

HALO[®]

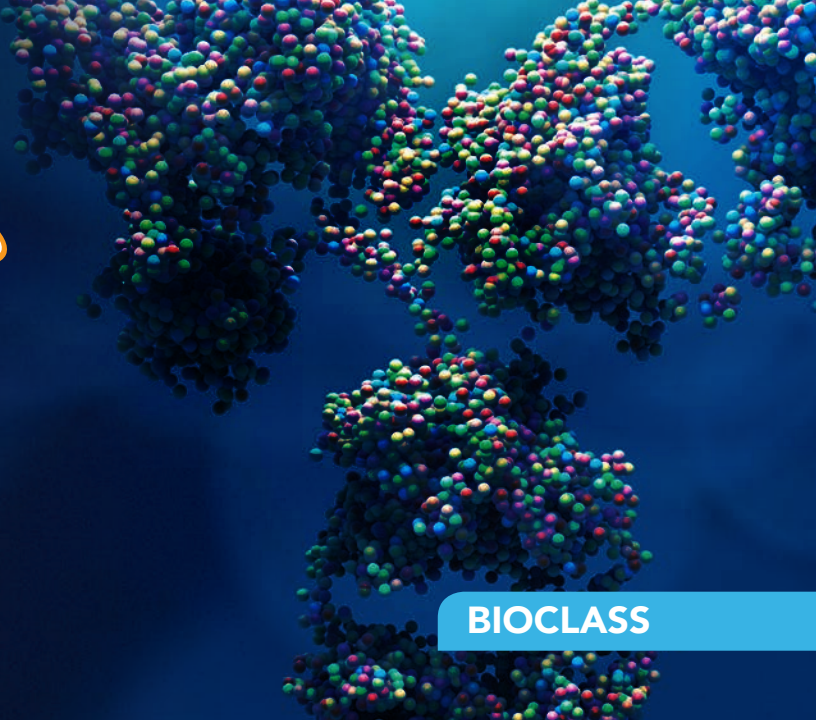
Biotherapeutic Method Development Guide

HALO[®] BIOCLASS 1000 Å PROTEIN SELECTIVITY KIT

Fused-Core[®] Particle Technology



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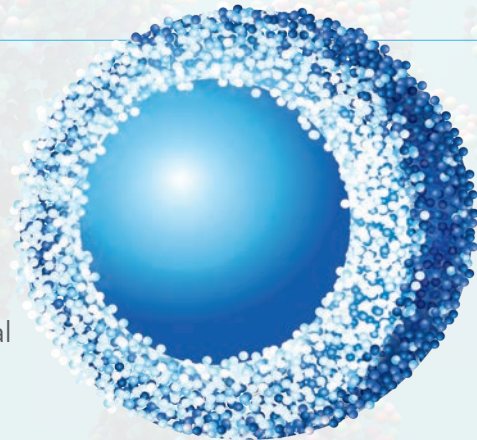
Strategy for Optimizing Protein Separations Using Reversed-phase Liquid Chromatography with Fused-Core® Particles.

- The advantages of using wide pore silica based superficially porous particles (SPP) for high resolution analysis of large proteins has been well established.
- Fused-Core® particles provide narrower peak widths and improved resolution of these biomolecules in comparison to fully porous particles (FPP).
- How are these improvements possible, when, by design, the SPP has less surface area than a FPP? Accessibility!



Reasons for a 1000 Å Pore Size

- Surface area only matters if the analytes can access it. With a 1000 Å pore size, large, bulky protein structures have unrestricted access to the bonded phase which resides primarily in the pores.
- Monoclonal antibodies (mAbs) are very large biomolecules. As a general guideline, the particle pore size should be ~ 10 fold larger than the analyte of interest in order to avoid (or minimize) restricted diffusion.
- Inadequate pore size results in broader peaks and lower resolution.



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Advantages of SPPs for Large Molecule Separations

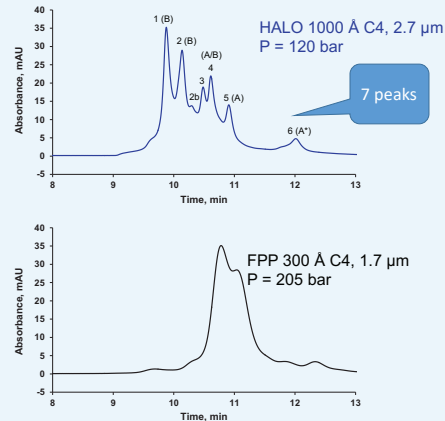
- Thanks to total pore access, improved resolution from HALO 1000 Å particles over fully porous particles is evident in this comparison of IgG2 variants.

Columns: 2.1 x 150 mm; Flow rate: 0.2 mL/min; Mobile Phase A: 88/10/2 H₂O/ACN/n-Propanol + 0.1% DFA; Mobile Phase B: 70/20/10 n-Propanol/ACN/H₂O + 0.1% DFA; Gradient: 14-24% B in 20 min; Injection Volume: 2 µL of 2 mg/mL denosumab in water + 0.1% DFA; Temp: 80 °C; Detection: PDA at 280 nm



Twice the peaks

Almost half the back pressure

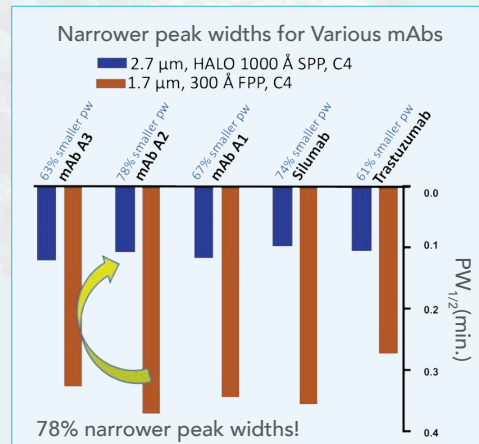


Comparative results presented here may not be representative for all applications.

Benefits of Very Large Pores with mAbs

- The benefits of SPPs for HPLC and UHPLC separations have been well established and include very high efficiencies without the consequence of high backpressure.
- For these five large biomolecules compared on a sub-2- μm FPP column and a 2.7 μm HALO 1000 Å column, the average peak width was 69% narrower using the HALO® SPP column.

Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 27-37% B in 20 min; Instrument: Shimadzu Nexera; Injection Volume: 2 μL (1 μg); Detection: 280 nm; Temp: 80 °C



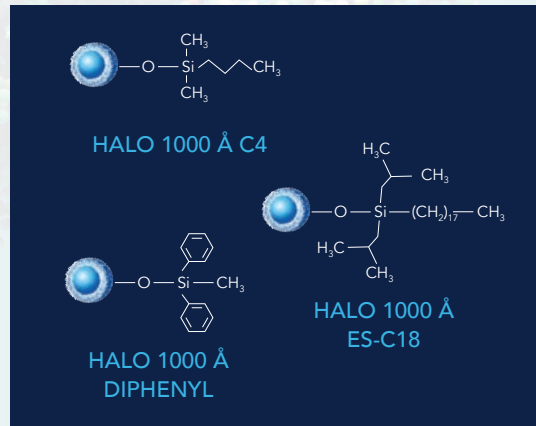
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1. Stationary Phase Selection

Your mAbs are unique – we provide options!

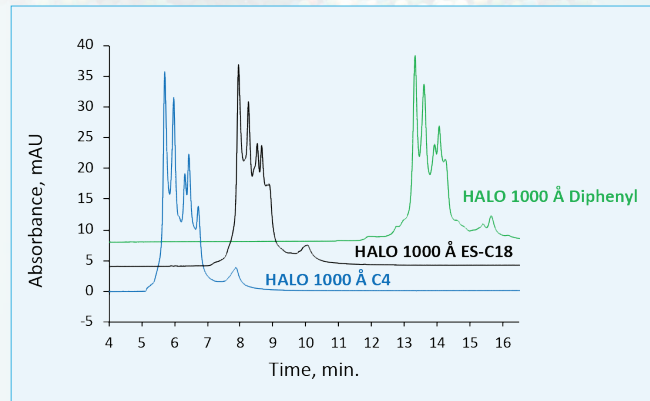
- As complex bio-therapeutics development continues to grow, understanding their structural modifications requires separation options. Often these minor variants consist of subtle differences in protein chains, glycosylation sites and free sulfhydryl groups.
- Only HALO® delivers three 1000 Å silica phase selectivities to choose from. Your reversed-phase LC method development just became more comprehensive!



Stationary Phase Selection

- Comparisons of short chain alkyl (C4), long chain alkyl (C18) and aromatic (diphenyl) stationary phases on a 1000 Å pore size demonstrate comparable resolving power, but also tunable selectivity for separations of highly similar mAbs.

Columns: 2.1 x 150 mm; Flow rate: 0.2 mL/min; Mobile Phase A: 2:10:88 n-propanol/ACN/H₂O + 0.1% difluoroacetic acid (DFA); Mobile Phase B: 70:20:10 n-propanol/ACN/H₂O + 0.1% difluoroacetic acid (DFA); Gradient: 16-26% B in 20 min; Injection Volume: 2 µL of 2 mg/mL denosumab in water/0.1% TFA; Temp: 80 °C; Detection: PDA at 280 nm



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2. Mobile Phase Additive Selection

FA? TFA? - DFA to the rescue!

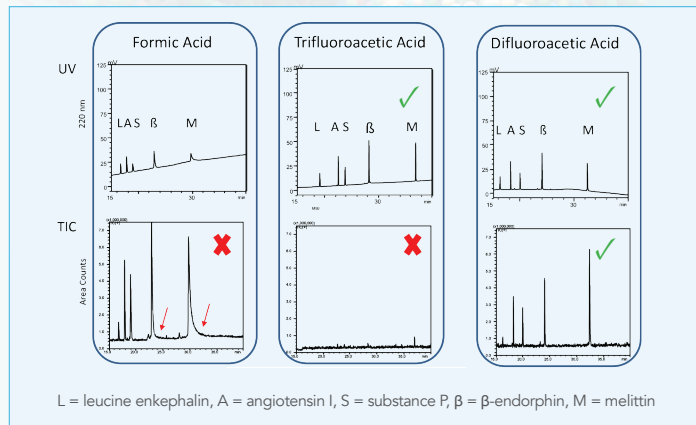
- Formic Acid (FA) is popular additive for increasing ionization efficiencies, however, for many large molecules, chromatography will suffer. Often, an increase in tailing, peak width, and poor recovery is observed.
- Trifluoroacetic Acid (TFA) is a popular additive, when not using mass spectral (MS) detection, providing excellent peak shape and high recoveries.

With LCMS methods, TFA can be tolerated at the expense of signal intensity. This is due to the well known ionization suppression inherent to TFA ion pairing with bases, and problems associated with TFA in the negative ion electrospray ionization (ESI) mode of operation. To restore the LCMS system to highest sensitivity operation it is usually necessary to flush the instrument components using 50:50 ACN/H₂O (1% acetic acid) for 18-24 hours!



Benefits of DFA

- Switching to Difluoroacetic Acid (DFA), a less fluorinated ion pairing acid mobile phase modifier provides MS sensitivity improvement relative to TFA, particularly with small to mid size molecules. DFA has the practical advantage of similar chromatographic benefits of TFA, (including excellent peak shape and recovery), along with easier removal from instrument components. DFA can be easily removed in 10-15 minutes with 50:50 ACN/H₂O.

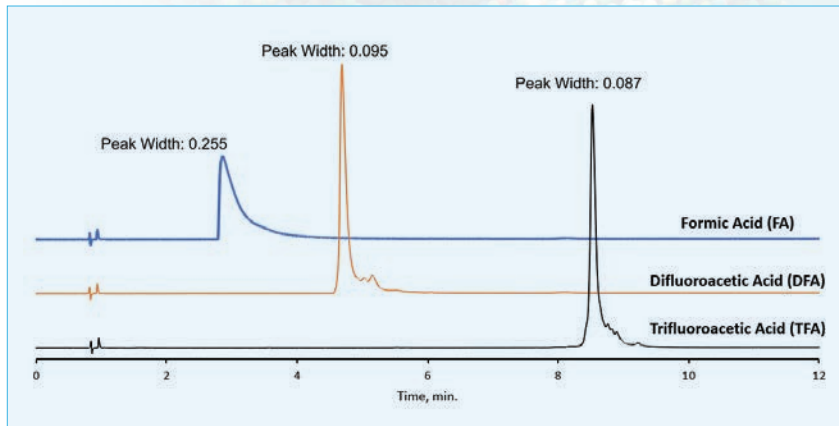


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Effect of Acid Modifiers on Intact mAb Peak Shape

- Both DFA and TFA provide improved peak shape and much narrower peak widths than formic acid (FA) for gradient separations of mAbs.

Column: HALO 1000 Å C4, 2.7 μm , 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water with 0.1% acid noted; Mobile Phase B: 80/20 ACN/water with 0.1% acid noted; Gradient: 35-47.5% B in 12 min; Injection Volume: 2 μL of trastuzumab; Temp: 80 °C; Detection: PDA at 280 nm



Use of Alcohol Organic Modifiers

Concerned about recovery? Interested in analysis speed? Just add alcohol.

- Using alcohol as an organic modifier allows separation at lower temperatures and in the case of Diphenyl, increased recoveries at these lower temperatures (Fig. 1). Notice the effect of alcohol as an organic modifier in terms of analysis speed (Fig. 2).

Figure 1.

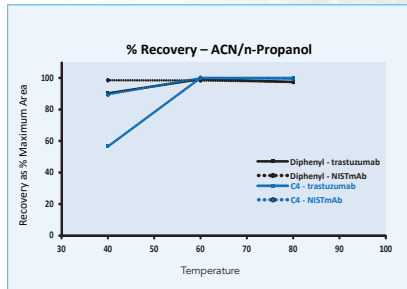
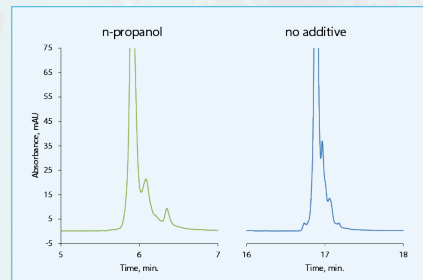


Figure 2.



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3. Gradient Condition Selection

Consider the Goal – Resolution, Speed, or Suitable Compromise?

- Once the ideal stationary phase and mobile phase composition is selected, adjust gradient steepness to assess its impact on selectivity and resolution.

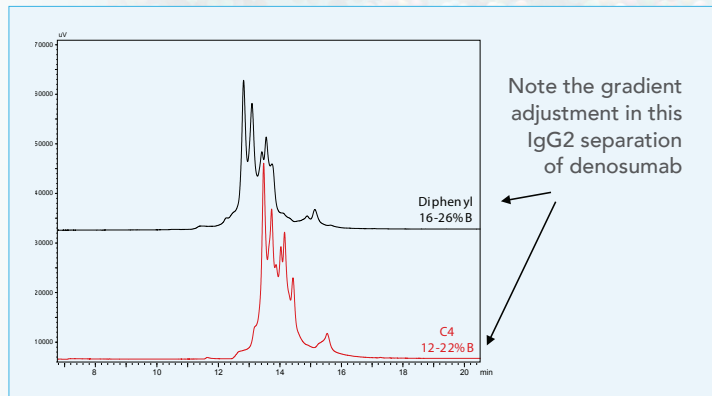


Goal: Resolution

- When comparing the HALO 1000 Å phases the appropriate initial and final %B and gradient conditions will likely be different for each phase type.
- Here, the same A and B solvent premixes are used, but different gradient programs are used to match retention time windows for comparison.

Columns: 2.1 x 150 mm; Flow rate: 0.2 mL/min; Mobile Phase A: 88/10/2 H₂O/ACN/nProp + 0.1% DFA; Mobile Phase B: 70/20/10 nProp/ACN/H₂O + 0.1% DFA; Gradient: 16-26 %B in 20 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL; Detection: 280 nm; Temp: 80 °C

Shallow gradient overlay comparison for optimization of candidate phases



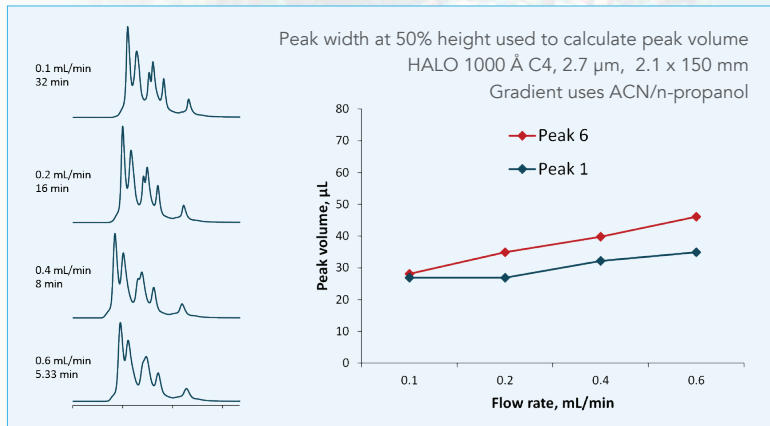
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Goal: Speed or Suitable Compromise?

Is it speed that is the goal? How much resolution is required?

By comparing different flow rates with scaled gradient times (to produce the same k^*) resolution and speed can be evaluated to strike the correct balance.

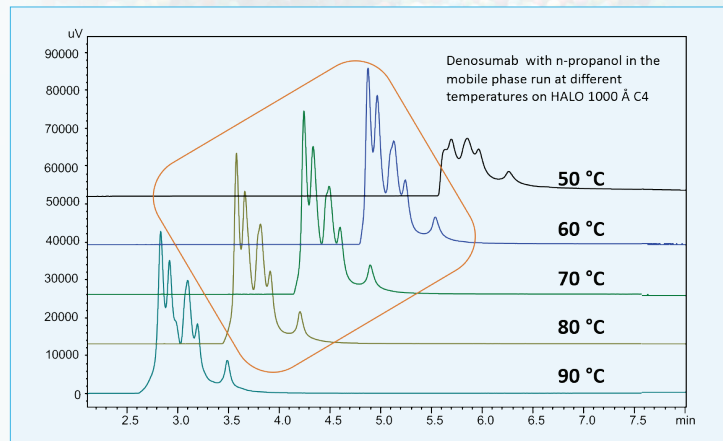
Separation of intact denosumab: Effect of Gradient Flow Rate. The longer gradient optimizes resolution, but is resolution adequate in $\frac{1}{4}$ of the analysis time?



4. Optimal Temperature Selection

- As part of any LC method development scheme, it is recommended to perform temperature studies for determining most robust and ideal separation conditions.
- As shown in this separation of denosumab, higher temperatures tend to be more favorable for resolution with protein separations.

HALO 1000 Å C4, 2.1 x 150 mm, A: 88/10/2 water/ACN/nProp/0.1% TFA,
B: 70/20/10 nProp/ACN/H₂O/0.1% TFA, 18–30% B in 8 min, 0.4 mL/min



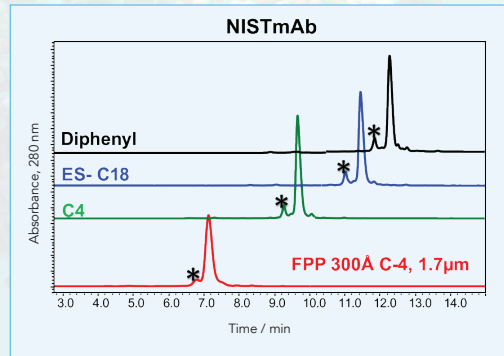
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Temperature –Too Hot to Handle?

- While most protein separations have traditionally taken place at elevated temperatures (60–80°C) for optimized peak area with commercially available bonded phase chemistries, evidence suggests this can lead to unintended consequences.
- In the case of NISTmAb, a commonly used representative

monoclonal antibody standard, temperature artifacts have been observed at higher temperatures for all columns (HALO® and non-HALO®) tested and all mobile phases examined.

- This observation has been confirmed by isolation and re-injection of the artifact peak. This artifact peak is absent when column temperatures at or below 60°C are used.



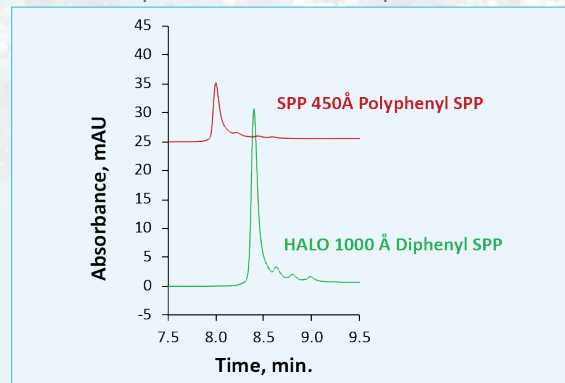
Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: H₂O/0.1% TFA; Mobile Phase B: ACN/0.1% TFA; Gradient: 34–42 %B in 16 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL; Detection: 280 nm; Temp: 80 °C

A New Phase to Turn Down the Heat

- The above suggests a case to develop methods using lower temperatures. Lower temperatures also enhance phase stability leading to longer column lifetimes, but operating at these lower temperatures has been avoided due to impaired recovery.
- The new HALO 1000 Å Diphenyl shows exemplary performance at lower temperatures, as in this example at 40°C.

Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% TFA; Mobile Phase B: ACN/0.1% TFA; Gradient: 30-45% B in 15 min; Injection Volume: 2 µL of 2 mg/mL trastuzumab in water/0.1% TFA; Temp: 40 °C; Detection: PDA at 280 nm

The HALO 1000 Å Diphenyl column exhibits improved resolution, retention and peak area under low temperature conditions



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Comparative results presented here may not be representative for all applications.

Is Bigger Pore Size Always Better?

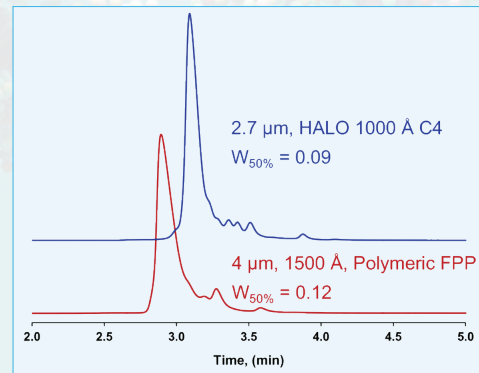
While the largest pore size may seem better, the proof is in the data. Important factors also include pressure limitations and pore morphology.

- Pressure limitations - Polymeric materials are only stable up to 275 bar (4000 psi) versus silica at 600 and 1000 bar (8,700 and 14,500 psi).

- Pore morphology – Accessibility of the analyte to the pore depends upon pore shape and volume.

Conditions: Columns: 2.1 x 100 mm; Mobile phase A: water (0.1% TFA); Mobile phase B: 80/20 ACN/water (0.085% TFA); Gradient: 40–47.5% B in 8 min; Flow rate: 0.4 mL/min; Temperature: 80 °C; Sample: trastuzumab; Injection volume: 2 µL of 2 mg/mL in water; Instrument: Shimadzu Nexera; Detection: UV at 280 nm with 350 nm reference wavelength

Intact trastuzumab separations using HALO 1000 Å C4 Fused-Core® technology SPP vs. a 1500 Å FPP columns.



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Stability

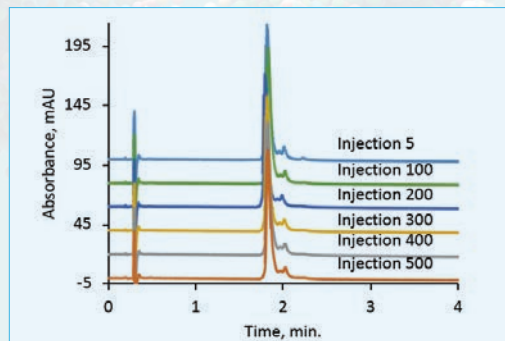
How rugged is your wide pore column?

- Advanced Materials Technology led the revolution in Fused-Core® particle technology with the development of the first commercially available sub-3 μm particle with the original HALO® 2.7 μm particle. Our manufacturing expertise has carried forward into being the wide pore leader.

- This innovative culture has been transferred to developing novel materials for the Biotherapeutic market. HALO 1000 Å particles are ready to solve the challenges of these complex separations.

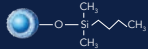
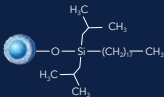
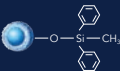
Column: HALO 1000 Å ES-C18, 2.7 μm , 2.1 x 50mm;
Flow Rate: 0.4 mL/min; Mobile Phase A: Water/ 0.1% TFA; Mobile Phase B: Acetonitrile/ 0.1% TFA; Gradient: 32-60% B in 6 min; Injection Volume: 1.0 μL ; Sample: trastuzumab; Temperature: 80°C; Detection: UV 280 nm, PDA

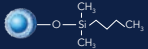
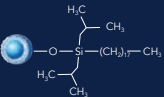
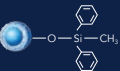
The HALO 1000 Å stationary phases offer rugged and reliable performance – every time. In the example of the HALO 1000 Å ES-C18, the retention times for trastuzumab show extreme phase stability for over 500 injections.



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HALO 1000 Å Specifications Table

			
	C4	ES-C18	Diphenyl
Functional Group	dimethylbutyl	diisobutyloctadecyl	diphenylmethyl
USP Listing	L26	L1	L11
Particle Size (µm)	2.7	2.7	2.7
Pore Size (Ångstroms)	1000	1000	1000
Carbon Load (%)	0.6	1.4	1.0
Surface Area (m2/g)	22	22	22

			
	C4	ES-C18	Diphenyl
Endcapped	Yes	Yes	Yes
pH Range	2-9	1-8	2-9
Maximum Temperature	90°C (pH 2) 40°C (pH 9)	90°C (pH 2) 40°C (pH 9)	90°C (pH 2) 40°C (pH 9)
Maximum Pressure	1000 bar (2.1 and 3.0 mm ID) 600 bar (4.6 mm ID)	1000 bar (2.1 and 3.0 mm ID) 600 bar (4.6 mm ID)	1000 bar (2.1 and 3.0 mm ID) 600 bar (4.6 mm ID)



HALO 1000 Å 2.7 µm Capillary and Microbore Columns

Dimensions (ID x Length, mm)	C4	ES-C18	Diphenyl
0.075 x 50	97219-414	97219-402	97219-426
0.075 x 100	97219-614	97219-602	97219-626
0.075 x 150	97219-714	97219-702	97219-726
0.1 x 50	97218-414	97218-402	97218-426
0.1 x 100	97218-614	97218-602	97218-626
0.1 x 150	97218-714	97218-702	97218-726
0.2 x 50	97217-414	97217-402	97217-426
0.2 x 100	97217-614	97217-602	97217-626
0.2 x 150	97217-714	97217-702	97217-726
0.3 x 50	97216-414	97216-402	97216-426

Dimensions (ID x Length, mm)	C4	ES-C18	Diphenyl
0.3 x 100	97216-614	97216-602	97216-626
0.3 x 150	97216-714	97216-702	97216-726
0.5 x 50	97215-414	97215-402	97215-426
0.5 x 100	97215-614	97215-602	97215-626
0.5 x 150	97215-714	97215-702	97215-726
1.0 x 30	92711-314	92711-302	92711-326
1.0 x 50	92711-414	92711-402	92711-426
1.0 x 75	92711-514	92711-502	92711-526
1.0 x 100	92711-614	92711-602	92711-626
1.0 x 150	92711-714	92711-702	92711-726



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HALO 1000 Å 2.7 µm Analytical Columns

Dimensions (ID x Length, mm)	C4	ES-C18	Diphenyl
2.1 x 20	92712-214	92712-202	92712-226
2.1 x 30	92712-314	92712-302	92712-326
2.1 x 50	92712-414	92712-402	92712-426
2.1 x 75	92712-514	92712-502	92712-526
2.1 x 100	92712-614	92712-602	92712-626
2.1 x 150	92712-714	92712-702	92712-726
2.1 x 250	92712-914	92712-902	92712-926
3.0 x 20	92713-214	92713-202	92713-226
3.0 x 30	92713-314	92713-302	92713-326
3.0 x 50	92713-414	92713-402	92713-426
3.0 x 75	92713-514	92713-502	92713-526
3.0 x 100	92713-614	92713-602	92713-626

Dimensions (ID x Length, mm)	C4	ES-C18	Diphenyl
3.0 x 150	92713-714	92713-702	92713-726
3.0 x 250	92713-914	92713-902	92713-926
4.6 x 20	92714-214	92714-202	92714-226
4.6 x 30	92714-314	92714-302	92714-326
4.6 x 50	92714-414	92714-402	92714-426
4.6 x 75	92714-514	92714-502	92714-526
4.6 x 100	92714-614	92714-602	92714-626
4.6 x 150	92714-714	92714-702	92714-726
4.6 x 250	92714-914	92714-902	92714-926
10.0 x 50	92710-414	92710-402	92710-426
10.0 x 75	92710-514	92710-502	92710-526
10.0 x 100	92710-614	92710-602	92710-626
10.0 x 150	92710-714	92710-702	92710-726



HALO 1000 Å 2.7 µm Guard Columns, 3-pack

Dimensions (ID x Length, mm)	C4	ES-C18	Diphenyl
2.1 x 5	92712-114	92712-102	92712-126
3.0 x 5	92713-114	92713-102	92713-126
4.6 x 5	92714-114	92714-102	92714-126

Guard Column Holder	94900-001
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