# APPLICATIONS

## Deep Proteome Coverage on HeLa Lysate using a bioZen™ Fractionation Column for High pH (Basic Reversed Phase) Fractionation in Combination with a bioZen Nano Peptide XB-C18 Column for Nano Flow LC-MS

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*Roxana likes to spend time with her dog and family. She has a German Shepperd mix named Cobi who was named after the official mascot of the 1992 Summer Olympics and not the basketball player. Besides the lab, you might find Roxana in old episodes of the Ghost Whisperer, MAD TV, The L word and others as she used to be a TV extra during her school years.*

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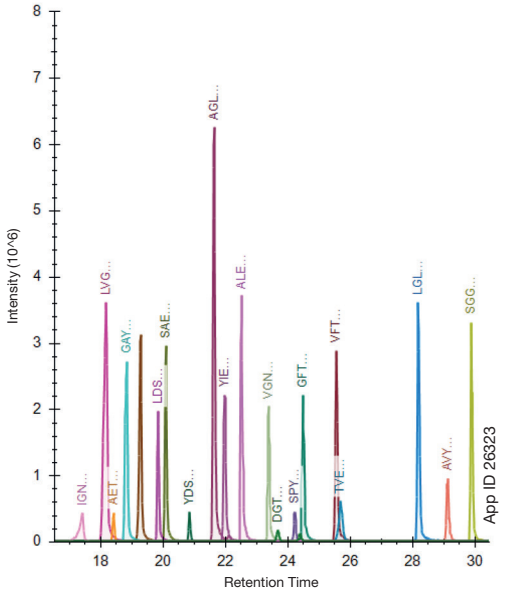
### Introduction

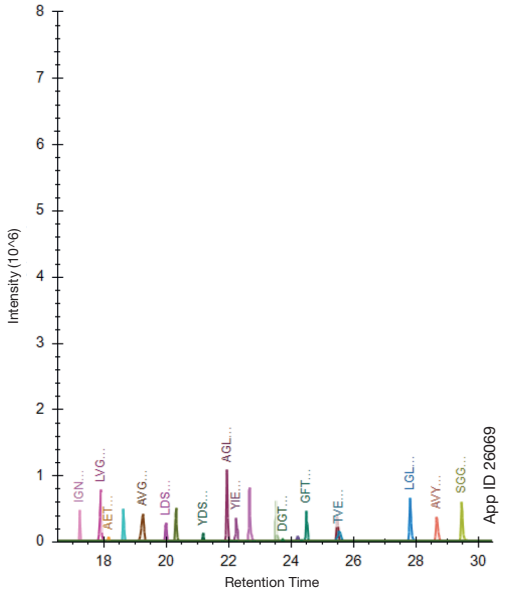
Basic reversed phase (pH > 9) or high pH fractionation is a strategy commonly used to increase proteome coverage and its main purpose is to maximize protein identification in complex sample matrices. This methodology generally includes a high pH reversed phase fractionation step together with a concatenation strategy followed by nano low pH reversed phase LC-MS for maximum sensitivity (**Figure 1**). The purpose of the concatenated strategy is to produce evenly distributed ion chromatograms that reduce analysis time while increasing proteome identification[1]. High pH fractionation can be problematic due to silica instability at high pH (>8) that results in degradation by dissolution and by the nonspecific adsorption of peptides into the column's hardware. bioZen Fractionation columns offer a unique combination of titanium column hardware to minimize nonspecific adsorption and TWIN-NX™ Technology, a patented organo-silica grafting process which incorporates highly stabilizing ethylene crosslinking. These organic groups are evenly incorporated into the grafted layers on the silica surface while maintaining a pure silica core. This not only provides resistance to high pH attack but also maintains the high efficiency and mechanical strength of a silica particle[2] (**Figures 2** and **3**). In this application note we show how to achieve deep proteome coverage on HeLa cell lysate using a high pH reversed phase fractionation strategy in combination with nano flow LC-MS.

**Figure 1.**
Example of sensitivity increase on a 20 peptide mixture (SCIEX® PepCalMix) when using a bioZen nano LC column (250 nL/min) (**Left**) as opposed to micro flow (10 µL/min) on a Kinetex® micro LC column (**Right**).

**bioZen Nano Peptide XB-C18 Column**




**Kinetex XB-C18 Micro Column**




**Column:** Kinetex 2.6 µm XB-C18
bioZen 2.6 µm Peptide XB-C18
**Dimensions:** 150 x 0.3 mm
150 x 0.075 mm
**Part No.:** 00F-4496-AC
00F-4768-AW-21
**Pressure (bar):** 200 bar
**Mobile Phase:** A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile

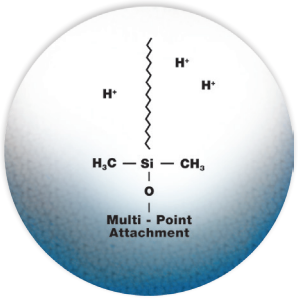
**Gradient:**

| Time (min) | %B |
|---|---|
| 0 | 3 |
| 30 | 40 |
| 35 | 70 |
| 40 | 80 |
| 41 | 3 |
| 45 | 3 |

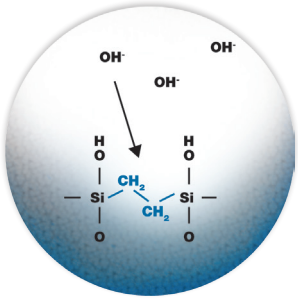
**Flow Rate:** Kinetex: 4 µL/min
bioZen: 250 nL/min
**Temperature:** 25 °C
**LC System:** NanoLC™ 425 (SCIEX)
**Detection:** MS/MS
**Detector:** 6500 QTRAP® (SCIEX)
**Injection Volume:** 2.5 µL
**µg on Column:** 1 µL

**Figure 2.**
The TWIN-NX™ Technology in the bioZen™ Fractionation column contains crosslinking that creates a high pH stable surface.

### Multi-Point Ligand Attachment Resists Low pH Ligand Cleavage



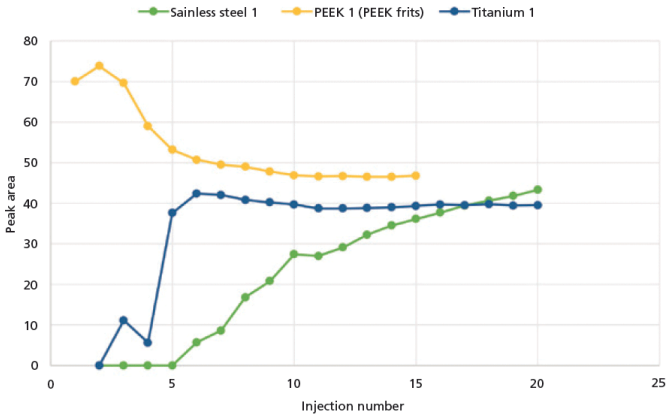

### Ethylene Crosslinking Resists High pH Attack




## Results and Discussion

High pH reversed phase fractionation UV response shows a mostly even separation of peptides through the chromatographic gradient (**Figure 4, Table 1**). Similarly, concatenated pooled fractions resulted in even distribution of peptides in the ion chromatogram, with normalized intensities higher than 1e9 throughout the nano-LC gradient, which results in higher identification of peptides and proteins (**Figure 5**). Comparing the number of identified peptides in the unfractionated HeLa lysate digest to the fractionated samples shows a significant increase in the number of peptides identified by fractionation/concatenation (**Figure 6**). This significant increase is attributed to the fact that fractionation reduces the complexity of a sample while increasing peak capacity. Additionally, the bioZen fractionation column with its titanium hardware is expected to minimize nonspecific adsorption resulting in higher peptide identifications than regular stainless steel hardware (**Figure 3**). Normally, a higher number of peptide identifications translates into a higher number of protein identifications. As shown in **Figure 7**, a significantly higher number of proteins were identified in the fractionated vs. the unfractionated sample. Besides the significant increase of protein and peptide identifications, using the bioZen 2.6 µm Peptide XB-C18 Nano LC column can be beneficial when injecting complex samples for this type of workflow. The core-shell technology in the bioZen 2.6 µm Peptide XB-C18 Nano LC column results in sharper peaks in comparison to columns packed with fully porous particles, while having a lower backpressure. Sharper peaks mean higher intensity and consequently higher peptide/protein identifications[3]. The lower backpressure helps to increase the column lifetime and allows for method development flexibility in terms of flow rate and temperature, aspects that are nearly impossible when using 1.6-1.9 µm particle sizes or smaller.

**Figure 3.**
**Left.** Peak area vs. injection number comparing titanium vs. stainless steel hardware for a SEC separation of γ-globulin and ovalbumin. In this case, SEC is similar to basic reversed phase chromatography in the fact that the basic environment prevents the displacement of metal ions in the hardware, which can result in peak tailing and analyte adsorption. **Right.** BioTi™ technology minimizes the need for priming with the titanium infused biocompatible hardware and frit that does not interfere with protein or peptide integrity.



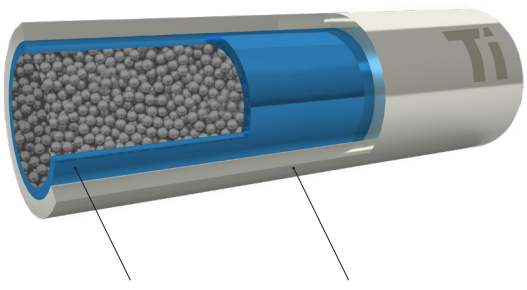
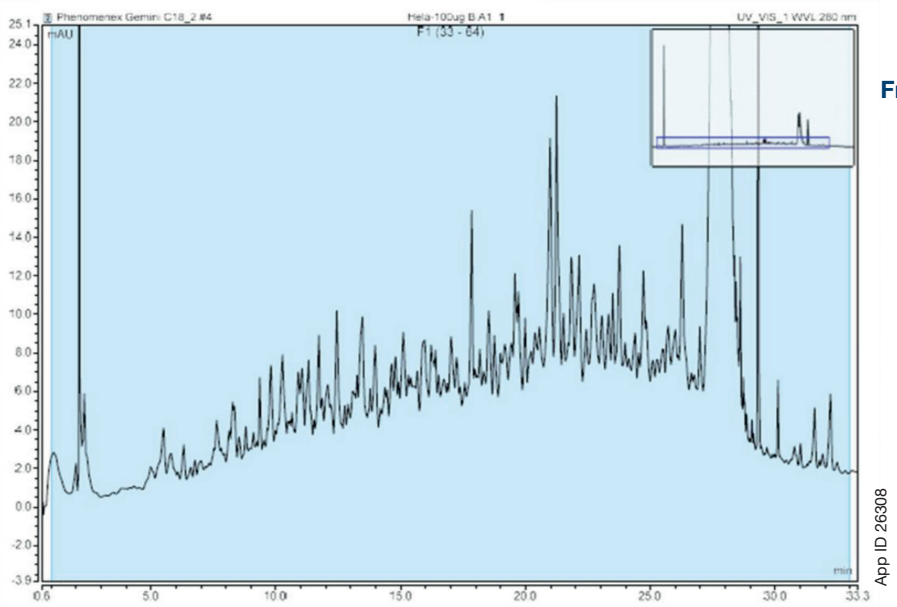



**Figure 4.**
UV trace of a 30 minute basic pH reversed phase separation using 100 µg of HeLa tryptic digest on a bioZen™ High pH Fractionation column.




## Fractionation LC Conditions

**Column:** bioZen 3 µm High pH Fractionation Column
**Dimensions:** 150 x 2.1 mm
**Part No.:** 00F-4793-AN
**Pressure (bar):** 150 bar
**Mobile Phase:** A: 10 mM Ammonium formate in Water
B: 10 mM Ammonium formate in 90 % Acetonitrile and 10% Water

**Gradient:**

| Time (min) | %B |
|---|---|
| 0 | 1 |
| 1 | 1 |
| 25 | 25 |
| 27 | 60 |
| 28 | 70 |
| 33 | 70 |
| 34 | 1 |

**Flow Rate:** 300 µL/min
**Temperature:** 50 °C
**LC System:** Vanquish™ Flex UHPLC
**Detection:** UV @ 280 nm
**Injection Volume:** 100 µL
**µg on Column:** 100 µg

### Fractionation Concatenation Strategy

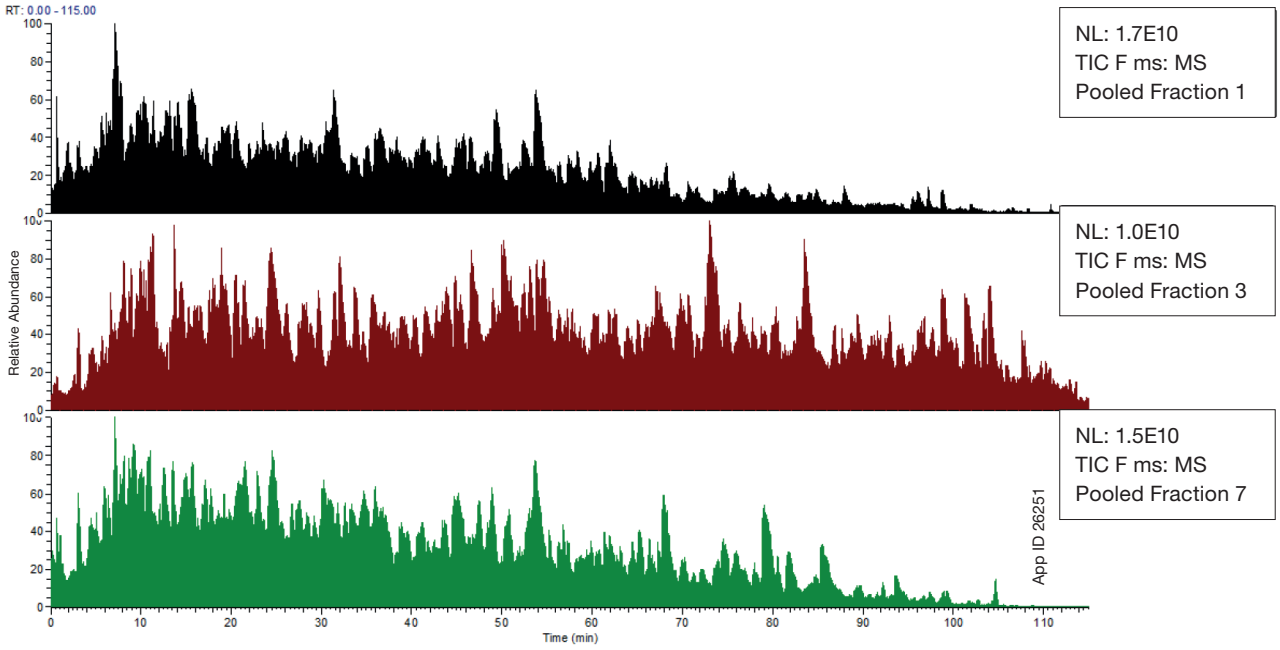
A total of 32 fractions resulted from the high pH reversed phase fractionation, concatenation of fractions followed as shown in the table below, resulting in a total of 8 fractions to be analyzed by nano flow LC-MS.

| Concatenated Fraction Number |
|---|
| 1 (pooled fractions 1, 9, 17, 25) |
| 2 (pooled fractions 2, 10, 18, 26) |
| 3 (pooled fractions 3, 11, 19, 27) |
| 4 (pooled fractions 4, 12, 20, 28) |
| 5 (pooled fractions 5, 13, 21, 29) |
| 6 (pooled fractions 6, 14, 22, 30) |
| 7 (pooled fractions 7, 15, 23, 31) |
| 8 (pooled fractions 8, 16, 24, 32) |

**Table 1.**
Amount of HeLa tryptic digest in each of the pooled fractions. Amount was determined using BCA assay. Each fraction was dissolved to a concentration of 400 ng/µL in LC-MS solvent A and 2.5 µL was injected onto the bioZen 2.6 µm Peptide XB-C18 Nano LC column for nano flow LC-MS analysis.

| Pooled Fraction Number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Total µg | 15 | 19 | 32 | 31 | 14 | 15 | 16 | 15 |

**Figure 5.**
Representative total ion chromatograms for fractions 1, 3 and 7




## LC Conditions

**Column:** bioZen™ 2.6 µm Peptide XB-C18 Nano LC
**Dimensions:** 250 x 0.075 mm
**Part No.:** 00G-4768-AW-21
**Pressure (bar):** 350 bar
**Mobile Phase:** A: 0.2 % Formic Acid in 98 % Water, 2 % Acetonitrile
B: 0.2 % Formic Acid in 80 % Acetonitrile, 20 % Water
**Gradient:**

| Time (min) | %B |
|---|---|
| 0 | 2 |
| 7.5 | 6 |
| 90 | 25 |
| 120 | 40 |
| 121 | 98 |
| 133 | 98 |

**Flow Rate:** 500 nL/min
**Temperature:** 50 °C
**LC System:** EASY-nLC™ 1200
**Detection:** nanoESI
**Detector:** Orbitrap™ HF
**Injection Volume:** 2.5 µL
**µg on Column:** 1 µg

## MS Conditions

**Ion Source:** Nanospray Flex™ Ion Source (NG)
**Ion Source Parameters:**
**Voltage:** 2.5kV
**Ion Mode:** Positive
**MS:** Q Exactive™ HF
**MS Parameters:**
**Acquisition Mode:** Data Dependent
**MS1 Resolution:** 60,000
**MS1 AGC Target:** 3e6
**MS1 Injection Time:** 15ms
**Scan Range:** 375-1500 m/z
**MS2 Resolution:** 30,000
**MS2 AGC Target:** 1e5
**MS2 INJECTION TIME:** 45ms
**Collision Dissociation Type:** HCD
**Collision Energy:** 28(N)

**Figure 6.**
Number of peptide identifications for each fraction, unique peptides found in all fractions (combined), and in unfractionated HeLa tryptic digest.

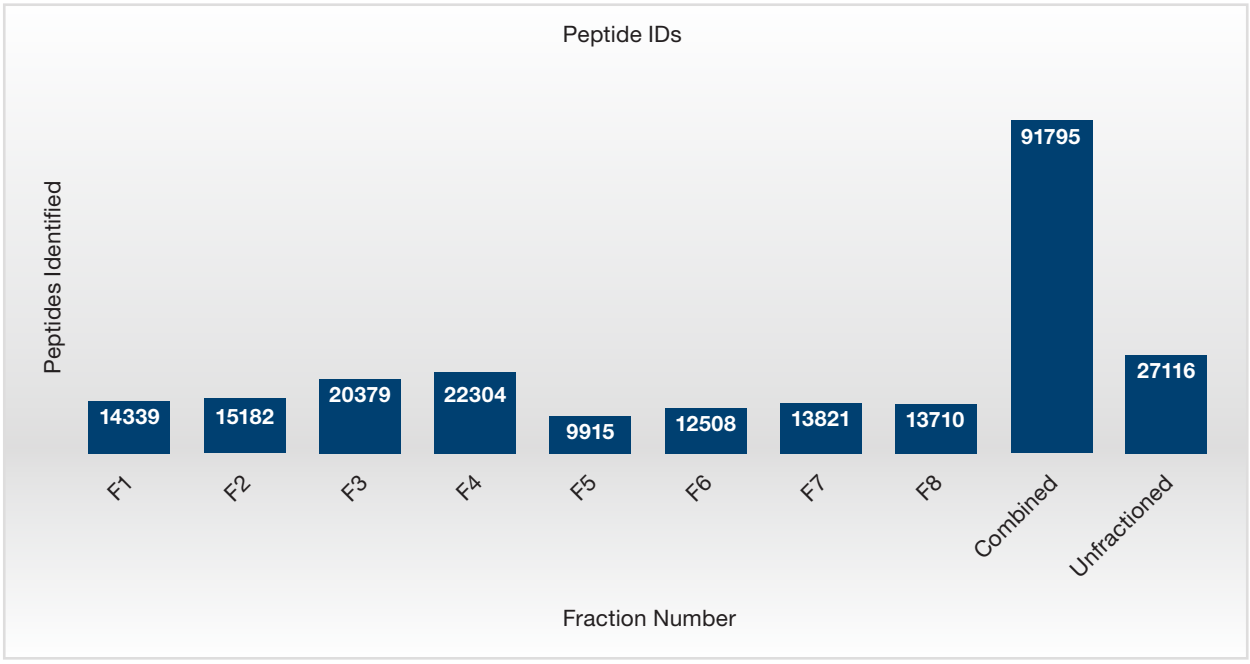
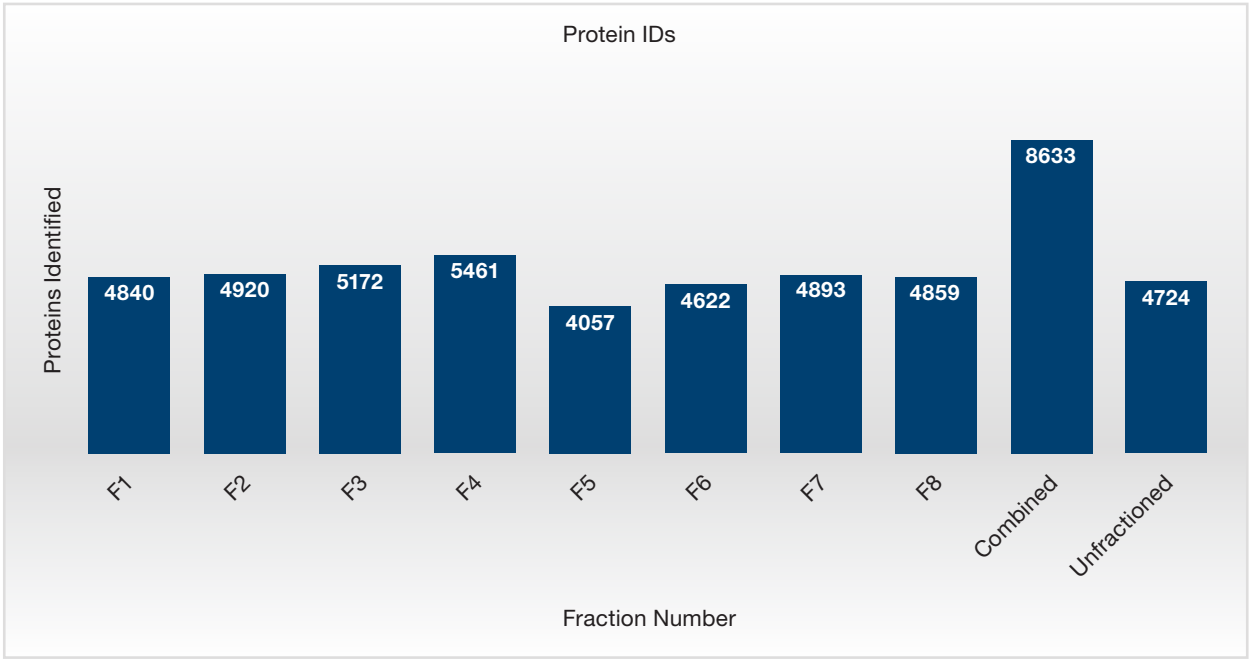



**Figure 7.**
Number of protein identifications for each fraction, unique proteins found in all fractions (combined), and in unfractionated HeLa tryptic digest.



## Conclusions

The current application shows that deep proteome coverage can be achieved by combining two strategies that are commonly used to improve peptide identification. By combining offline high pH fractionation, using the bioZen™ 3 µm High pH Fractionation column, with the highly sensitive bioZen 2.6 µm Peptide XB-C18 Nano LC column for nano LC-MS, a 70% increase in peptide identifications and a close to 50% increase in protein identifications was accomplished. For users in need of obtaining high proteome coverage, this strategy is strongly encouraged.

## References


1) Batth, T. S. (2017). An Optimized Shotgun Strategy for the Rapid Generation of Comprehensive Human Proteomes. Cell Syst., 587-599.
2) Anspach J, Rivera B, Srinivasa Rao. (2018, June 1st). LCGC. Retrieved from www.chromatographyonline.com: https://www.chromatographyonline.com/view/bioinert-versus-biocompatible-benefits-different-column-materials-liquid-chromatography-separations
3) Joshua J. Coon1, A. S. (2016). Now, More Than Ever, Proteomics Needs Better Chromatography. Cell Systems, 321-324.


## Ordering Information

### bioZen Nano LC Columns with Integrated SecurityLINK™ Fitting

| Phases | 150 x .075 | 250 x .075 | 500 x .075 |
|---|---|---|---|
| bioZen 3 µm Peptide PS-C18 | 00F-4771-AW-21 | 00G-4771-AW-21 | – |
| bioZen 2.6 µm Peptide XB-C18 | 00F-4768-AW-21 | 00G-4768-AW-21 | – |
| bioZen 3 µm Polar C18 | 00F-4782-AW-21 | 00G-4782-AW-21 | – |
| bioZen 5 µm Peptide XB-C18 | – | – | 00J-4792-AW-11 |

### bioZen Nano LC Columns with Open Fused-Silica Inlet/Outlet Fitting

| Phases | 150 x .075 | 250 x .075 | 500 x .075 |
|---|---|---|---|
| bioZen 3 µm Peptide PS-C18 | 00F-4771-AW-11 | 00G-4771-AW-11 | – |
| bioZen 2.6 µm Peptide XB-C18 | 00F-4768-AW-11 | 00G-4768-AW-11 | – |
| bioZen 3 µm Polar C18 | 00F-4782-AW-11 | 00G-4782-AW-11 | – |
| bioZen 5 µm Peptide XB-C18 | – | – | 00J-4792-AW-21 |



### bioZen High pH Fractionation Column

| Fractionation Column | | |
|---|---|---|
| Part No. | Description | Dimension |
| 00F-4793-AN | bioZen 3 µm High pH Fractionation Column | 150 x 2.1 mm |

### Nano Trap Columns

| Trap Columns | | |
|---|---|---|
| | 10 x 0.075 mm | Unit |
| RP-1 | 05N-4252-AW | 3/pk |
| RP-2 | 05N-4754-AW | 3/pk |

### Trap Fittings

| Trap Fittings | | |
|---|---|---|
| Part No. | Description | Unit |
| AQ0-7602 | PEEKLok™ fittings with 6-40 thread for 1/32" OD tubing (2 x fittings, 6 x ferrules and 1 x tightening tool) | ea |
| AQ0-7603 | PEEKLok fittings with 6-32 thread for 1/32" OD tubing (2 x fittings, 6 x ferrules and 1 x tightening tool) | ea |
| AQ0-7600 | PEEKLok fittings with 10-32 thread for 1/32" OD tubing with low profile hex head (2 x fittings, 6 x ferrules and 1 x wrench) | ea |

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