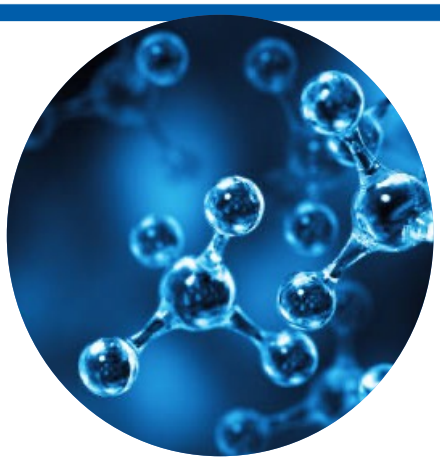


TN-1367

High-Resolution LC-MS Characterization of Antibody Drug Conjugates with PNGase F Enzymatic Cleavage of N-linked Oligosaccharides using Biozen™ Native-RP-5

James Song and Carl Sanchez
Phenomenex Inc., 411 Madrid Ave., Torrance, CA 90501, USA

Introduction

Antibody drug conjugates (ADCs) remain a highly researched biotherapeutic modality due to their success in targeting and killing tumor cells without compromising healthy ones. Cysteine-linked ADCs have improved pharmacokinetics, efficacy and reduced toxicity. Examples of approved cysteine-linked ADCs are Adcetris® and Tivdak®.

Characterization of ADCs is still an ongoing challenge. Hydrophobic Interaction Chromatography (HIC) is a traditional approach but requires sample treatment for MS friendly analysis and although reversed analysis is an acceptable approach, LC conditions cause the antibody subunits with reduced disulfide bonds to dissociate.

In this technical note, we demonstrate the performance of Biozen™ Native RP-5, a non-porous, hydrophilic particle designed to preserve the native forms of intact biomolecules under reversed phase conditions, for the intact mass measurement resulting from deglycosylation via PNGase F, as well as the separation of intact ADC drug-to-antibody ratio (DAR) species, of four cysteine-linked ADCs.

Sample Preparation

Four commercial cysteine linked ADC drugs were tested: 1) Adcetris (brentuximab vedotin), 2) Padcev® (enfortumab vedotin), 3) Polivy® (polartuzumab vedotin-piiq), 4) Tivdak (tisotumab vedotin-tftv).

N-linked oligosaccharides of ADC samples were enzymatically cleaved using PNGase F from Promega® (cat# V4831) according to Promega protocol without the use of surfactants.

Enzymatically treated ADC samples were buffer exchanged into 20 mM Ammonium Acetate pH 5.2 using Vivaspin® 500 30 kDa centrifugal concentrators prior to analysis. Buffer exchange was performed to reduce fouling of the MS source by components of the ADC formulation and to improve sensitivity for the DAR0 species which elutes close to the void time of the column. The relatively high concentration of salts and other formulation components will cause MS signal suppression for any components eluting near the void time.

LC Conditions

Column: Biozen Native-RP-5
Dimensions: 50 x 2.1 mm
Part No.: 00B-4800-AN
Mobile Phase: A: 50 mM Ammonium Acetate in Water
B: 25mM Ammonium Acetate in 50/50 Water/Isopropanol (v/v)

Gradient:

Time (min)	% B
0	0
2	0
5	15
20	50
24	50
26	100
27	100
27.1	0
30	0

Flow Rate: 0.2 mL/min
Injection Volume: 20 µg ADC on-column
Temperature: 30 °C
LC System: Agilent 1290 Infinity II UHPLC
Detection: HRMS, UV (280 nm)
Detector: SCIEX® X500B HRMS, Agilent® DAD (UV)

Note: The Agilent 1290 Infinity II UHPLC system contained the following components: G7120A high speed binary pump with JetWeaver V35, G7167B multisampler, G7116B multicolumn thermostat and G7117B diode array detector (10mm pathlength). Also, plastic mobile phase reservoirs were used to reduce any metallic impurities (especially sodium and potassium) that may leach from standard borosilicate glass reservoirs. Finally, to ensure ADCs remained in their native, intact state the mobile phase organic composition was limited to a maximum of 25 v/v% IPA except for a brief column clean-up step (50 v/v% IPA) at the end of the gradient.

HRMS Conditions

Ionization Mode: ESI
Polarity: Positive
Source Temperature: 450 °C
Declustering Potential: 200 V (50 V spread)
CE ± CE Spread: 5 ± 0V
Ion Spray Voltage: 5000 V
Scan Range: 1000-8000 m/z

Results and Discussion

The PNGase treated ADC samples compared to the non-treated samples show loss of mass approximate to N-linked glycans commonly associated with monoclonal antibodies (see **Figures 1-4** and **Table 1** below).

It was also observed that the PNGase treated ADC DAR species showed loss of multiple peaks separated by 162 Da and a simplified mass fingerprint compared to the non-treated samples. This mass shift of 162 Da corresponds to ADC species that have lost N-linked glycans with various glycoforms with added galactose or mannose molecules further confirming that N-linked glycans were cleaved from the ADCs.

Table 1. PNGase Treated ADC Samples Comparing Loss of Mass to Approximate N-linked Glycans

Sample	Observed Average loss Da	Probable Glycan	Theoretical Average Mass Da
Adcetris®	3030.3	G0F/G1F	3052.8
Padcev®	2875.1	G0F/G0F	2890.7
Polivy®	2858.6	G0F/G0F	2890.7
Tivdak®	2875.0	G0F/G0F	2890.7

Figure 1. A280nm Chromatogram of Adcetris ADC Separation by Biozen™ Native RP-5 Column

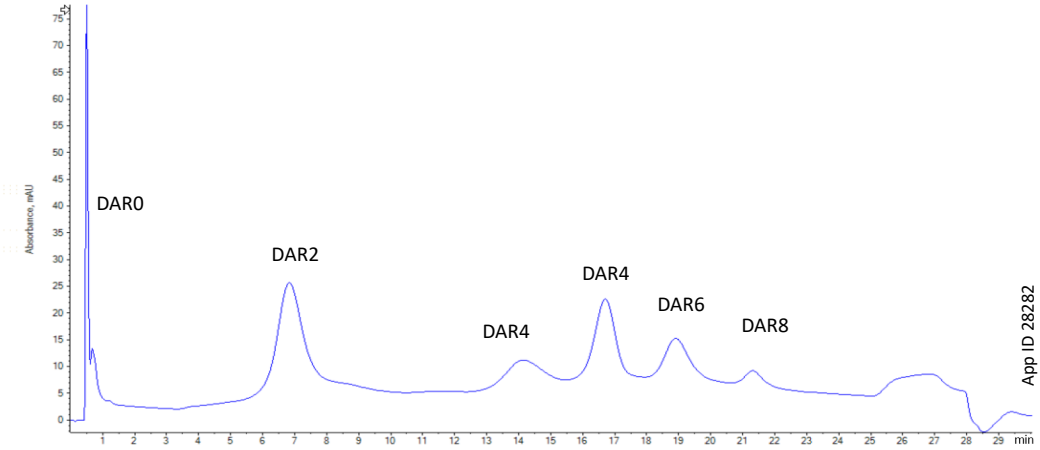


Figure 2. Mass Spectra Deconvolution of Adcetris DAR0-DAR8 (top); Adcetris DAR0-DAR8 Deglycosylated (bottom)

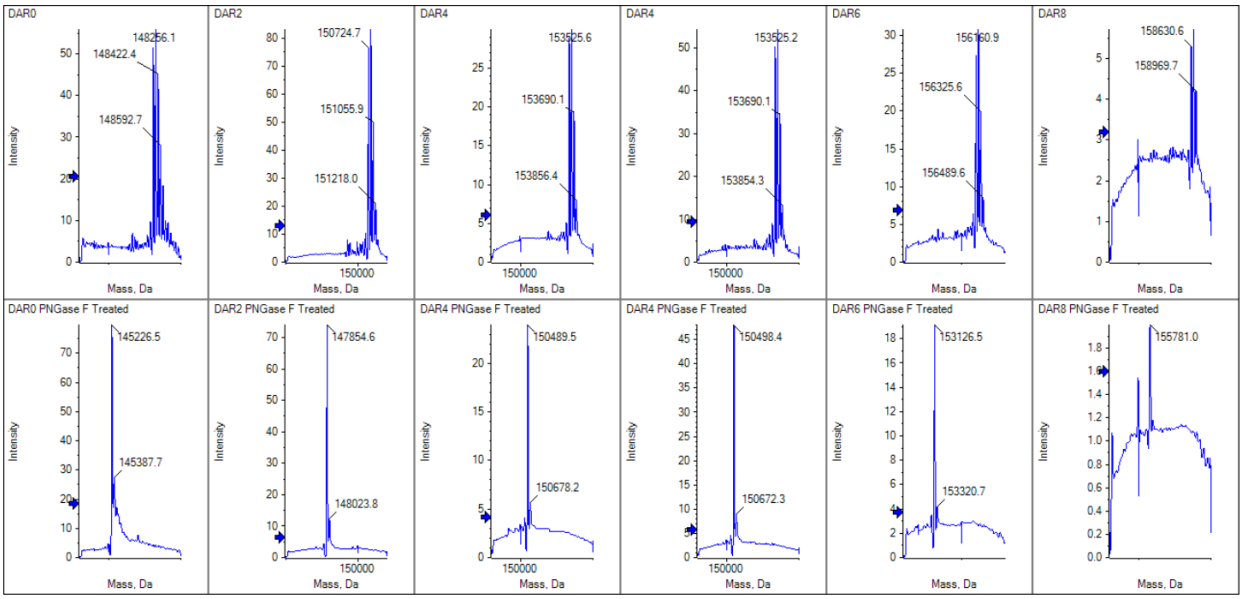


Figure 3. A280nm Chromatogram of Padcev® ADC Separation by Biozen™ Native RP-5 Column

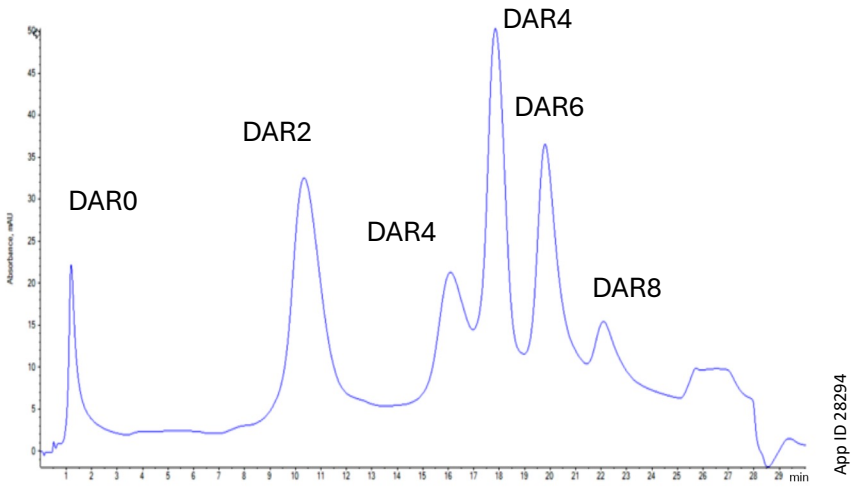


Figure 4. Mass Spectra Deconvolution of Padcev DAR0-DAR8 (top); Padcev DAR0-DAR8 Deglycosylated (bottom)

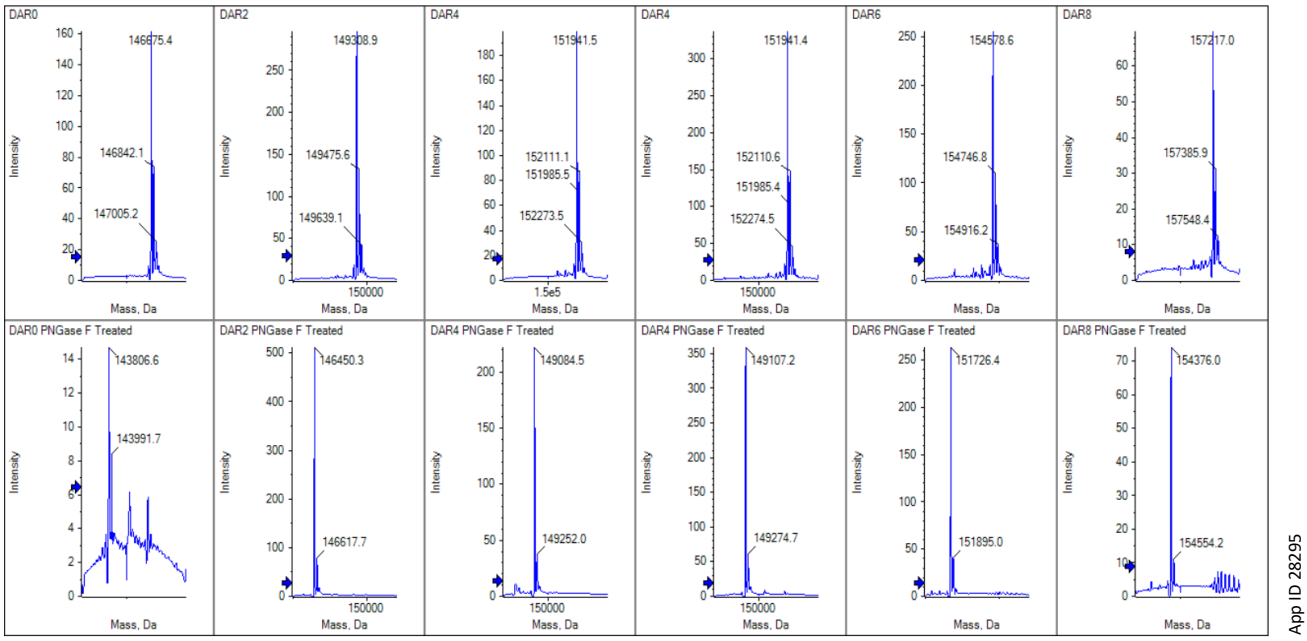


Figure 5. A280nm Chromatogram of Polivy® ADC Separation by Biozen Native RP-5 Column

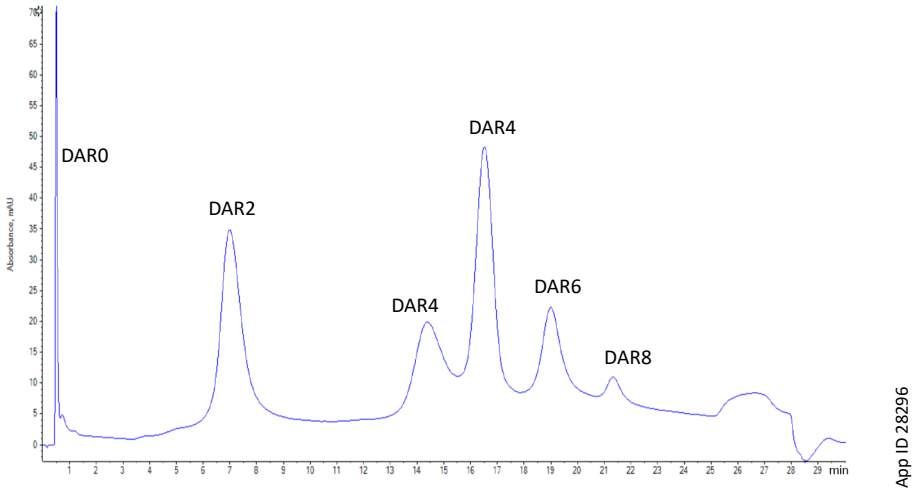


Figure 6 . Mass Spectra Deconvolution of Polivy® DAR0-DAR8 (top); Polivy DAR0-DAR8 Deglycosylated (bottom)

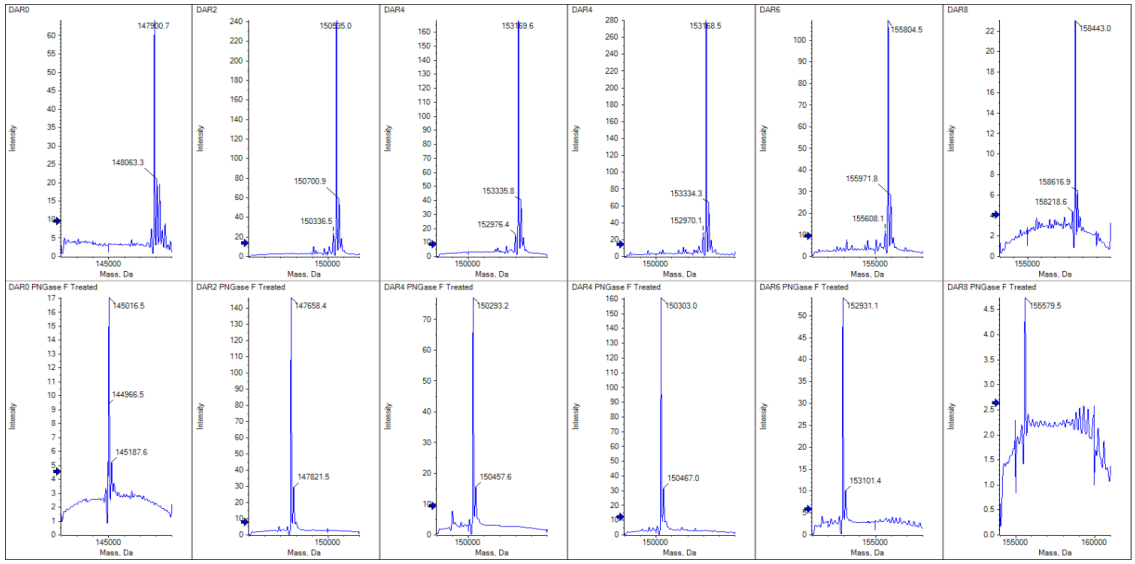


Figure 7 . A280nm Chromatogram of Tivadak® ADC Separation by Biozen™ Native RP-5 Column

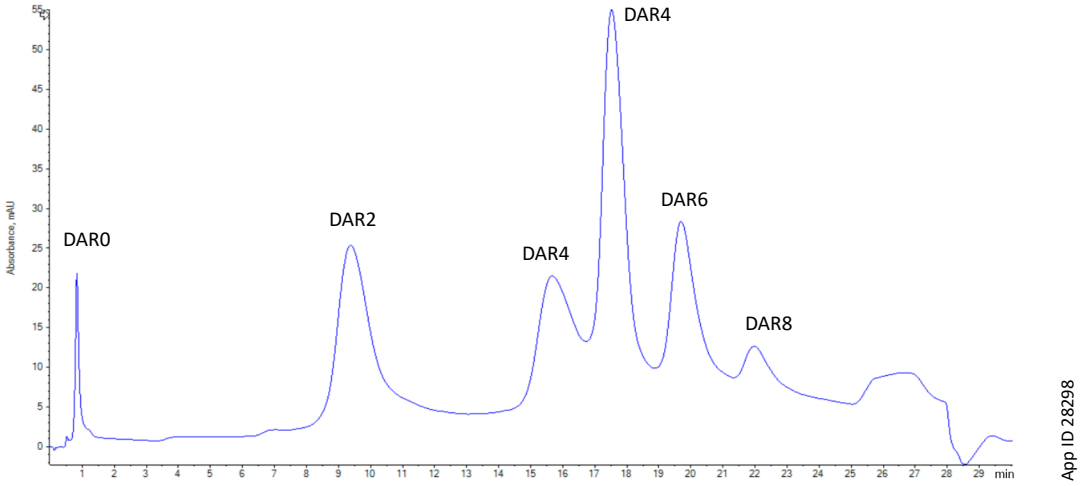
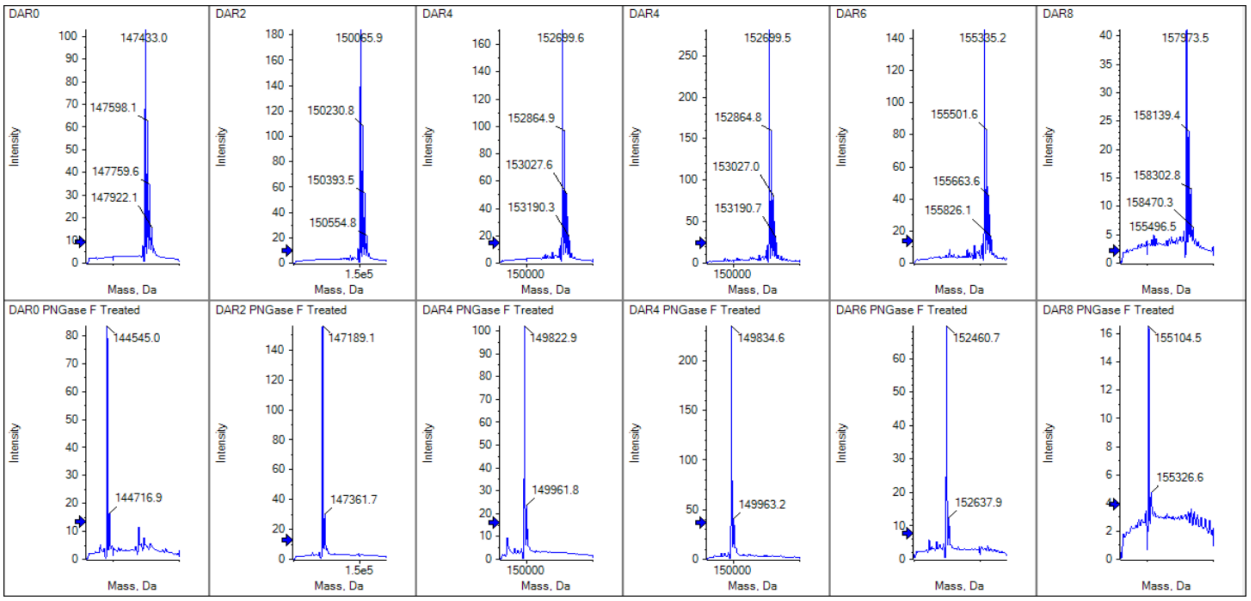


Figure 8 . Mass Spectra Deconvolution of Tivadak DAR0-DAR8 (top); Tivadak DAR0-DAR8 Deglycosylated (bottom)



Have questions or want more details on implementing this method? We would love to help!
*Visit **www.phenomenex.com/Chat** to get in touch with one of our Technical Specialists*

Conclusion

The challenges in characterizing ADCs necessitate advanced techniques, such as the use of Biozen™ Native RP-5 columns, which enable intact mass measurement under native reverse phase conditions and separation of cysteine linked ADC species without dissociating the antibody subunits. The experimental results confirm successful deglycosylation of N-linked glycans from the ADCs, providing a clearer mass fingerprint and simplifying the analysis. The Biozen Native RP-5 LC column is suitable for LC HRMS analysis of intact ADC for drug-to-antibody ratio (DAR), and PNGase F treated ADCs.

Biozen Ordering Information

Biozen Columns (mm)		
	50 x 2.1	**50 x 4.6**
Biozen Native-RP-5	00B-4800-AN	00B-4800-E0
Biozen Native-RP-1	00B-4799-AN	00B-4799-E0

Need a different column size or sample preparation format?

No problem! We have a majority of our available dimensions up on www.phenomenex.com, but if you can't find what you need right away, our super helpful Technical Specialists can guide you to the solution via our online chat portal www.phenomenex.com/Chat.

Australia
t: +61 (0)2-9428-6444
auinfo@phenomenex.com

Austria
t: +43 (0)1-319-1301
anfrage@phenomenex.com

Belgium
t: +32 (0)2 503 4015 (French)
t: +32 (0)2 511 8666 (Dutch)
beinfo@phenomenex.com

Canada
t: +1 (800) 543-3681
info@phenomenex.com

China
t: +86 400-606-8099
cninfo@phenomenex.com

Czech Republic
t: +420 272 017 077
cz-info@phenomenex.com

Denmark
t: +45 4824 8048
nordicinfo@phenomenex.com

Finland
t: +358 (0)9 4789 0063
nordicinfo@phenomenex.com

France
t: +33 (0)1 30 09 21 10
franceinfo@phenomenex.com

Germany
t: +49 (0)6021-58830-0
anfrage@phenomenex.com

Hong Kong
t: +852 6012 8162
hkinfo@phenomenex.com

India
t: +91 (0)40-3012 2400
indiainfo@phenomenex.com

Indonesia
t: +62 21 3952 5747
indoinfo@phenomenex.com

Ireland
t: +353 (0)1 247 5405
eireinfo@phenomenex.com

Italy
t: +39 051 6327511
italiainfo@phenomenex.com

Japan
t: +81 (0) 120-149-262
jpinfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
nlinfo@phenomenex.com

Mexico
t: 01-800-844-5226
tecnicomx@phenomenex.com

The Netherlands
t: +31 (0)30-2418700
nlinfo@phenomenex.com

New Zealand
t: +64 (0)9-4780951
nzinfo@phenomenex.com

Norway
t: +47 810 02 005
nordicinfo@phenomenex.com

Poland
t: +48 22 51 02 180
pl-info@phenomenex.com

Portugal
t: +351 221 450 488
ptinfo@phenomenex.com

Singapore
t: 800-852-3944
sginfo@phenomenex.com

Slovakia
t: +420 272 017 077
sk-info@phenomenex.com

Spain
t: +34 91-413-8613
espinfo@phenomenex.com

Sweden
t: +46 (0)8 611 6950
nordicinfo@phenomenex.com

Switzerland
t: +41 (0)61 692 20 20
swissinfo@phenomenex.com

Taiwan
t: +886 (0) 0801-49-1246
twinfo@phenomenex.com

Thailand
t: +66 (0) 2 566 0287
thaiinfo@phenomenex.com

United Kingdom
t: +44 (0)1625-501367
ukinfo@phenomenex.com

USA
t: +1 (310) 212-0555
info@phenomenex.com

All other countries/regions
Corporate Office USA
t: +1 (310) 212-0555
www.phenomenex.com/chat

www.phenomenex.com

Phenomenex products are available worldwide. For the distributor in your country/region, contact Phenomenex USA, International Department at international@phenomenex.com