Agilent AdvanceBio Columns

ADVANCE ACCURACY AND PRODUCTIVITY FOR FASTER ANALYSIS

The Measure of Confidence

with Agilent ZORBAX RRHD 300Å 1.8 µm columns

Rapid resolution high definition columns for UHPLC protein and peptide separations

ZORBAX RRHD 300Å 1.8 µm columns provide UHPLC performance for separations of intact proteins and peptide digests. When used with UHPLC instruments such as the Agilent 1290 Infinity LC, they enable higher order characterization with reduced analysis time.

The columns are available with reversed-phase C18, C8, C3, and diphenyl functionalities to provide more options for protein primary structure analysis.

These products are an extension of Agilent 300Å pore columns, which include fully porous ZORBAX 300Å, superficially porous Poroshell 300, and polymeric PLRP-S 300Å for reversed-phase separations of peptides and proteins. So, you can use ZORBAX StableBond to achieve UHPLC power for protein analysis. Also available in HILIC, for fast, high resolution separation of polar glycopeptides (for more information see Agilent pub #5991-1435EN).

Proven reversed-phase technology

The columns are packed with ZORBAX StableBond technology with C18, C8, and C3 functionalities, and end-capped diphenyl to give you:

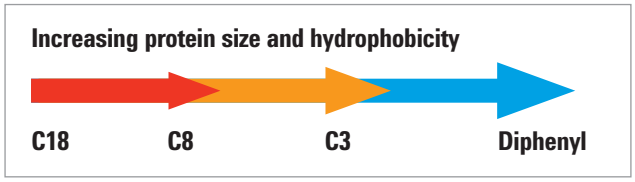
- **Stability at low pH** – you can run your protein and peptide separations down to pH 1 using trifluoroacetic acid (TFA) and formic acid eluents with complete confidence.
- **Temperature stability** – you can run your separations up to 80 °C to improve efficiency and reduce eluent viscosity, without compromising column lifetime.

StableBond media is based on the ZORBAX silica, designed to minimize strong adsorption of basic peptides and proteins, and deliver symmetrical peaks with minimal peak tailing.

The diphenyl phase is a proven chemistry taken from the Pursuit 200Å columns and applied to the wider pore ZORBAX 300Å columns to enable the benefit of this unique selectivity to be used for the analysis of larger proteins.

C18, C8, C3, and diphenyl functionalities

- **C18** – bonding a sterically-protected C18 functionality, diisobutyl *n*-octadecylsilane, to the ZORBAX particle produces a chemically stable stationary phase for long column lifetimes when using acidic mobile phases. This is important for the analysis of peptide fragments from protein digests and small proteins.
- **C8** – the sterically protected C8 functionality, diisopropyl *n*-octylsilane, gives improved separation and recovery for larger proteins, including antibodies.
- **C3** – broadens the selectivity, peak shape and recovery options for larger proteins, including antibodies.
- **Diphenyl** – gain the benefit of additional selectivity through pi-pi interactions with aromatic amino acids.



Faster separation

The ZORBAX RRHD 300SB-C18 columns are packed with 1.8 µm particles and, as with UHPLC of small molecules, performance is maintained with proteins at higher flow rates (**Figure 1**).

Column:	ZORBAX RRHD 300SB-C18 2.1 x 50 mm, 1.8 µm
Sample:	Insulin (bovine-sigma, 1 mg/mL)
Injection:	3 µL
Flow rate:	1.5 mL/min, 1.0 mL/min, 0.5 mL/min, 0.3 mL/min
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA + 80% ACN
Run time:	5 min
Gradient:	5 to 100% B, 0 to 4 min
Detection:	1290 Infinity LC at 280 nm

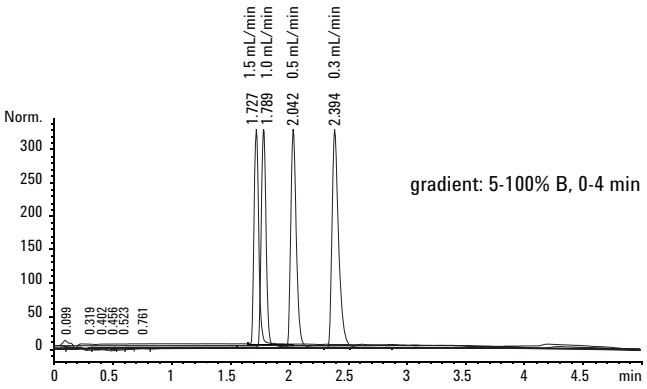


Figure 1. *An overlay of four different flow rates on an Agilent ZORBAX 300SB-C18 shows how retention times are achieved with higher flow rates*

Table 1. *Agilent ZORBAX 300SB-C18 performance as a function of flow rate. Peak symmetry improves with minimal reduction in efficiency, as the flow rate increases*

Flow rate (mL)	Pressure (bar)	Retention time (min)	Tailing factor (5%)	Plate count
0.3	230 to 150	2.39	1.47	8855
0.5	350 to 250	2.04	1.27	9226
1.0	680 to 520	1.78	1.09	8980
1.5	890 to 670	1.72	1.13	8912

Reproducibility

The analysis of complex protein structures requires that the biocolumn used for the separation not only provides the resolution needed, but also demonstrates reproducibility from batch-to-batch, column-to-column, and run-to-run. The batch-to-batch and column-to-column reproducibility, is achieved by using the ZORBAX 300Å particle, the proven StableBond and diphenyl bonding technology, and robust column loading/packing protocols. To achieve the run-to-run reproducibility sample recovery is essential. Sample loss on-column will not only compromise the accuracy of the analysis, but incomplete recovery will also alter the column performance and reduce reproducibility of the separation. **Figure 2** demonstrates the run-to-run reproducibility over 200 injections and shows reproducibility of peak retention, height and symmetry.

Column:	ZORBAX RRHD 300SB-C18 2.1 x 50 mm, 1.8 µm
Sample:	Insulin (bovine-sigma, 1 mg/mL)
Injection:	3 µL
Flow rate:	1.0 mL/min
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA + 80% ACN
Run time:	5 min
Gradient:	25 to 50% B, 0 to 4 min
Detection:	1290 Infinity LC at 280 nm

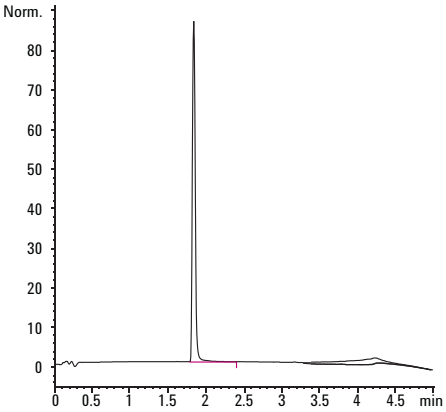


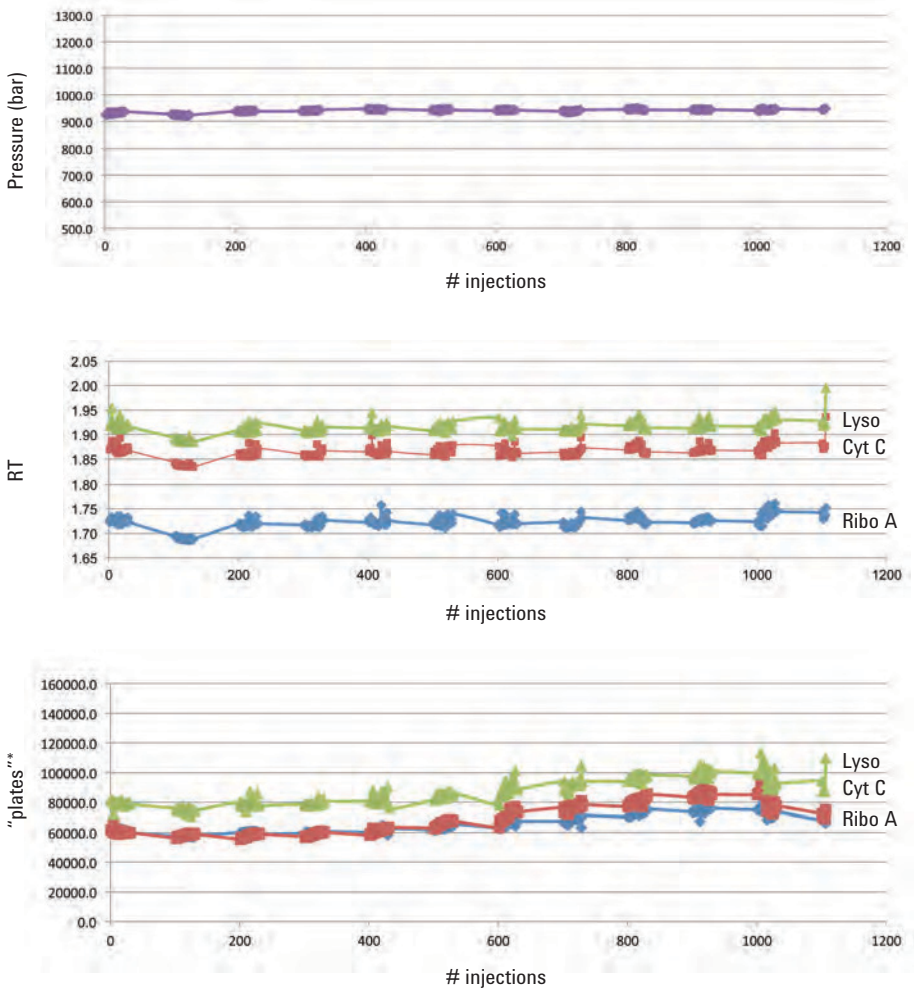
Figure 2. *Overlay of run 1, 50, 100 and 200 in an insulin separation demonstrates excellent retention time reproducibility and no reduction of efficiency or deterioration of peak shape*

Table 2*. Agilent ZORBAX 300SB-C18 performance characteristics at intervals during the 200 run reproducibility test*

Flow rate (mL)	Run number	Pressure (bar)	Retention time (min)	Tailing factor (5%)	Plate count
1	1	680 to 520	1.789	1.08	9258
1	50	680 to 520	1.790	1.06	9241
1	100	680 to 520	1.788	1.07	9252
1	200	680 to 520	1.789	1.10	9305

Lifetime

The ZORBAX RRHD 300Å 1.8 µm columns are stable to 1200 bar. Although the optimum analytical conditions, flow rate, temperature and mobile phase often mean that the actual operating pressure is below 1000 bar, the columns are packed to withstand the higher operating pressure and to provide stable column beds over multiple injections at these high pressures. **Figure 3** shows ZORBAX RRHD 300SB-C3 2.1 x 100 mm, 1.8 µm is stable under the conditions routinely used for the analysis of proteins and peptides and that there is no deterioration in peak shape or change in retention times over the number of injections monitored.



Column:	ZORBAX RRHD 300SB-C3 2.1 x 100 mm, 1.8 µm
Sample:	3 mg/mL (ribonuclease A, cytochrome C, and lysozyme)
Injection:	1 µL
Temperature:	74 °C
Mobile phase A:	0.1% TFA in water
Mobile phase B:	0.01% TFA in ACN
Pressure:	900-950 bar
Gradient:	0-10% B, 2.5-70% B, 3.0-90% B then 4 min-25% to 5 min
Detection:	UV225

**Plates are undefined in gradient chromatography. However, in this case, "plates" is a representation of resolving power, and was calculated using peak width and retention time as if the run was isocratic*

Figure 3. *Life tests of ZORBAX RRHD 300SB-C3, 1.8 µm using a test mixture of three proteins: lysozyme, cytochrome C, and ribonuclease A. Pressure, retention times, and resolving power (i.e. "plates") are shown for a continuous run of more than 1000 injections*

Recovery

Shorter 50 mm columns are often used for the analysis of large proteins to maximize recovery. This is demonstrated in **Figure 4** in the analysis of a MAb using the ZORBAX RRHD 300SB-C8, 1.8 µm column. The top chromatogram compares the separation of the first and 150 analysis of the MAb and shows that there is no change in the elution time or peak shape and resolution. The bottom chromatogram shows the pressure and baseline before the first analysis and after run 150 with the wash region highlighted. There is no change between the two runs, indicating good recovery.

Column:	ZORBAX RRHD 300SB-C8 2.1 x 50 mm, 1.8 µm
Flow rate:	1.0 mL/min
Temperature:	74 °C
Mobile Phase:	A: 98/2 water/n-propanol (0.08% TFA) B: 70/20/10 n-propanol/ACN/water (0.08% TFA)
DAD:	215 nm

Table 3. *Gradient timescale*

Time (min)	% Solvent B
0	10
2.5	25
4.5	35
4.56	90
5.0	90
6.0	10

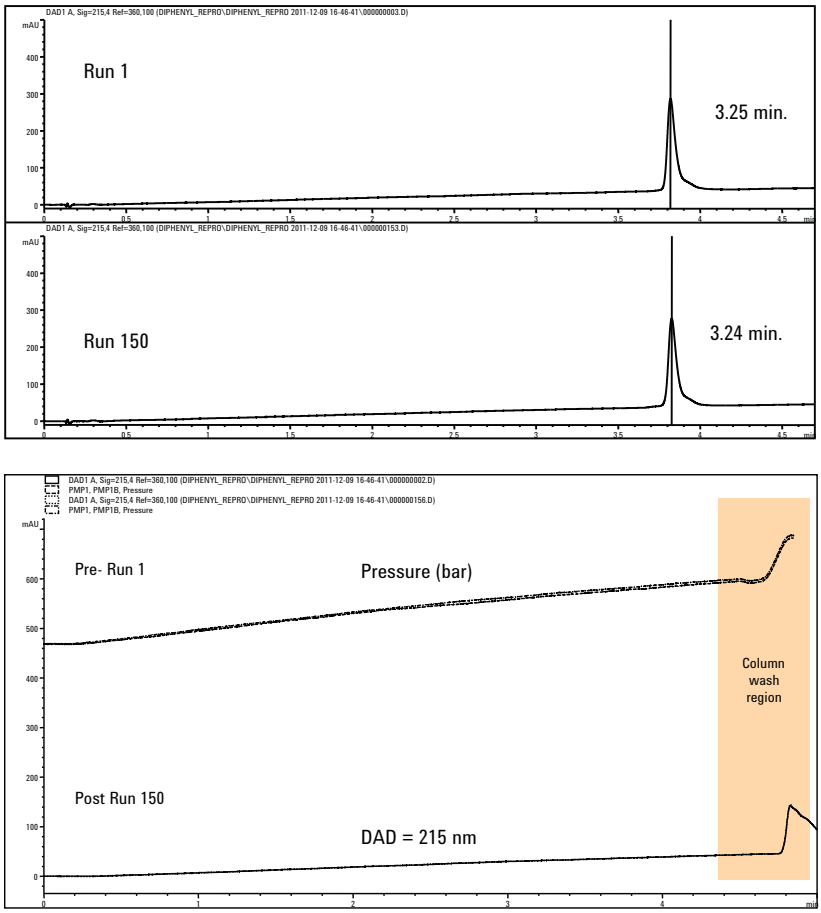
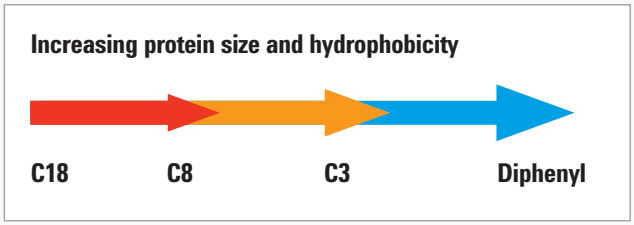


Figure 4. *Good recovery with ZORBAX RRHD 300SB-C8, 1.8 µm*

Applications



With four different ligand types, C18, C8, and C3 alkyl chains, and the diphenyl to provide additional selectivity based on pi-pi aromatic amino acids, Agilent has the widest range of reversed-phase columns for all peptide and protein UHPLC separations.

Increased resolution for peptide mapping

For peptide mapping applications, the longer ZORBAX RRHD 300SB-C18 2.1 x 100 mm, 1.8 µm column is recommended as it provides increased resolution of the peptide fragments from the protein enzymatic digest. The higher efficiency of the 1.8 µm particles provide increased resolution of the individual peptide fragments for rapid identification of post translational modifications to the amino acids (**Figure 5**).

Column:	ZORBAX RRHD 300SB-C18 2.1 x 100 mm, 1.8 µm
Sample:	Enzymatic protein digest (MAb)
Injection:	5 µL
Flow rate:	0.5 mL/min
Temperature:	50 °C
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA + 80% ACN
Gradient:	2% B for 1 min, 2 to 45% B for 8.8 min, 45 to 95% B for 0.2 min, 95% B for 2 min, 95 to 2% B for 0.2 min
Detection:	1290 Infinity LC at 280 nm

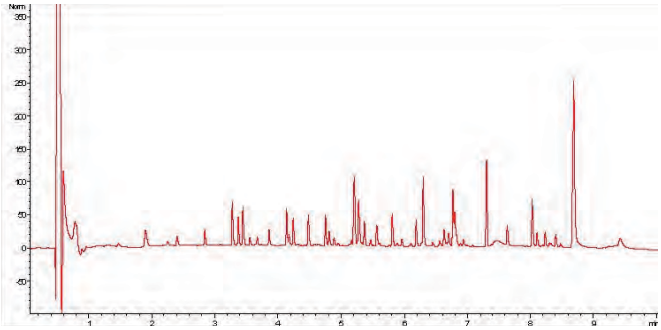


Figure 5. *The longer 100 mm Agilent ZORBAX 300SB-C18 column provides maximum resolution for protein digests – in this sample, the total run time, including washing and equilibration, is* ***under fifteen minutes***

High resolution of small intact proteins

When analyzing intact proteins, the shorter ZORBAX RRHD 300SB-C18 2.1 x 50 mm, 1.8 µm column is ideal. The recovery of intact protein is likely to be higher with the shorter column length as the protein will have a shorter distance to migrate through the column to elute. The C18 ligand is the most hydrophobic of the alkyl chains used for peptide and protein separations and is better suited to the analysis of small globular intact proteins, insulin, a small protein of 5,800 Daltons (**Figure 6**).

Column:	ZORBAX RRHD 300SB-C18 2.1 x 50 mm, 1.8 µm
Sample:	Insulin, insulin chain A and chain B, oxidized (bovine-sigma, 1 mg/mL)
Injection:	2 µL
Flow rate:	1.0 mL/min
Mobile phase A:	0.1% TFA
Mobile phase B:	0.01% TFA + 80% ACN
Run time:	8 min
Gradient:	33 to 50% B, 0 to 4 min
Detection:	1290 Infinity LC at 280 nm

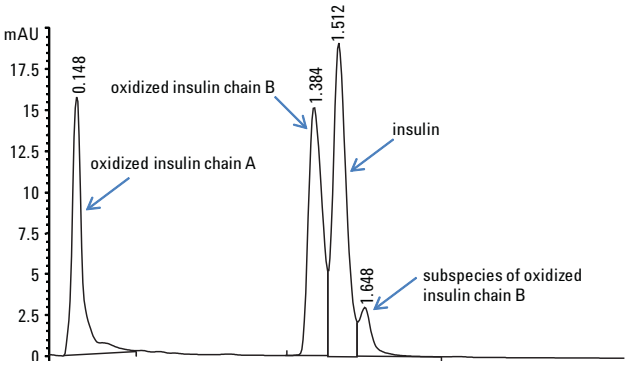


Figure 6. *It is evident that the oxidized insulin chains are resolved from insulin in under 2 minutes using the Agilent ZORBAX RRHD 300SB-C18 2.1 x 50 mm, 1.8 µm column*

Reproducibility and recovery of monoclonal antibodies

For larger proteins, including monoclonal antibodies, a shorter, less hydrophobic C8 functionality is used. This provides improved resolution and high recovery. **Figure 7** demonstrates the reproducibility and lifetime of the ZORBAX RRHD 300SB-C8 column over 150 injections, with no retention time or peak abnormalities. The bottom chromatogram shows the pre- and post-150 injection blank runs and gradient pressure curves, demonstrating that there is no ghosting or increase in pressure after 150 injections, therefore no column failure.

Column:	ZORBAX RRHD 300SB-C8 2.1 x 50 mm, 1.8 µm
Sample:	MAb
Mobile phase A:	$H_2$0:IPA (98:2), 0.1% TFA
Mobile phase B:	IPA:ACN:$H_2$0 (70:20:10), 0.1% TFA
Flow rate:	1.0 mL/min
Temperature:	80 °C
Detection:	1290 Infinity LC at 225 nm

Table 4. *Gradient timescale*

Time (min)	% Solvent B
0.00	25
3.00	35
4.00	90
5.00	25

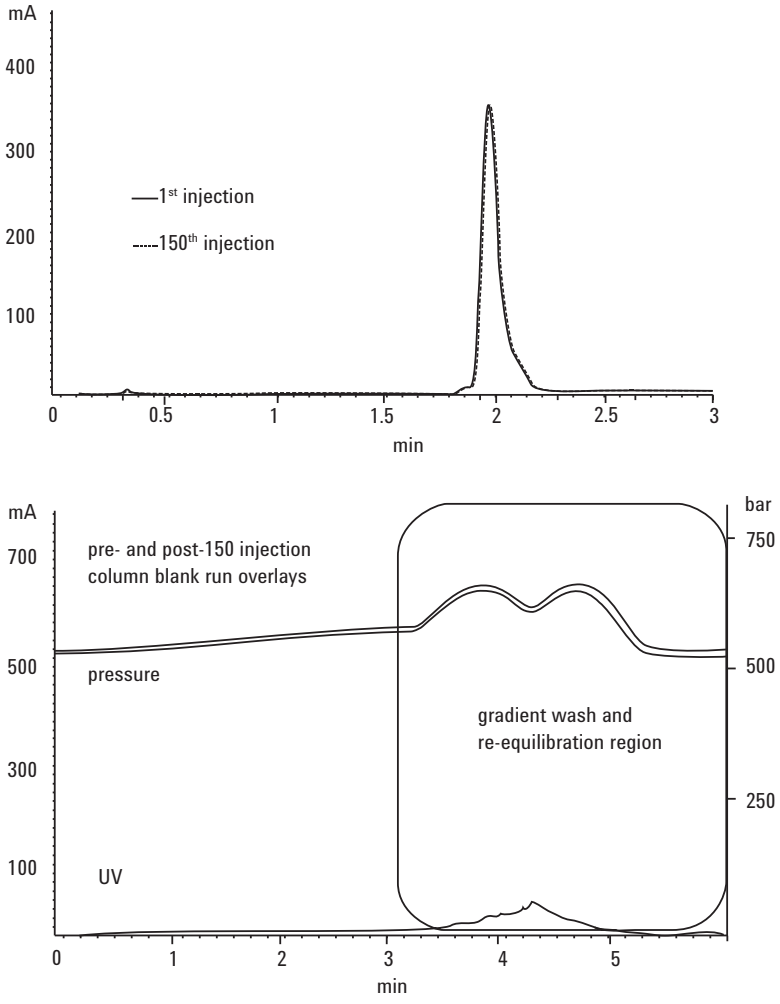


Figure 7. *Excellent column reproducibility and protein recovery using Agilent ZORBAX 300SB-C8*

Additional application note resources

Analysis of oxidized insulin chains using reversed-phase Agilent ZORBAX RRHD 300SB-C18
Agilent pub #5990-7988EN

Analyze MAb and BSA digests by UHPLC with UV detection and Agilent ZORBAX RRHD 300SB-C18
Agilent pub #5990-8244EN

Reversed-phase separation of intact monoclonal antibodies using Agilent ZORBAX RRHD 300SB-C8, 1.8 µm column
Agilent pub #5990-9016EN

Fast separation of recombinant human erythropoietin using reversed-phased Agilent ZORBAX RRHD 300SB-C18, 1.8 µm column
Agilent pub #5990-9248EN

Rapid UHPLC analysis of reduced monoclonal antibodies using an Agilent ZORBAX RRHD 300SB-C8 column
Agilent pub #5990-9631EN

Optimization of ultra-fast separations for intact and reduced monoclonal antibodies analysis using an Agilent ZORBAX RRHD 300SB-C3 column
Agilent pub #5990-9667EN

Ultra-high speed and high resolution performance for reduced and intact monoclonal antibodies with use of an Agilent ZORBAX RRHD sub-2 µm 300-Diphenyl column
Agilent pub #5990-9668EN

Disulfide linkage analysis of IgG1 using an Agilent 1260 Infinity Bio-inert system with an Agilent ZORBAX RRHD Diphenyl sub-2 µm column
Agilent pub #5991-1694EN

The Agilent 1290 Infinity LC

Agilent AdvanceBio columns:

For faster, more consistent biopharmaceutical analysis

ZORBAX RRHD 300Å 1.8 µm columns are part of Agilent's growing state-of-the-art AdvanceBio family of biocolumns. They are designed to deliver consistent, exceptional performance for the separation and characterization of peptides and proteins, antibodies, conjugates, new biological entities, and biopharmaceuticals. The science behind AdvanceBio columns helps to advance accuracy and productivity that support faster analysis and efficiency in your lab. To learn more, visit **www.agilent.com/chem/AdvanceBio**

Ordering information

Description	Part number
ZORBAX RRHD 300SB-C18 2.1 x 50 mm, 1.8 µm	857750-902
ZORBAX RRHD 300SB-C18 2.1 x 100 mm, 1.8 µm	858750-902
ZORBAX RRHD 300SB-C18 2.1 x 150 mm, 1.8 µm	863750-902
ZORBAX RRHD 300SB-C8 2.1 x 50 mm, 1.8 µm	857750-906
ZORBAX RRHD 300SB-C8 2.1 x 100 mm, 1.8 µm	858750-906
ZORBAX RRHD 300SB-C8 2.1 x 150 mm, 1.8 µm	866750-906
ZORBAX RRHD 300SB-C3 2.1 x 50 mm, 1.8 µm	857750-909
ZORBAX RRHD 300SB-C3 2.1 x 100 mm, 1.8 µm	858750-909
ZORBAX RRHD 300SB-C3 2.1 x 150 mm, 1.8 µm	863750-914
ZORBAX RRHD 300-Diphenyl 2.1 x 50 mm, 1.8 µm	857750-944
ZORBAX RRHD 300-Diphenyl 2.1 x 100 mm, 1.8 µm	858750-944
ZORBAX RRHD 300-Diphenyl 2.1 x 150 mm, 1.8 µm	863750-944
ZORBAX RRHD 300-HILIC 2.1 x 50 mm, 1.8 µm	857750-901
ZORBAX RRHD 300-HILIC 2.1 x 100 mm, 1.8 µm	858750-901

For more information on ZORBAX RRHD 300-HILIC, see Agilent pub #5991-1435EN

Find out how to take your protein analysis to the next level

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