TN-1250

Development of a 2-Step Liraglutide Purification Process on a Single Stationary Phase

Genevieve Hodson[1], Darren Stewardson[1], Olivier Drouet[2], and Bryan Tackett, PhD[1]
[1]Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA
[2]Phenomenex, Parc des Grillons, Bât. 3, 60 route de Sartrouville, 78232 Le Pecq Cedex, France

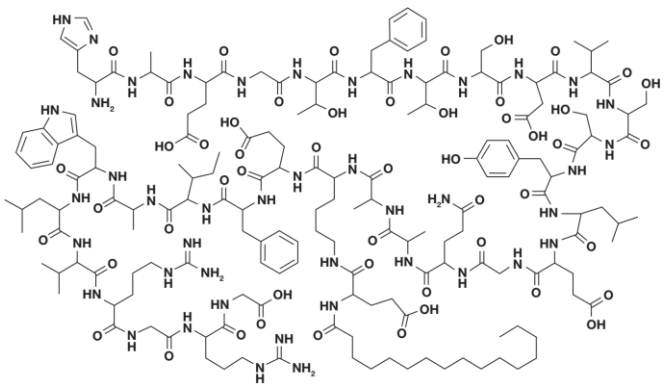
Introduction

Liraglutide is a human glucagon-like peptide-1 (GLP-1) analogue with a 31 amino acids sequence that is 97 % similar to endogenous human GLP-1 (Figure 1). Liraglutide was approved in the EU in 2009, followed closely by approval in the U.S. in 2010. Currently, Liraglutide is commercially available in more than 95 countries and has been approved for the treatment of type 2 diabetes and obesity in adults with related comorbidity.

Manufacturing a commercially successful synthetic peptide API often requires a multistep purification process to achieve the necessary purity, yield and throughput. The first step will typically isolate the desired component from the crude mixture but not achieve the purity level required. A "polishing" step is needed to achieve the desired purity. In order to keep manufacturing costs down, the purification process needs to be optimized. In particular, the number of steps and chromatographic stationary phases used should be kept to a minimum.

Peptides are chains of amino acid monomers linked by amide bonds. Unlike proteins, their smaller size allows certain polarity and ionization properties to be predicted from its amino acid sequence. These properties can provide insight into the selection of chromatographic stationary phases and mobile phases used for the purification process development. A useful attribute of peptide chromatography is that selectivity can be altered by several means. The typical changing of the stationary phase is effective but can be costly for a preparative process. Changing chromatographic selectivity by adjusting the pH, buffer composition or organic modifier can be effective, relatively simple and inexpensive. By modifying these variables, a cost-effective multistep purification process can be developed for the purpose of achieving a high purity peptide product.

Figure 1. Chemical Structure for Liraglutide.



H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys(γ-Glu-palmitoyl)-Glu-Phe-Ile-Ala-Trp-Leu-Val-Arg-Gly-Arg-Gly-OH

Table 1. Characterized Liraglutide Sequence.

Positively charged residues (basic):	Non-polar aliphatic residues:	Aromatic residues:
Arginine	Glycine	Phenylalanine
Histidine	Alanine	Tyrosine
Lysine	Valine	Tryptophan
	Leucine	
	Isoleucine	
Negatively charged residues (acidic):	**Polar non-charged residues:**	
Glutamic Acid	Threonine	
Aspartic Acid	Glutamine	
Serine		

Table 2. Liraglutide Sequence Table.

	Isoelectric Point	Acidic Side Chains	Basic Side Chains	Non-Polar Side Chain	Aromatic Side Chains	Polar (Uncharged) Side Chains
Liraglutide	4.9	5	4	13	4	6
	Neutral pH	pH Sensitivity	Aliphatic Character	Interactions	Polar Interactions	

Results and Discussion

Examination of the Liraglutide sequence of amino acids identified several chemical properties that are useful for chromatographic development (**Table 1**). These properties include aliphatic, pi-pi, and polar interactions. With over a third of the amino acids being nonpolar and aromatic, stationary phases such as phenyl or aliphatic hydrocarbon stationary phases are suitable for this peptide. The isoelectric point for Liraglutide is 4.9 and the amino acid sequence includes 4 acidic and 4 basic side chains. This would indicate that pH could have a significant effect on the chromatography. From the perspective of resolving power, this method development initially evaluated a C18 stationary phase (**Figure 2**). With acidic eluent conditions, sufficient resolution was difficult to obtain between the main component and a significant impurity that eluted just after the main peak. The ionization state of this peptide and impurity was altered by a buffer of Ammonium Bicarbonate, pH to 6.9 with Acetic Acid as the aqueous component. This change in pH altered the chromatographic selectivity and reversed the elution order so this impurity eluted before the main peak. There was also a change in selectivity when using Acetonitrile versus a mixture of Acetonitrile - Alcohol as the organic component. The gradient conditions were adjusted for initial conditions and rate of change.

Organic modifiers were evaluated for their impact for the separation (**Figure 3**). First, 10 % of the Acetonitrile was replaced with Methanol, which provided different selectivity. The amount of Methanol was increased to 20 % but this was still not enough to fully separate the impurity. Therefore, a slightly higher polarity alcohol, Ethanol, was used in place of Methanol as the organic modifier. Good separation was achieved using the combination of Ethanol and Acetonitrile as the organic components, even with an increase in the flow rate.

The most optimal conditions were evaluated with a 0.5 % crude load on a Luna™ C8(3) column and Liraglutide was collected as a series of fractions (**Figure 4**). Not a single fraction collected was able to meet the required 98 % purity. It was determined at this time that a polishing step would be necessary to achieve the level of purity needed for the Liraglutide. The pooled fractions gave a purity of 91 % with a yield of 91 % and was taken forward to the polishing step. The final polishing step was performed on the same column, Luna C8(3) (**Figure 5**). The polishing step had different selectivity for the impurities of Liraglutide since acidic conditions were used with Acetonitrile. Material isolated from the first step methodology was processed with the polishing step. Fractions were collected and a pool of these fractions provided material with a final purity of 98.2 % with a yield of 80 %.

Figure 2. Effect of pH on Post Impurity Separation.

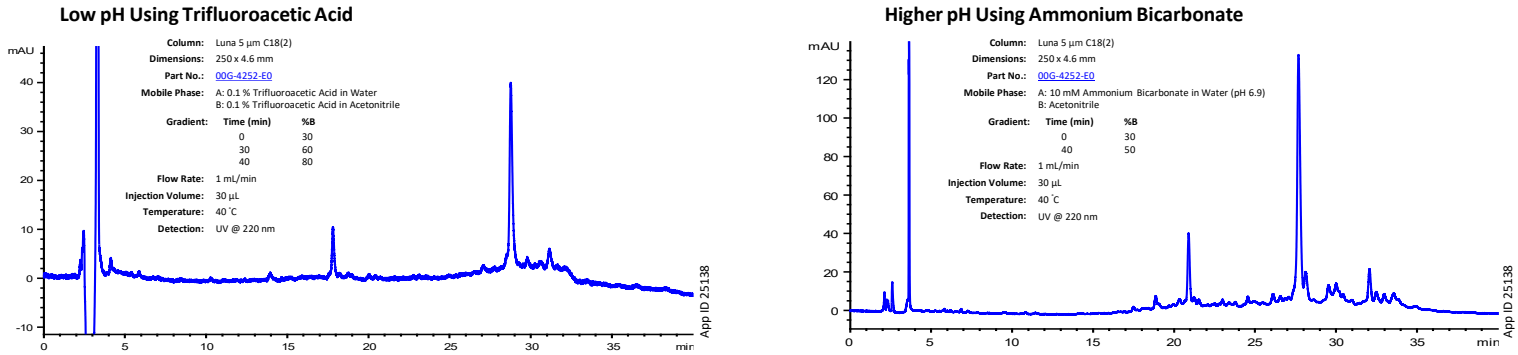


Figure 3 . Effect of Type of Organic Modifiers on Separation.

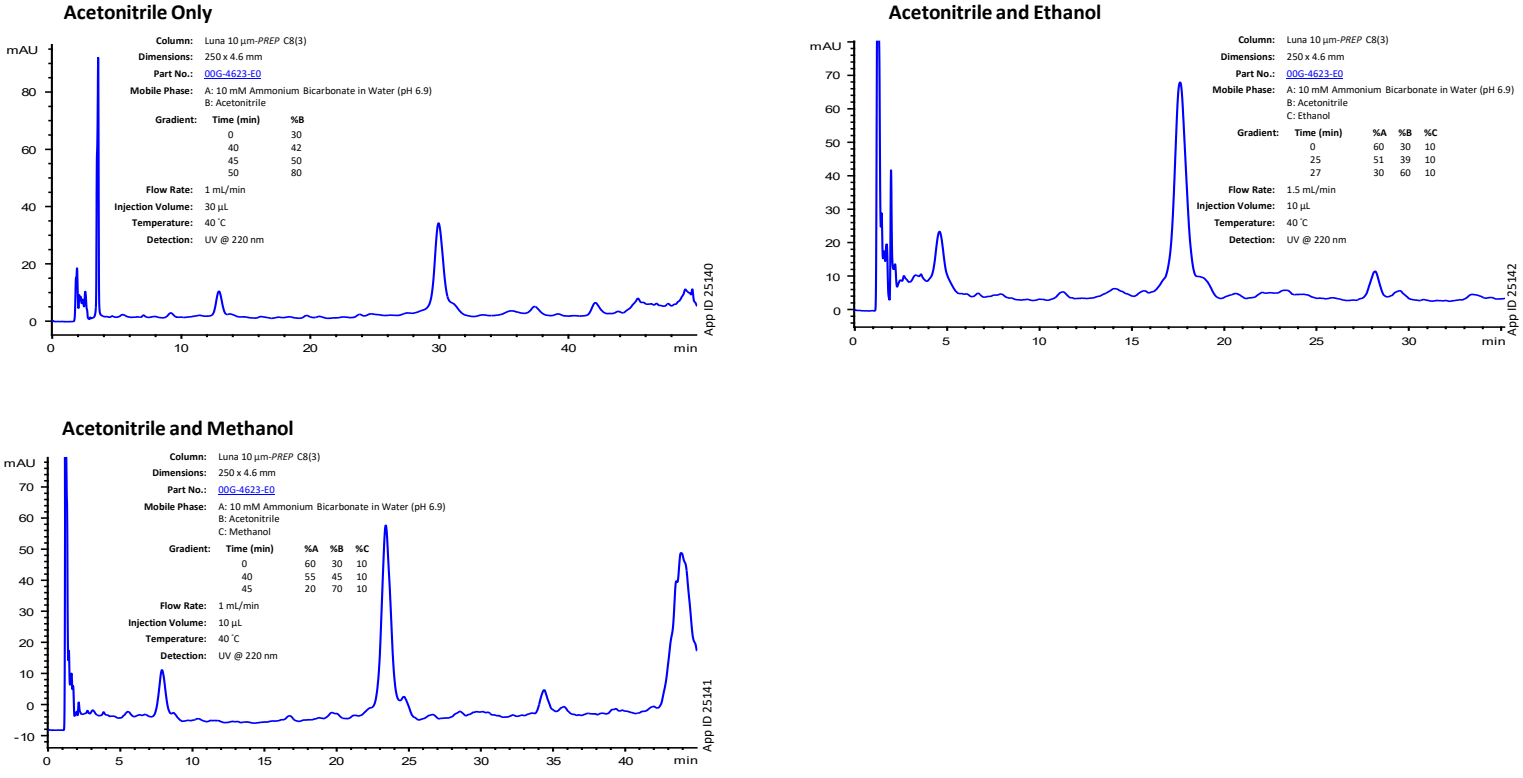
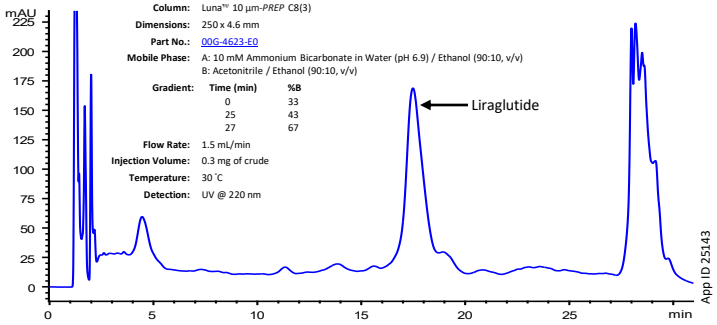
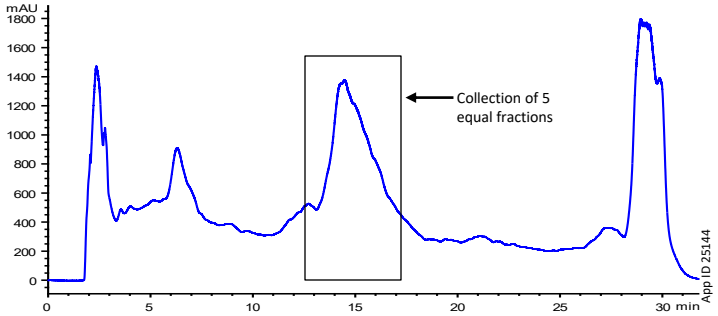


Figure 4 . Step 1 Methodology, Loading Chromatogram, Fraction Analysis, and Pooled Results.

Prep Methodology for Crude Liraglutide Sample

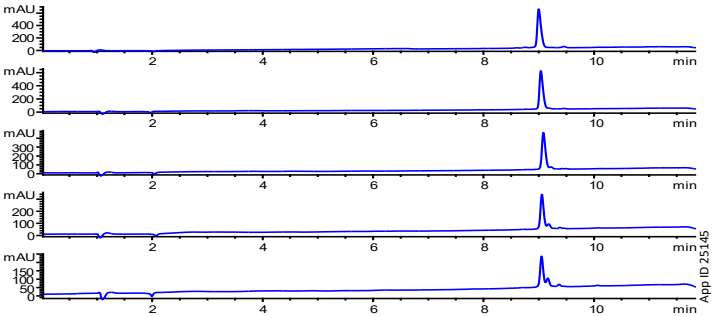


Loading Chromatogram: 0.5 % Loading



Fraction %	1	2	3	4	5
% Purity	94.7	95.9	91.6	81.3	74.4
Yield	28	25	18	12	7

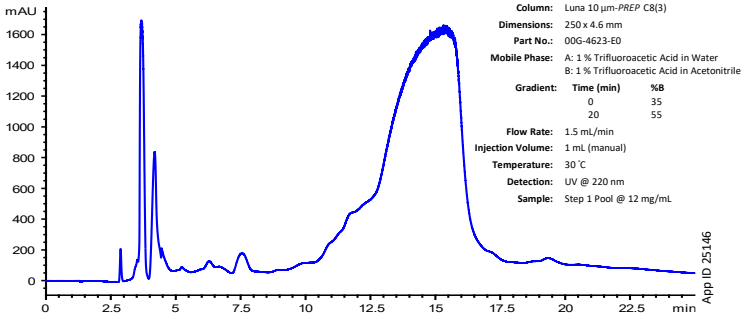
Collected Fractions 1-5



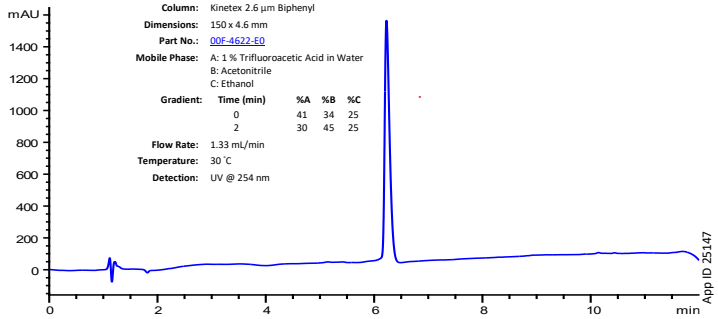
Column: Kinetex™ 5 µm Biphenyl
Dimensions: 150 x 4.6 mm
Part No.: 00F-4627-E0
Mobile Phase: A: 1 % Trifluoroacetic Acid in Water
B: 1 % Trifluoroacetic Acid in Methanol
Gradient: Time (min) %B
0 50
10 60
Flow Rate: 1 mL/min
Injection Volume: 0.3 mg of crude
Temperature: 30 ˚C
Detection: UV @ 254 nm

Figure 5.

Polishing Step



Example Fraction



Conclusions

A 2-step process was successfully developed for the purification of Liraglutide. Both steps used Luna 10µm-*PREP* C8(3) as the stationary phase. The crude Liraglutide sample for this study had an initial purity of 30 %. The first step upgraded the purity to 91 %. A second polishing step was needed and elevated the purity to the desired 98.5 %. During the development process, the use of a single stationary phase was a primary objective to minimize the overall cost of the methodology.

The Luna 10 µm-*PREP* C8(3) column was shown to be effective in the purification of Liraglutide as part of a multi-step process. This phase is available in prepacked preparative HPLC columns and in large quantities for packing in dynamic axial compression columns. The viability of Gemini™ C8(3) media was also confirmed to be suitable with this final optimized methodology. This stationary phase is suitable for high pH applications and has shown good stability for caustic washes (data not shown). This can be significant in a large-scale purifications with synthetic processes that are susceptible to aggregation as well as other impurities which may remain on the column after the purification.

Kinetex™ Ordering Information

2.6 µm Analytical Columns (mm)							SecurityGuard™ ULTRA Cartridges*
Phases	**30 x 4.6**	**50 x 4.6**	**75 x 4.6**	**100 x 4.6**	**150 x 4.6**	**250 x 4.6**	**3/pk**
EVO C18	00A-4725-E0	00B-4725-E0	—	00D-4725-E0	00F-4725-E0	00G-4725-E0	AJ0-9296
PS C18	00A-4780-E0	00B-4780-E0	—	00D-4780-E0	00F-4780-E0	00G-4780-E0	AJ0-8949
Polar C18	00A-4759-E0	00B-4759-E0	—	00D-4759-E0	00F-4759-E0	—	AJ0-9530
Biphenyl	—	00B-4622-E0	—	00D-4622-E0	00F-4622-E0	—	AJ0-9207
XB-C18	—	00B-4496-E0	00C-4496-E0	00D-4496-E0	00F-4496-E0	—	AJ0-8768
C18	00A-4462-E0	00B-4462-E0	00C-4462-E0	00D-4462-E0	00F-4462-E0	—	AJ0-8768
C8	—	00B-4497-E0	00C-4497-E0	00D-4497-E0	00F-4497-E0	—	AJ0-8770
HILIC	—	00B-4461-E0	00C-4461-E0	00D-4461-E0	00F-4461-E0	—	AJ0-8772
Phenyl-Hexyl	—	00B-4495-E0	00C-4495-E0	00D-4495-E0	00F-4495-E0	—	AJ0-8774
F5	00A-4723-E0	00B-4723-E0	—	00D-4723-E0	00F-4723-E0	—	AJ0-9320

for 4.6 mm ID

5 µm Analytical Columns (mm)					SecurityGuard ULTRA Cartridges*
Phases	**50 x 4.6**	**100 x 4.6**	**150 x 4.6**	**250 x 4.6**	**3/pk**
EVO C18	00B-4633-E0	00D-4633-E0	00F-4633-E0	00G-4633-E0	AJ0-9296
F5	00B-4724-E0	00D-4724-E0	00F-4724-E0	00G-4724-E0	AJ0-9320
Biphenyl	00B-4627-E0	00D-4627-E0	00F-4627-E0	00G-4627-E0	AJ0-9207
XB-C18	00B-4605-E0	00D-4605-E0	00F-4605-E0	00G-4605-E0	AJ0-8768
C18	00B-4601-E0	00D-4601-E0	00F-4601-E0	00G-4601-E0	AJ0-8768
C8	00B-4608-E0	00D-4608-E0	00F-4608-E0	00G-4608-E0	AJ0-8770
Phenyl-Hexyl	00B-4603-E0	00D-4603-E0	00F-4603-E0	00G-4603-E0	AJ0-8774
HILIC	—	—	00F-4606-E0	00G-4606-E0	AJ0-8772

for 4.6 mm ID

*SecurityGuard ULTRA Cartridges require holder, Part No.: AJ0-9000

Luna™ Ordering Information

5 µm Analytical Columns (mm)							SecurityGuard Cartridges (mm)
Phases	**30 x 4.6**	**50 x 4.6**	**75 x 4.6**	**100 x 4.6**	**150 x 4.6**	**250 x 4.6**	**4 x 3.0** /10pk**
Silica(2)	—	00B-4274-E0	—	00D-4274-E0	00F-4274-E0	00G-4274-E0	AJ0-4348
C5	—	00B-4043-E0	—	00D-4043-E0	00F-4043-E0	00G-4043-E0	AJ0-4293
C8(2)	00A-4249-E0	00B-4249-E0	00C-4249-E0	00D-4249-E0	00F-4249-E0	00G-4249-E0	AJ0-4290
C18(2)	00A-4252-E0	00B-4252-E0	00C-4252-E0	00D-4252-E0	00F-4252-E0	00G-4252-E0	AJ0-4287
CN	00A-4255-E0	00B-4255-E0	00C-4255-E0	00D-4255-E0	00F-4255-E0	00G-4255-E0	AJ0-4305
Phenyl-Hexyl	00A-4257-E0	00B-4257-E0	—	00D-4257-E0	00F-4257-E0	00G-4257-E0	AJ0-4351
NH_2	—	00B-4378-E0	—	00D-4378-E0	00F-4378-E0	00G-4378-E0	AJ0-4302
SCX	—	00B-4398-E0	—	00D-4398-E0	00F-4398-E0	00G-4398-E0	AJ0-4308
HILIC	—	—	—	00D-4450-E0	00F-4450-E0	00G-4450-E0	AJ0-8329
PFP(2)	—	00B-4448-E0	—	—	—	—	AJ0-8327

for ID: 3.2 – 8.0 mm

**SecurityGuard Analytical Cartridges require holder, Part No.: KJ0-4282

10 µm-*PREP* Columns (mm)		
Phases	**250 x 4.6**	**250 x 10**
Silica(3)	00G-4617-E0	00G-4617-N0
C8(3)	00G-4623-E0	00G4623-N0
C18(3)	00G-4616-E0	00G-4616-N0

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*

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