

Capillary Chromatography: Extreme Resolution and Sensitivity in Proteomics LC/MS

The new line of VYDAC® MS capillary columns allows reversed-phase separation of smaller samples, at lower flow rates, thus allowing direct 100% feed of column eluates to mass spectrometric detectors. VYDAC MS capillary columns are packed with a derivatized proprietary grade of high-purity synthetic 300 Å silica. The MS-grade silica allows you to reduce TFA in peptide and protein separations from the commonly required 0.1% TFA to as low as 0.01%, or even completely eliminate it and substitute acetic acid or formic acid. This dramatically improves ion generation – for mass spectrometry with far better signal to noise ratios and sensitivity. A whole new line of capillary columns is now being introduced with 75 µm, 150 µm, 300 µm and 500 µm diameters available.

The following data was generously provided by Applied Biosystems (www.appliedbiosystems.com). A C18 capillary column – 300 µm i.d. x 50 mm long (Grace Vydac 218MS5.305) – was used to feed an API QSTAR™ Pulsar Hybrid LC/MS/MS System (Applied Biosystems/MDS SCIEX) with IonSpray™ source. Here tandem quadrupole and time-of-flight (TOF) mass spectrometers are combined, specifically to enable protein identification and characterization as well as drug metabolism studies.

LC/MS/MS is a very powerful analytical technique. It can even dissect and analyze regions of chromatograms with overlapping peaks, common when separating complex samples such as enzymatic digests. Figure 1 shows the separation of a tryptic digest of bovine serum albumin (BSA) with MS detection. The amplitude of the trace in

Figure 1a is the total ion count (TIC) measured by TOF MS. The chromatogram contains many peaks, quite a few of which are overlapping.

Using the data processing capabilities of the API QSTAR instrument, significant additional information can be derived.

Continued on next page

VYDAC 218MS5.305 Tryptic Digest of Bovine Serum Albumin

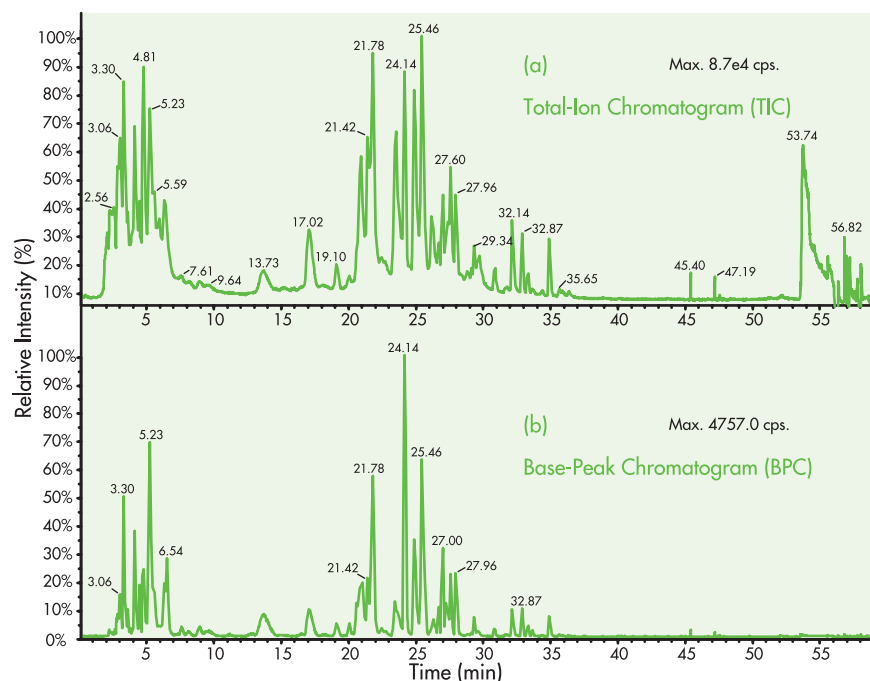


Figure 1. Chromatographic separation of tryptic digest of bovine serum albumin (BSA). Sample: 3 pmole. Column: VYDAC 218MS5.305 5 µm, 300 Å, polymeric-C18 reversed-phase (300 µm i.d. x 50 mm L). Flow: 5 µL/min. Mobile phase: A = 0.1% formic acid, 98% water, 2% ACN. B = 0.1% formic acid, 98% ACN, 2% water. Gradient: Hold 3% B from 0 to 5 minutes. Then ramp from 3% B to 50% B at 65 minutes. Final ramp to 75% B at 70 minutes. Detection: MS. (a) Total ion count. (b) Base peak intensity.

Continued from previous page

Figure 1b shows the base-peak chromatogram (BPC) corresponding to Figure 1a. The base peak is defined as the single mass peak with maximum amplitude at each time in the chromatogram. The BPC emphasizes peaks containing a single predominant molecular species and deemphasizes heterogeneous peaks and noise.

Another technique – total ion count based on dependent MS/MS data – is shown in Figure 2. Chromatogram 2a is identical to the chromatogram of Figure 1a. The instrument counts all ions from the TOF mass spectrometer with no ion selection by the quadrupole. In the dependent-data traces, the first quadrupole selects and passes only the most abundant single ion (Fig. 2b) or the second most abundant single ion (Fig. 2c) into the collision cell, just a few seconds each. The TOF mass spectrometer then produces fragment spectra suitable for peptide sequence determination (Figs. 3 and 4). The total ion counts from the TOF spectrum are displayed as amplitude in Figure 2b and 2c. Peptide peaks only partially resolved by the HPLC column are easily revealed by detection dependent on the second most abundant ion (Fig. 2c).

In essence the reversed-phase column and the quadrupole act as orthogonal separation mechanisms to produce pure peptide molecular ions for fragmentation. Complete chromatographic resolution of all peptides is not required. However, the resolution of the chromatographic column simplifies the task of the MS/MS system in selecting ions for sequencing, and the low-TFA capability of the

VYDAC 218MS5.305 Tryptic Digest of Bovine Serum Albumin

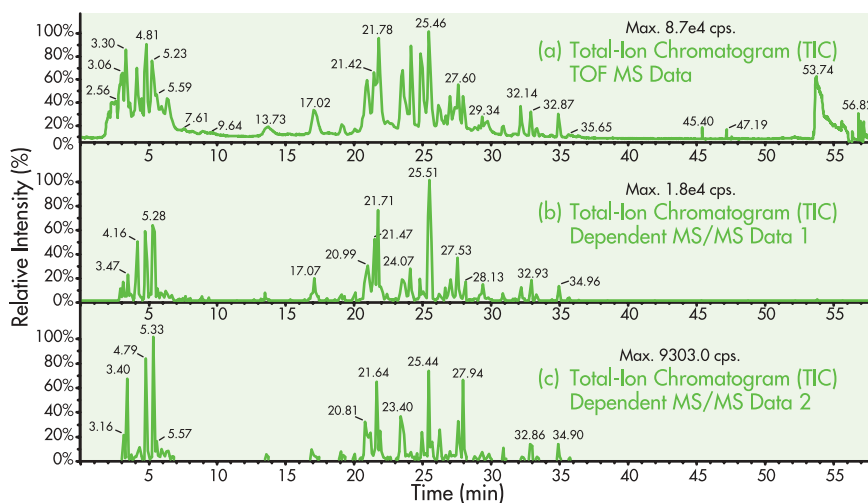


Figure 2. Chromatographic separation of tryptic digest of BSA. Column: VYDAC 218MS5.305 5 μm , 300 \AA , polymeric-C18 reversed-phase (300 μm i.d. x 50 mm L). Conditions: Same as Figure 1. Detection: MS/MS. (a) Total ion count. (b) MS dependent total ion count, base peak. (c) MS dependent total ion count, second base peak.

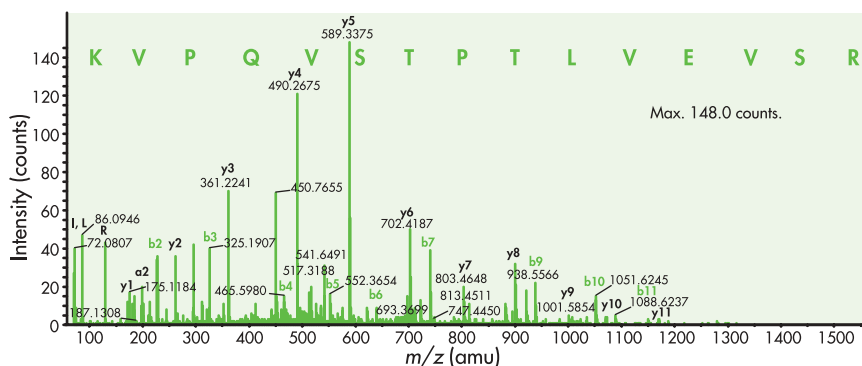


Figure 3. MS/MS data for BSA T61-T62 peptide (437-451), 1639.93 amu. Column and conditions as in Figure 1. Retention time: 21.642 minutes.

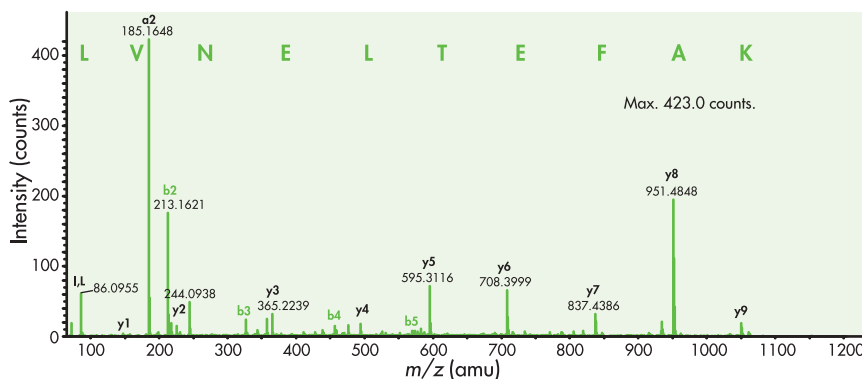


Figure 4. MS/MS data for BSA T10 peptide (66-75), 1162.62 amu. Column and conditions as in Figure 1. Retention time: 24.070 minutes.

VYDAC 218MS5.305 Tryptic Digest of Bovine Serum Albumin

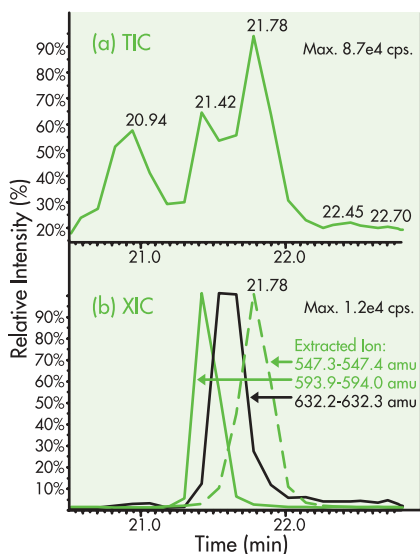


Figure 5. Extracted ion chromatograms showing separation of different components in a TIC peak. Column and conditions as in Figure 1. Detection: TOF MS. (a) Total ion count. (b) Extracted ion counts.

VYDAC MS column assures the best possible MS sensitivity.

The API QSTAR™ Pulsar System will also display extracted ion chromatograms (XICs). By recording the amplitude of single mass peaks as a function of HPLC retention time, XICs reveal the distribution of individual peptide components in TIC peaks, as shown in Figure 5.

Our appreciation to

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Practical Application

In a poster presented at the meeting of the American Society for Mass Spectrometry in Chicago, May 27-31, 2001, scientists* from the Protein Characterization and Proteomics Laboratory at University of Cincinnati College of Medicine reported using a VYDAC reversed-phase capillary column (Cat. No. 214MS5.310, C4, 300 μm i.d. x 100 mm) together with an Eldex Micropro HPLC system and an Applied Biosystems/MDS SCIEX API 3000™ triple-quadrupole mass spectrometer for detection and identification of expressed sequence tags to identify gene products in *Pseudomonas aeruginosa*. One objective of this work has been to identify proteins that are up-regulated and perhaps essential for anaerobic growth. Such proteins would be potential therapeutic targets for mediation of *P. aeruginosa* biofilms that do not respond to conventional antibiotic therapy and are severely problematic in a number of human diseases including cystic fibrosis.

Proteins from *P. aeruginosa* cultures grown aerobically and anaerobically were separated by 1-D electrophoresis on polyacrylamide gels (Fig. 6). Bands of interest were reduced, alkylated, and then digested in the gel with trypsin. Extracted peptides were applied to the C4 capillary column for separation and analysis. A sample chromatogram is shown in Figure 7, with intensities corresponding to the total ion count (TIC) from the third quadrupole (Q3). Examples of the corresponding mass spectra are shown in Figure 8.

*Chris L. Stumpf, Daniel J. Hassett, Ying-Qing Yu, Abby Newland, Tara Mitchell, Amy Whitescarver, and George M. Hilliard

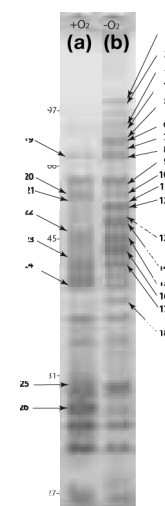


Figure 6. Separation of proteins from aerobic (a) and anaerobic (b) cultures of *Pseudomonas aeruginosa* on polyacrylamide gel electrophoresis (SDS-PAGE).

VYDAC 214MS5.305, PAGE Band 16

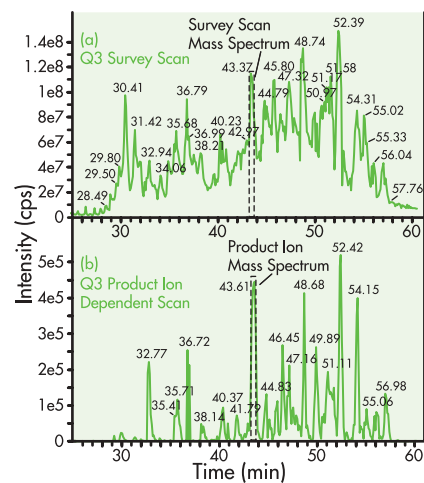


Figure 7. Chromatogram of tryptic peptides from PAGE band 16. Column: VYDAC 214MS5.310 5 μm , 300 \AA , C4 reversed-phase (300 μm i.d. x 100 mm L). Flow: 1 $\mu\text{L}/\text{min}$. Mobile phase: A = 0.1% TFA (w/v) in water. B = 0.085% TFA (w/v) in 90:10 ACN:water. Gradient: Ramp from 3%B to 65%B over 50 minutes. Detection: MS/MS. (a) Total ion count. (b) MS dependent total ion count.

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LC/MS Elongation Factor Tu, Gene PA4265, Band 16, *Pseudomonas aeruginosa*

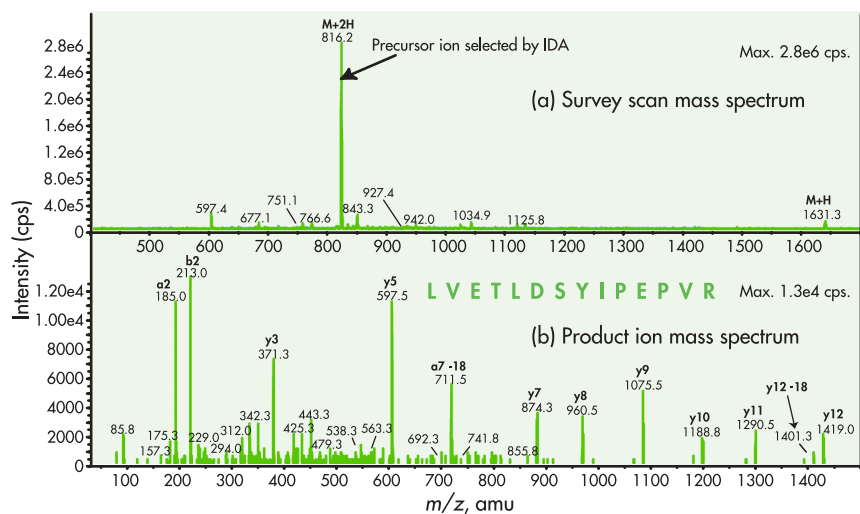


Figure 8. Mass spectra of selected peptide from PAGE band 16 digest. (a) Precursor-ion candidates at 43.37 minutes. No fragmentation. (b) Product-ion spectrum from fragmentation of selected m/z 816.2 precursor.

The API 3000 LC/MS system equipped with Analyst® 1.1 software was used in information-dependent-acquisition (IDA) mode, cycling repeatedly through two experiments: a two-second survey scan during which all ions are passed to Q3 to automatically identify the most intense peptide ion in the 500-1500 m/z range (Figs. 7a and 8a), followed by a four-second MS dependent product-ion scan during which the precursor ion thus identified is selected by the first quadrupole (Q1), fragmented in the collision cell (Q2), and the mass spectrum of the fragments displayed by the Q3 scan (Figs 7b and 8b).

To arrive at peptide sequences and gene assignments, database search software (Proteomics Sonar MS/MS database search engine) was used to compare the expressed-sequence-tag mass spectra obtained from these experiments to

predicted mass spectra using a custom database constructed from DNA sequences for the 5,570 open reading frames encoded in the *P. aeruginosa* genome. Sensitivity using the VYDAC 300 μm i.d. x 100 mm column was estimated from experiments using a tryptic digest of BSA and [Glu1]-Fibrinopeptide B as samples and query to a current NCBI non-redundant database. Reliable sequence assignments were possible with as little as 40 pmole of BSA before digestion and 6.25 pmole of a standard peptide.

Data in this article courtesy of

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VYDAC® Columns in Protein Oxidation Studies

Tissue damage upon reperfusion following cardiac ischemia is of significant concern in relation to heart attack survival. Myoglobin, a 17-kDa heme protein which normally stores oxygen in muscle tissues has been implicated in lipid peroxidation and protein oxidation during reperfusion. Angelo Filosa, working with Ann M. English at Concordia University, reports using two VYDAC columns in LC/MS applications to investigate oxidation sites in myoglobin (Mb).

Reaction of metMb with H_2O_2 forms both an oxyferryl heme and an unstable protein-based radical, P^\bullet . The specific location of the radical on Mb has been the subject of earlier studies using the spin trap 2-methyl-2-nitrosopropane (MNP).

In this experiment, Mb was incubated with H_2O_2 at various pHs in the presence of a different spin trap, 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS), then further reacted with ascorbate to reduce spin adducts to more stable spin-paired species. The resulting modified Mb was purified on a 4.6 mm diameter

Purification on VYDAC 218TP54

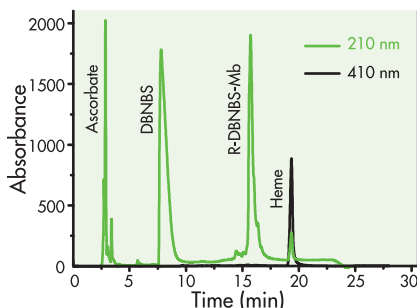


Figure 9. Purification of myoglobin from reaction mixture by reversed-phase HPLC. Column: VYDAC 218TP54, 5 μ m, 300 \AA , C18 (4.6 mm i.d. x 250 mm). Flow: 1.0 mL/min. Mobile phase: A = 5:95 ACN: water, 0.05% TFA. B = 55:45 ACN:water, 0.05% TFA. Gradient: Linear, 0% to 100% B over 20 minutes. Detection: UV absorbance with stream splitting (1:50) to MS (Fig. 10).

VYDAC 300 \AA C18 column (Fig. 9). Post-column splitting directed 40 μ L per minute of the column effluent to the ESI source of a Finnigan SSQ7000 single-quadrupole mass spectrometer for molecular weight determination. Deconvoluted mass spectra (Fig. 10) show the effects of H_2O_2 concentration and pH on the products obtained.

Effects of Oxidation Conditions

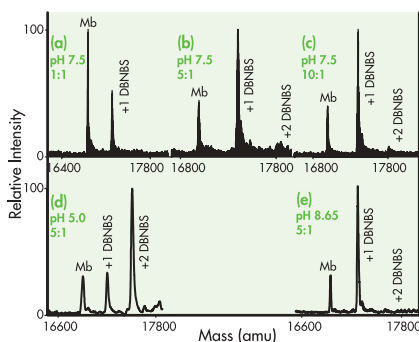


Figure 10. Deconvoluted mass spectra of reaction products. (a) Reaction at pH 7.5, 1:1 molar ratio of H_2O_2 :Mb. (b) pH 7.5, 5:1. (c) pH 7.5, 10:1. (d) pH 5.0, 5:1. (e) pH 8.65, 5:1.

Following digestion with trypsin, tryptic peptides were separated on a 1.0 mm diameter VYDAC C18 column with LC/MS/MS detection using a Waters Alliance/Micromass Quattro spectrometer in positive ion mode between 80-2000 m/z . Peptide mass mapping revealed modified peptides not observed in the native Mb digest (Fig. 11), suggesting radical forma-

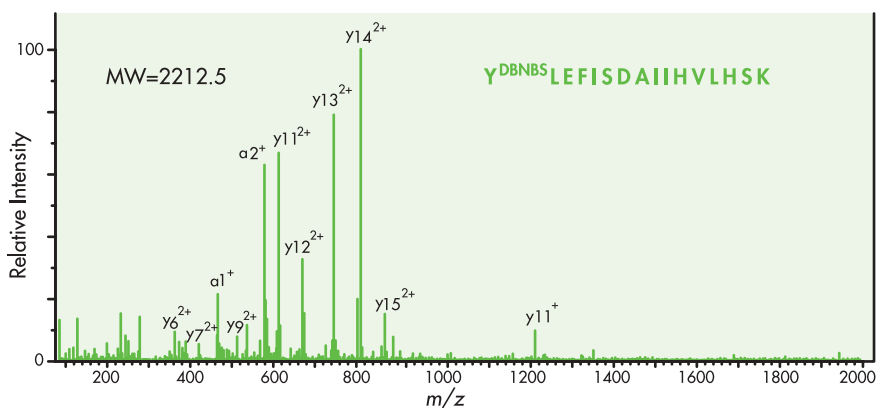


Figure 12. LC/MS/MS sequencing of R-DBNBS-modified peptide 103-118.

Analysis on VYDAC 218TP51

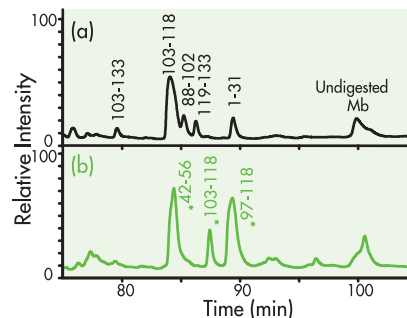


Figure 11. Analytical separation of tryptic peptides by microbore HPLC. Column: VYDAC 218TP51 5 μ m, 300 \AA , C18 (1.0 mm i.d. x 250 mm). Flow: 40 μ L/min. Mobile phase: A = 5:95 ACN: water, 0.05% TFA. B = 55:45 ACN:water, 0.05% TFA. Gradient: Linear, 0% to 100% B over 120 minutes. Detection: LC/MS/MS. (a) Native Mb. (b) Reduced-DBNBS-Mb tryptic peptide. R-DBNBS-modified peptides 97-118, 103-118, and 42-56 are highlighted (*).

tion on Tyr103 and Lys42. Sequencing by ESI MS/MS (Fig. 12) confirmed Tyr103 as one site of radical formation.

Courtesy of

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75 µm i.d. x 150 mm	214MS5.07515	208MS5.07515	218MS5.07515	238MS5.07515	219MS5.07515
75 µm i.d. x 250 mm	214MS5.07525	208MS5.07525	218MS5.07525	238MS5.07525	219MS5.07525
150 µm i.d. x 50 mm	214MS5.1505	208MS5.1505	218MS5.1505	238MS5.1505	219MS5.1505
150 µm i.d. x 100 mm	214MS5.1510	208MS5.1510	218MS5.1510	238MS5.1510	219MS5.1510
150 µm i.d. x 150 mm	214MS5.1515	208MS5.1515	218MS5.1515	238MS5.1515	219MS5.1515
150 µm i.d. x 250 mm	214MS5.1525	208MS5.1525	218MS5.1525	238MS5.1525	219MS5.1525
300 µm i.d. x 50 mm	214MS5.305	208MS5.305	218MS5.305	238MS5.305	219MS5.305
300 µm i.d. x 100 mm	214MS5.310	208MS5.310	218MS5.310	238MS5.310	219MS5.310
300 µm i.d. x 150 mm	214MS5.315	208MS5.315	218MS5.315	238MS5.315	219MS5.315
300 µm i.d. x 250 mm	214MS5.325	208MS5.325	218MS5.325	238MS5.325	219MS5.325
500 µm i.d. x 50 mm	214MS5.505	208MS5.505	218MS5.505	238MS5.505	219MS5.505
500 µm i.d. x 100 mm	214MS5.510	208MS5.510	218MS5.510	238MS5.510	219MS5.510
500 µm i.d. x 150 mm	214MS5.515	208MS5.515	218MS5.515	238MS5.515	219MS5.515
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