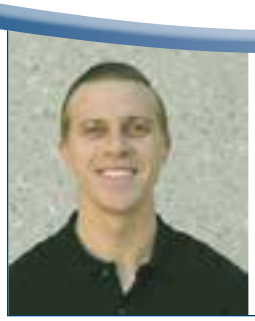
APPLICATION

Optimizing Methods for Drugs of Abuse Using β-Gone™ β-Glucuronidase Removal

Matthew Brusius
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA



Matt Brusius
Product Manager, Sample Preparation

Matt Brusius is an avid ice hockey player. He likes skating backwards and taking slapshots from the point.

Introduction

During metabolism, drugs are tagged with a glucuronic acid that helps change the polarity of the drug compound and aids in the absorption into the kidneys. When the drugs exit the body through urine, they are still in their glucuronide form and before chromatographic analysis can occur, the glucuronide bond must be cleaved through hydrolysis. Enzymatic hydrolysis, using β-glucuronidase, is preferred over acid hydrolysis because the bond is cleaved without introducing harsh solvents into the sample. Now the sample contains drug compounds and residual β-glucuronidase enzyme, which if the enzyme is not removed can precipitate out in the LC column during the run. The column's selectivity and lifetime is negatively affected and can result in buildup in the mass spectrometer (MS). "Dilute-and-shoot" is a common method that is used to prepare hydrolyzed urine samples for LC/MS analysis. This method can cause issues with the sensitivity because it dilutes the sample 10x up to 30x before injection onto the column. In this technical note we will compare signal responses and recoveries of various classes of drugs of abuse using β-Gone β-Glucuronidase Removal Products with our standard protocol involving a 0.1% formic acid in methanol dilution and a protocol without a dilution prior to filtration. Both of these protocols will also be compared to the "dilute-and-shoot" protocol to further understand how dilution affects sensitivity.

Materials and Methods

All reagents and solvents were HPLC or analytical grade. Analyses were performed using an API 4000™ LC/MS/MS (SCIEX, Framingham, MA)

Sample Preparation

Enzymatic Hydrolysis

IMCSzyme® Hydrolysate Mix was prepared as follows:

1) Add 10 µL of analyte spike (1 µg/mg) to 140 µL of urine
2) Dilute with 80 µL of IMCS buffer (IMCS Part No.: 04-EZ-RHB-20) and add 30 µL IMCS Enzyme (IMCS Part No.: 04-E1F-010). Vortex for 15 seconds
3) Proceed to β-Glucuronidase Removal Protocols for IMCS and "dilute-and-shoot"

Campbell Enzyme Hydrolysate Mix was prepared as follows:

1) Add 10 µL of analyte spike (1 µg/mL) to 200 µL of urine
2) Dilute with 100 µL of 0.1 M ammonium acetate buffer
3) Add 40 µL of Campbell Science β-Glucuronidase Enzyme Solution (Campbell Part No.: DR2102)
4) Add 400 µL of 0.1% formic acid in water and vortex for 15 seconds
5) Proceed to β-Glucuronidase Removal Protocols for Campbell Protocol

β-Glucuronidase Removal

IMCS Protocol 1:
β-Gone Recombinant Enzyme – Methanol Dilution

1) Dilute 200 µL urine hydrolysate with 133 µL with 0.1% formic acid in methanol
2) Load diluted IMCSzyme solution onto the Recombinant β-Gone 96-Well Plate (Phenomenex Part No.: 8E-S139-TGA)
3) Collect eluent and inject 10 µL

IMCS Protocol 2:
β-Gone Recombinant Enzyme – No Dilution

1) Transfer 200 µL of IMCSzyme solution onto the Recombinant β-Gone Well Plate (Part No.: 8E-S139-TGA)
2) Collect eluent and inject 10 µL

Campbell Protocol 1:
β-Gone Non-Recombinant Enzyme – Methanol Dilution

1) Dilute 200 µL urine hydrolysate solution with 133 µL of 0.1% formic acid in methanol
2) Transfer Campbell Enzyme solution to Non-Recombinant β-Gone Well Plate (Part No.: 8E-S322-DGA) and apply 2-5" Hg of vacuum
3) Collect eluent and inject 10 µL

Campbell Protocol 2:
β-Gone Non-Recombinant Enzyme – No Dilution

1) Transfer 200 µL of Campbell Enzyme solution to Non-Recombinant β-Gone 96-Well Plate (Part No.: 8E-S322-DGA) and apply 2-5" Hg of vacuum
2) Collect eluent and inject 10 µL

"Dilute-and-Shoot" Protocol:

1) Transfer 100 µL of urine hydrolysate to vial
2) Add 900 µL 0.1% formic acid in water and vortex for 15 seconds
3) Inject 10 µL onto column

Results and Discussion

IMCSzyme Enzyme (Recombinant):

Table 1 provides the absolute recovery values for the IMCSzyme prepared samples that were subject to IMCS Protocol 1, a 40% dilution with 0.1% formic acid in methanol, prior to filtration through the β-Gone β-Glucuronidase Removal 96-Well plate. **Table 2** provides absolute recovery values for the IMCS prepared samples that were filtered through β-Gone with no prior dilution (Protocol 2). While these recoveries were generally lower than the methanol diluted samples, these samples show an increase in sensitivity.

Figure 1 and Figure 2 compare the differences in sensitivity using a methanol dilution, no dilution, and "dilute-and-shoot" protocols for morphine and lorazepam. Without the methanol dilution, both of these compounds show lower recovery (86% vs. 97% for morphine, 81% vs 109% for lorazepam), but show better signal response, as the loss in recovery is overcome by omitting the 40% dilution; without it, these samples are simply more concentrated. All compounds in this suite followed the same trend, with the exception of THC-COOH and buprenorphine, which show better sensitivity with the methanol dilution.

Figure 3 compares the different signal response using a methanol dilution, no dilution, and the "dilute-and-shoot" protocols for buprenorphine. For this analyte, it shows that the methanol dilution yields the best recovery and a higher response. The sample with no dilution still produces a signal greater than 3X more than the "dilute-and-shoot" sample. The methanol dilution proves to be more beneficial for THC-COOH than it is for buprenorphine since the non-methanol diluted sample produces a response that is similar to "dilute-and-shoot" (**Figure 4**).

Campbell Enzyme (Non-Recombinant):

Table 3 provides the absolute recovery values of the Campbell Enzyme prepared samples that were subject to Protocol 1, a 40% dilution with 0.1% formic acid in methanol, prior to filtration through the β-Gone β-Glucuronidase Removal 96-Well Plates. **Table 4** provides absolute recovery values for Campbell Enzyme prepared samples that were simply filtered through β-Gone with no prior dilution (Protocol 2). In contrast to the IMC samples, all but one compound (THC-COOH) show improved sensitivity when comparing the methanol diluted samples to the undiluted filtrate. These results also indicate that if THC-COOH is included in the suite, a 40% methanol dilution must be used to achieve acceptable results.

Table 1:
IMCS Protocol 1 Recovery Data with Dilution

Analyte	Average Recovery %	%CV (n=8)
Benzoylecognine	101	3
Buprenorphine	75	7
Codeine	109	7
Lorazepam	109	4
Methamphetamine	102	3
Morphine	97	5
Norbuprenorphine	101	5
PCP	97	4
THC-COOH	97	2

Table 2:
IMCS Protocol 2 Recovery Data without Dilution

Analyte	Average Recovery %	%CV (n=8)
Benzoylecognine	95	4
Buprenorphine	40	16
Codeine	102	6
Lorazepam	81	6
Methamphetamine	96	3
Morphine	86	4
Norbuprenorphine	72	3
PCP	95	4
THC-COOH	23	13

HPLC Conditions

Column: Kinetex® 2.6 µm Biphenyl
Dimensions: 50 x 3.0 mm
Part No.: 00B-4622-Y0
Mobile Phase: A: 0.1 % Formic acid in Water
B: 0.1 % Formic acid in Methanol

Gradient:

Time (min)	% B
0	10
1	10
4	100
5	100
5.01	10
6	10

Flow Rate: 0.7 mL/min
Injection: 5 µL
Temperature: Ambient
Detection: UV @ 220 nm

Figure 1.
Morphine

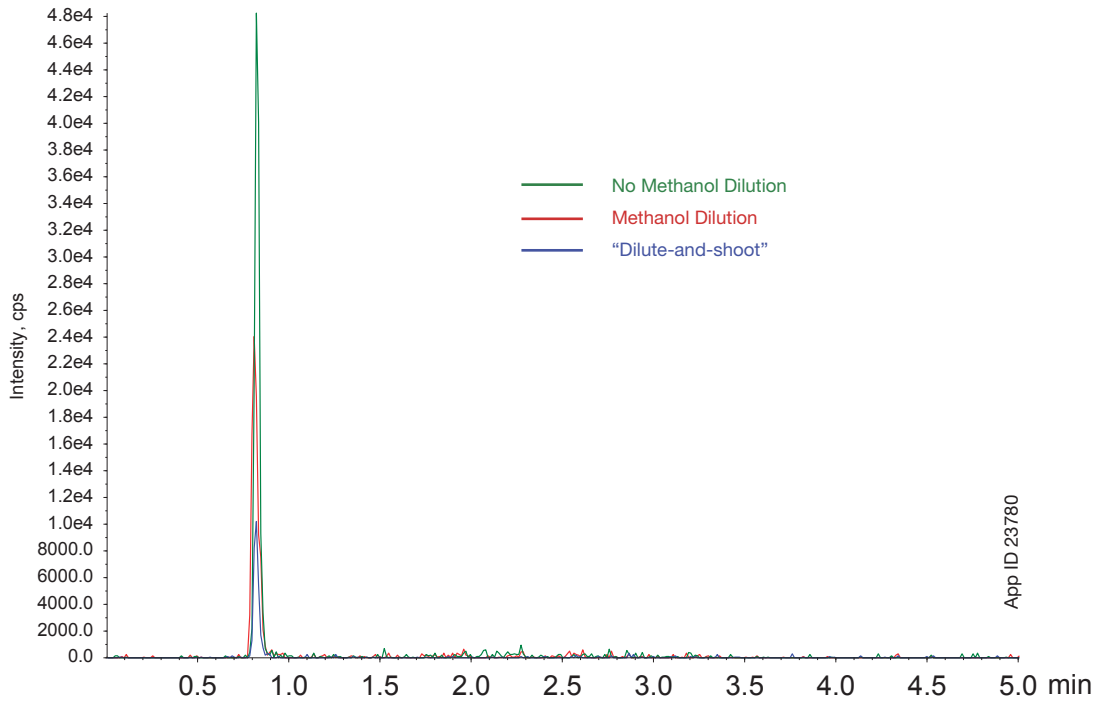


Figure 2.
Lorazepam

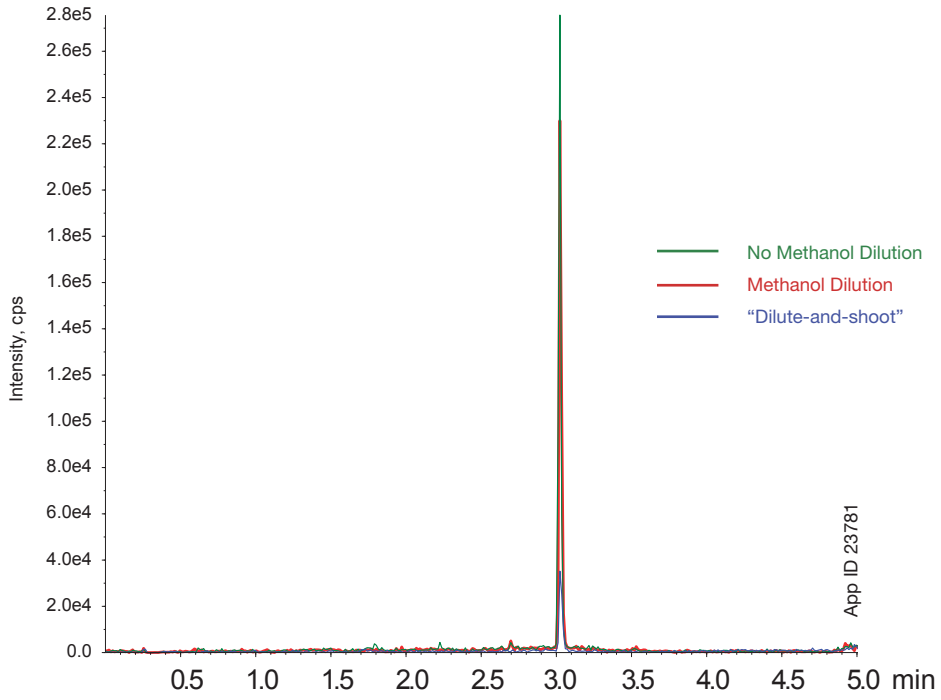


Figure 3.
Buprenorphine

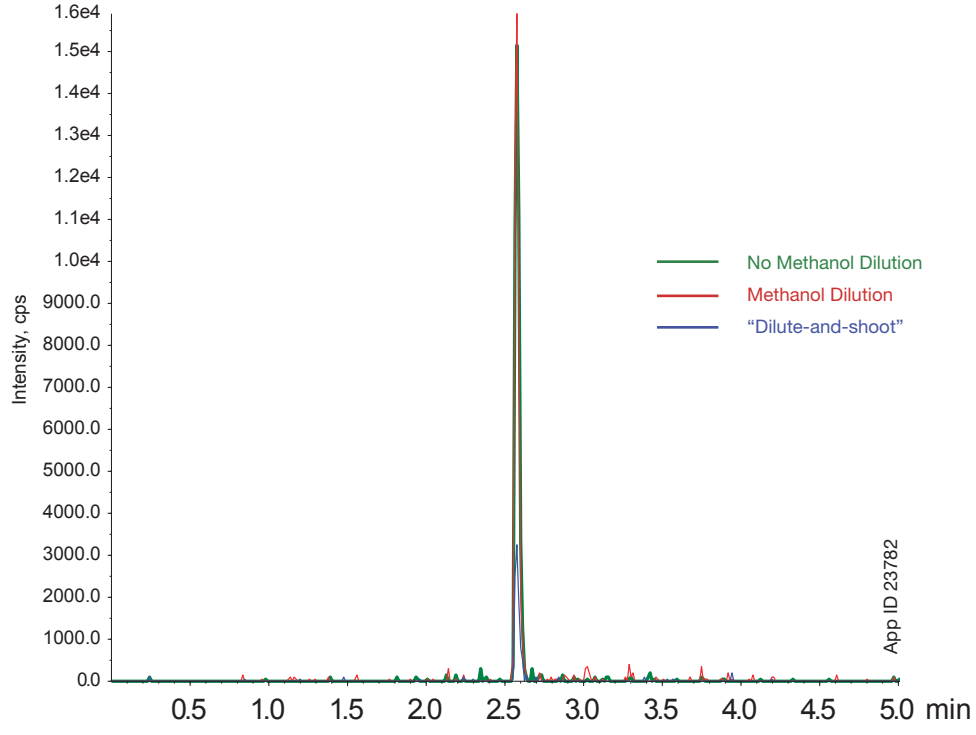


Figure 4.
THC-COOH

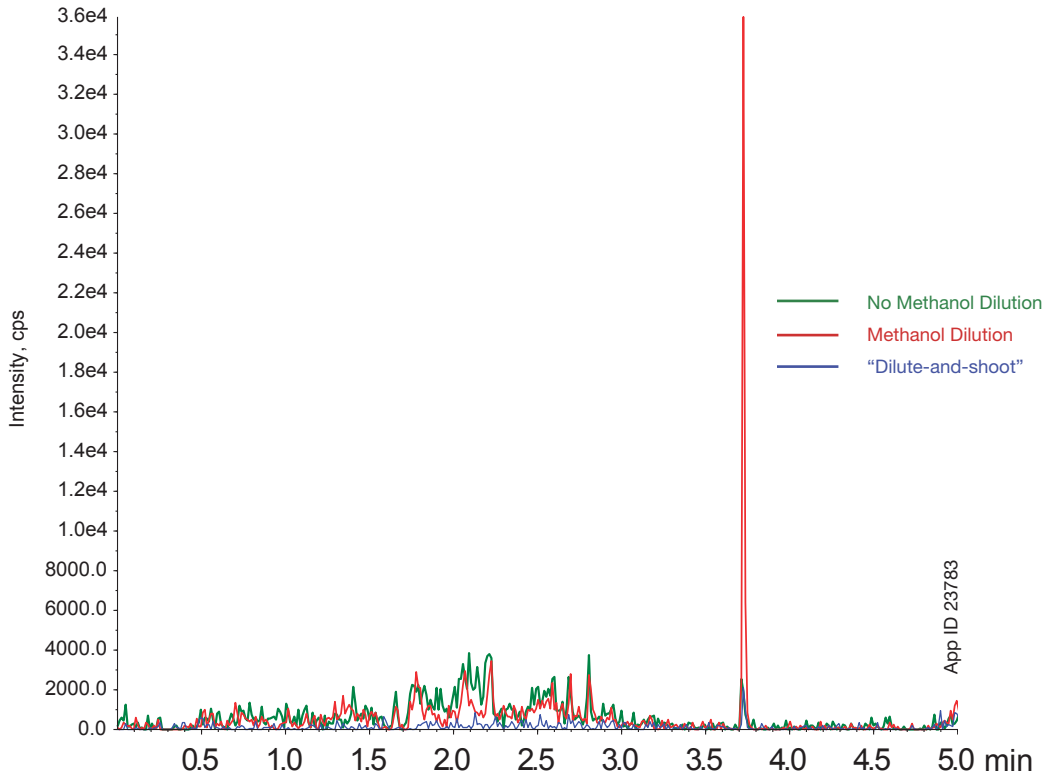


Table 3:
Campbell Protocol 1 Recovery Data with Dilution:

Analyte	Average Recovery %	%CV (n=8)
Benzoylecognine	109	3
Buprenorphine	108	10
Codeine	107	12
Lorazepam	96	7
Methamphetamine	106	3
Morphine	104	6
Norbuprenorphine	109	5
PCP	102	3
THC-COOH	74	8

Table 4:
Campbell Protocol 2 Recovery Data without Dilution:

Analyte	Average Recovery %	%CV (n=8)
Benzoylecognine	78	4
Buprenorphine	92	4
Codeine	99	2
Lorazepam	78	4
Methamphetamine	82	4
Morphine	103	3
Norbuprenorphine	95	4
PCP	85	3
THC-COOH	0	

Conclusion

This study shows that when using IMCSzyme®, it is possible to omit the methanol dilution to achieve better sensitivity for all compounds in this suite, excluding THC-COOH and buprenorphine. Both analytes show a better response with the standard protocol involving a 40% dilution with 0.1% formic acid in methanol. With no methanol dilution, both compounds still provide sensitivity comparable to “dilute-and-shoot”. Therefore, most comprehensive drug research panel suites hydrolyzed by IMCSzyme can be used with a recombinant β-Gone β-Glucuronidase Removal product effectively with or without the dilution that is recommended in the general protocol.

By contrast, when working with the Campbell Enzyme, simply loading the hydrolysate solution onto the plate (no dilution) yields an increase in sensitivity for all compounds relative to the ones prepared by the standard protocol, except for THC-COOH, which shows no recovery without the methanol dilution. When working with the Campbell Enzyme in a suite that contains THC-COOH, a 40% dilution with 0.1% formic acid in methanol is necessary to achieve acceptable results.

Ordering Information

β-Gone™ β-Glucuronidase Removal Products

Part No.	Description	Unit
8B-S139-TAK	1 mL Tubes, Recombinant Enzyme	100/Box
8B-S322-DAK	1 mL Tubes, Non-Recombinant Enzyme	100/Box
8E-S139-TGA	96-Well Plate, Recombinant Enzyme	1/Box
8E-S322-DGA	96-Well Plate, Non-Recombinant Enzyme	1/Box

Kinetex® 2.6 µm Minibore Columns (mm)

					SecurityGuard™ ULTRA Cartridges‡
Phases	30 x 2.1	50 x 2.1	100 x 2.1	150 x 2.1	3/pk
Biphenyl	00A-4622-AN	00B-4622-AN	00D-4622-AN	00F-4622-AN	AJ0-9209 for 2.1 mm ID

Kinetex 2.6 µm MidBore™ Columns (mm)

				SecurityGuard™ ULTRA Cartridges‡
Phases	50 x 3.0	100 x 3.0	150 x 3.0	3/pk
Biphenyl	00B-4622-YO	00D-4622-YO	00F-4622-YO	AJ0-9208 for 3.0 mm ID

‡ SecurityGuard ULTRA Cartridges required holder, Part No.: AJ0-9000.

Vacuum Manifolds

Part No.	Description	Unit
12–Position Vacuum Manifold for Tubes*		
AH0-6023	12-Position Vacuum Manifold Set, complete assembly	ea
24–Position Vacuum Manifold for Tubes*		
AH0-6024	24-Position Vacuum Manifold Set, complete assembly	ea
96-Well Plate Manifold		
AH0-8950	96-Well Plate Manifold, Universal with vacuum gauge	ea

* Manifolds include: Vacuum-tight glass chamber, vacuum gauge assembly, polypropylene lid with gasket, male and female luers and yellow end plugs, stopcock valves, collection rack assemblies, polypropylene needles, lid support legs. Waste container included with 12-position manifold.

Presston™ 100 Positive Pressure Manifold

Part No.	Description	Unit
AH0-9334	Presston 100 Positive Pressure Manifold, 96-Well Plate	ea
AH0-9342	Presston 100 Positive Pressure Manifold, 1 mL Tube Complete Assembly	ea
AH0-9347	Presston 100 Positive Pressure Manifold, 3 mL Tube Complete Assembly	ea
AH0-9343	Presston 100 Positive Pressure Manifold, 6 mL Tube Complete Assembly	ea

The Presston 100 96-Well Positive Pressure Manifold can also process 1, 3, and 6 mL tubes using the following adapter kits

Presston 100 Tube Adapter Kits (for AH0-9334)

Part No.	Description	Unit
AH0-9344	1 mL Tube Adapter Kit	ea
AH0-9345	3 mL Tube Adapter Kit	ea
AH0-9346	6 mL Tube Adapter Kit	ea

WARRANTY Phenomenex warrants that for a period of 12 months following delivery, the Presston 100 Positive Pressure Manifold you have purchased will perform in accordance with the published specifications and will be free from defects in materials or workmanship. In the event that the Presston 100 Positive Pressure Manifold does not meet this warranty, Phenomenex will repair or replace defective parts. Please visit www.phenomenex.com/Presston for complete warranty information.

APPLICATION

Australia
t: +61 (0)2-9428-6444
f: +61 (0)2-9428-6445
auinfo@phenomenex.com

Austria
t: +43 (0)1-319-1301
f: +43 (0)1-319-1300
anfrage@phenomenex.com

Belgium
t: +32 (0)2 503 4015 (French)
t: +32 (0)2 511 8666 (Dutch)
f: +31 (0)30-2383749
beinfo@phenomenex.com

Canada
t: +1 (800) 543-3681
f: +1 (310) 328-7768
info@phenomenex.com

China
t: +86 (0)20 2282-6668
f: +86 (0)20 2809-8130
chinainfo@phenomenex.com

Denmark
t: +45 4824 8048
f: +45 4810 6265
nordicinfo@phenomenex.com

Finland
t: +358 (0)9 4789 0063
f: +45 4810 6265
nordicinfo@phenomenex.com

France
t: +33 (0)1 30 09 21 10
f: +33 (0)1 30 09 21 11
franceinfo@phenomenex.com

Germany
t: +49 (0)6021-58830-0
f: +49 (0)6021-58830-11
anfrage@phenomenex.com

India
t: +91 (0)40-3012 2400
f: +91 (0)40-3012 2411
indiainfo@phenomenex.com

Ireland
t: +353 (0)1 247 5405
f: +44 1625-501796
eireinfo@phenomenex.com

Italy
t: +39 051 6327511
f: +39 051 6327555
italiainfo@phenomenex.com

Luxembourg
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

Mexico
t: 01-800-844-5226
f: 001-310-328-7768
tecnicomx@phenomenex.com

The Netherlands
t: +31 (0)30-2418700
f: +31 (0)30-2383749
nlinfo@phenomenex.com

New Zealand
t: +64 (0)9-4780951
f: +64 (0)9-4780952
nzinfo@phenomenex.com

Norway
t: +47 810 02 005
f: +45 4810 6265
nordicinfo@phenomenex.com

Puerto Rico
t: +1 (800) 541-HPLC
f: +1 (310) 328-7768
info@phenomenex.com

Spain
t: +34 91-413-8613
f: +34 91-413-2290
espinfo@phenomenex.com

Sweden
t: +46 (0)8 611 6950
f: +45 4810 6265
nordicinfo@phenomenex.com

United Kingdom
t: +44 (0)1625-501367
f: +44 (0)1625-501796
ukinfo@phenomenex.com

USA
t: +1 (310) 212-0555
f: +1 (310) 328-7768
info@phenomenex.com

All other countries
Corporate Office USA
t: +1 (310) 212-0555
f: +1 (310) 328-7768
info@phenomenex.com

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