm (for Quick Turn fitting)	5500-1596
Mounting tool for QuickTurn fittings	5043-0915
Capillary MP35N 0.12 x 90 mm SL/SL ns/ns (for connecting guard and column)	5004-0018
Sample containment	
A-line screw top vial, 2 mL, amber, write-on spot, 100/pk vial size 12 x 32 mm (12 mm cap)	5190-9590
Screw cap, bonded, blue, PTFE/white silicone septa, 100/pk. Cap size 12 mm	5190-7021
Vial insert, 250 µL, deactivated glass with polymer feet, 100/pk. Insert size 5.6 x 30 mm	5181-8872
InfinityLab 96-well plate, 0.5 mL, 30/pk	5043-9310
InfinityLab 96-well plate, 1 mL, 50/pk	5043-9305
InfinityLab 96-well plate, 1.2 mL, 25/pk Recommended for wash steps with p/n 1980-1104	5043-9308
InfinityLab 96-well plate, 2 mL, 30/pk	5043-9302
InfinityLab 96-well plate, 2.2 mL, 30/pk	5043-9300
InfinityLab 96-well plate closing mat, 50/pk (for 5043-9310, 5043-9305, 5043-9308, 5043-9302)	5042-1389
InfinityLab 96-well plate closing mat, 50/pk (for 5043-9300)	5043-9319

Description	Part No.
1260 Infinity II/1260 Infinity II Bio-Inert analytical fraction collection (G1364F and G5664B)	
Glass test tubes, 12 x 48 mm, 5 mL, 100/pk	5022-6534
Glass test tubes, 16 x 48 mm, 9 mL, 100/pk	5022-6533
Glass test tubes, 30 x 48 mm, 20 mL, 100/pk	5042-6470

MyList 4: Preparative scale PL-SAX columns

Agilent PL-SAX preparative columns		Part No.	Part No.
Dimensions (mm)	Particle Size (µm)	PL-SAX 1000 Å	PL-SAX 4000 Å
7.5 x 50	8	PL1151-1802	PL1151-1803
7.5 x 150		PL1151-3802	PL1151-3803
25 x 50	10	PL1251-1102	PL1251-1103
25 x 150		PL1251-3102	PL1251-3103
50 x 150		PL1751-3102	PL1751-3103
100 x 300		PL1851-2102	PL1851-2103
25 x 150	30	PL1251-3702	PL1251-3703
50 x 150		PL1751-3702	PL1751-3703
100 x 300		PL1851-3102	PL1851-3103

MyList 5: Preparative scale supplies

Description	Part No.
Solvent and sample preparation	
AdvanceBio Spin columns for desalting or buffer exchange, <1000 µL samples, 50/pk columns, plus 4 reusable adapters	1980-1105
AdvanceBio Spin column re-usable adapters, 8/pk For optional use with p/n 1980-1105	1980-1106
Centrifuge tube, polypropylene, graduated, 29 mm od, 115 mm, 50 mL, conical base, wide neck, threaded top, 25/pk	5610-2049
Centrifuge tube, polypropylene, graduated, 29 mm od, 115 mm, 50 mL, skirted conical base, wide neck, threaded top, 500/pk	190065200
Captiva disposable syringe, 5 mL, 100/pk	9301-6476
Captiva disposable syringe, 10 mL, 100/pk	9301-6474
Captiva disposable syringe, 20 mL, 100/pk	5190-5103
Captiva premium syringe filter, PES, 15 mm, 0.2 µm, 100/pk (1–15 mL sample volume)	5190-5096
Captiva premium syringe filter, PES, 15 mm, 0.45 µm, 100/pk (1–15 mL sample volume)	5190-5097
Captiva Econofilter, polypropylene, PES, 25 mm, 0.2 µm, 100/pk (15–100 mL sample volume)	5190-5098
Captiva Econofilter, polypropylene, PES, 25 mm, 0.45 µm, 100/pk (15–100 mL sample volume)	5190-5099
Semiprep filter, 0.5 µm, 12.7 mm id, 1–5 mL/min (replacement frit 5022-2185)	5064-8273
High pressure semiprep filter, 10 µm, 19 mm id, 5–10 mL/min (replacement frit: 5022-2166)	5022-2165

Description	Part No.
Sample containment	
A-line screw top vial, 2 mL, amber, write-on spot, 100/pk. Vial size 12 x 32 mm (12 mm cap)	5190-9590
Screw cap, bonded, blue, PTFE/white silicone septa, 100/pk. Cap size 12 mm	5190-7021
Vial, screw top, clear, high recovery, 5 mL, for LC, 30/pk	5188-5369
Septum, preslit PTFE/silicone, 16 mm, 100/pk	5188-2758
Cap, screw, for 6 mL vials, 100/pk	9301-1379
InfinityLab 96-well plate, 2 mL, 30/pk	5043-9302
InfinityLab 96-well plate, 2.2 mL, 30/pk	5043-9300
InfinityLab 96-well plate closing mat, 50/pk (for 5043-9302)	5042-1389
InfinityLab 96-well plate closing mat, 50/pk (for 5043-9300)	5043-9319
1260 and 1290 Infinity II Preparative LC System	
System capillary kit, 15–40 mL/min	5067-7016
System capillary kit, 40–80 mL/min	5067-7017
System capillary kit, 80–200 mL/min	5067-7018

MyList 6: Infinity II Preparative Open-Bed Fraction collection

Description	Part No.
Glass test tubes, 12 x 48 mm, 5 mL, 100/pk	5022-6534
Glass test tubes, 12 x 100 mm, 7 mL, 250/pk	5022-6531
Glass test tubes, 16 x 48 mm, 9 mL, 100/pk	5022-6533
Glass test tubes, 16 x 100 mm, 14 mL, 250/pk	5022-6532
Glass test tubes, 25 x 100 mm, 35 mL, 100/pk	5042-6459
Glass test tubes, 30 x 48 mm, 20 mL, 100/pk	5042-6470
Glass test tubes, 30 x 100 mm, 45 mL, 100/pk	5042-6458
Glass test tubes, 12 x 150 mm, 11 mL, 250/pk	5190-9093
Glass test tubes, 16 x 150 mm, 21 mL, 250/pk	5190-9092
Glass test tubes, 25 x 150 mm, 55 mL, 100/pk	5190-9091
Glass test tubes, 30 x 150 mm, 85 mL, 100/pk	5190-9090

MyList 7: PL-SAX bulk media and columns

Agilent PL-SAX bulk media		Part No.	Part No.
Particle Size (µm)	Unit	PL-SAX 1000 Å	PL-SAX 4000 Å
10	10 g	PL1451-2102	PL1451-2103
	100 g	PL1451-2103	PL1451-4103
	1 kg	PL1451-6102	PL1451-6103
30	10 g	PL1451-2702	PL1451-2703
	100 g	PL1451-4702	PL1451-4703
	1 kg	PL1451-6702	PL1451-6703
Load & Lock columns for bulk media			
Load & Lock column, 27 id x 500 mm L		PCG93LL500X25WJ	
Load & Lock column, 50 id x 500 mm L		PCG93LL500X50WJ	
Load & Lock column, 75 id x 500 mm L		PCG93LL500X75WJ	
Mobile packing station (air driven hydraulic)		PCG93LLSTAND123	
Load & Lock low pressure upgrade kit for mobile packing station		PCG93LLSTAND123LPU*	

** Not available for purchase online. Please contact your local sales representative for details.*

MyList 8: Solvent filtration supplies

Description	Part No.
Solvent filtration	
InfinityLab solvent filtration assembly	5191-6776
InfinityLab solvent filtration flask, glass, 2 L	5191-6781
Filter membrane, Nylon 47 mm, pore size 0.2 µm, 100/pk	5191-4341
Filter membrane, regenerated cellulose 47 mm, pore size 0.2 µm, 100/pk	5191-4340
Solvent bottle glass filter, solvent inlet, 20 µm	5041-2168

MyList 9: Solvent handling supplies

Description	Part No.
Solvent handling	
InfinityLab Stay Safe cap starter kit	5043-1222
InfinityLab solvent bottle, clear, 1 L	9301-6524
InfinityLab solvent bottle, amber, 1 L	9301-6526
Solvent bottle, clear, 2 L	9301-6342
Solvent bottle, amber, 2 L	9301-6341
InfinityLab Stay Safe purging bottle	5043-1339
InfinityLab waste can, GL45, 6 L with Stay Safe cap (charcoal filter 5043-1193 not included)	5043-1221
InfinityLab charcoal filter with time strip, 58 g (use with 5043-1221)	5043-1193

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