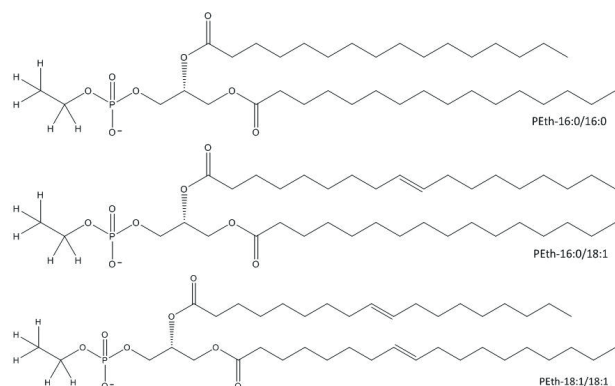


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# Extraction of Phosphatidylethanol (PEth) Species from Whole Blood Using ISOLUTE® SLE+ Prior to HPLC-MS/MS Analysis



**Figure 1.** Chemical structures of three common PEth species.

## Introduction

Phosphatidylethanol is an alcohol biomarker with a high degree of specificity; blood concentration of PEth correlates to the amount of alcohol consumed. This application note describes the extraction of 3 common species of PEth from whole blood using ISOLUTE® SLE+ supported liquid extraction prior to HPLC-MS/MS analysis.

ISOLUTE SLE+ supported liquid extraction fixed-well plates offer an efficient alternative to traditional liquid-liquid extraction (LLE) for bioanalytical sample preparation, providing high analyte recoveries, no emulsion formation, and significantly reduced sample preparation time.

This simple sample preparation procedure produces clean extracts, good recoveries with low RSD, and LOQ from 20 ng mL<sup>-1</sup>. This method can be automated using Biotage® Extrahera™, see appendix for details.



## Analytes

PEth is a group of phospholipids comprising a phosphoethanol head group and 2 fatty acid tails of varying length and saturation. This application note covers 3 commonly occurring species:

- » 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanol (PEth-16:0/16:0)
- » 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol (PEth-16:0/18:1)
- » 1,2-dioleoyl-*sn*-glycero-3-phosphoethanol (PEth-18:1/18:1)
- » *D*<sub>5</sub>-1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanol (*D*<sub>5</sub>-PEth-16:0/18:1) was used as an internal standard.

See Figure 1 for chemical structures.

## Sample Preparation Procedure

### Format

ISOLUTE® SLE+ 200 µL supported liquid extraction plate, part number 820-0200-P01.

### Sample Pretreatment

To 20 µL of whole blood, add 300 µL of 6.25% (v/v) aqueous ammonium hydroxide in 30% aqueous methanol. Add an appropriate amount of internal standard separately or mix internal standard into the ammonium hydroxide pre-treatment solution prior to adding to sample. Mix thoroughly and allow to equilibrate.

### Sample Loading

Load 140 µL of the pre-treated whole blood into each well of the ISOLUTE SLE+ plate (equivalent to 8.75 µL whole blood). Ensure the surface of the well frit is completely covered by the pre-treated sample. Using a Biotage® PRESSURE+96 Positive Pressure Manifold, apply 2–5 psi of pressure to load samples onto the sorbent. Wait 5 minutes for the sample to equilibrate on the sorbent.

### Analyte Extraction

Apply 750 µL of ethyl acetate and allow to flow under gravity for 5 minutes. Apply pressure (5–10 seconds) to remove any remaining extraction solvent.

### Post Elution and Reconstitution

Dry the extract in a stream of air or nitrogen using a Biotage® SPE Dry 96 (40 °C at 60 L min<sup>-1</sup>). Reconstitute the extracts with 150 µL mobile phase A:B (85:15 v/v). Mix thoroughly.

## HPLC Conditions

### Instrument

Waters Alliance 2795 HPLC with a 20  $\mu$ L loop.

### Column

Agilent Poroshell 120 EC-C8 2.1 x 50 mm, 2.7  $\mu$ m analytical column; and Poroshell 120 EC-C8 2.1 x 5 mm, 2.7  $\mu$ m UHPLC guard column.

### Mobile Phase

**A:** acetonitrile : 2 mM ammonium acetate (aq) 80:20 v/v;

**B:** propan-2-ol.

### Flow Rate

0.25 mL min<sup>-1</sup>.

**Table 1.** Gradient Conditions.

Time	% A	% B	Curve
0.00	85	15	1
0.40	85	15	1
3.50	15	85	6
3.51	5	95	6
5.50	5	95	6
6.00	85	15	6
8.90	85	15	1

### Injection Volume

10  $\mu$ L (partial loop)

### Sample Temperature

12 °C

### Column Temperature

Room temperature

## Mass Spectrometry Conditions

### Instrument

Waters Ultima Pt triple quadrupole mass spectrometer using electrospray ionization.

### Capillary Voltage

3.2 kV

### Desolvation Temperature

350 °C

### Ion Source Temperature

100 °C

Negative ions acquired in multiple reaction monitoring (MRM) mode:

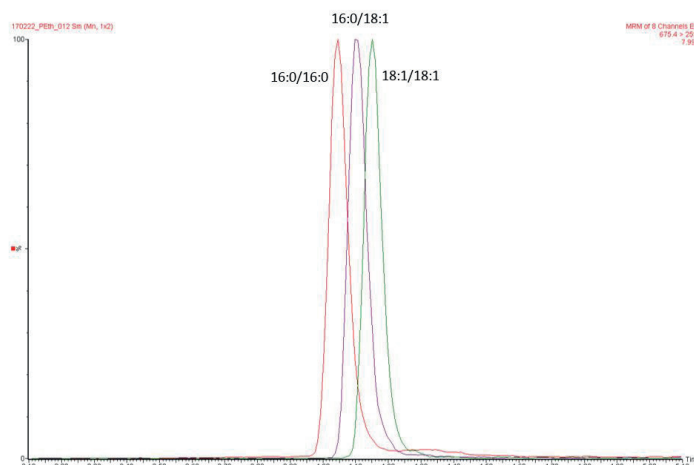
**Table 2.** MRM Conditions.

Compound	MRM Transition	Cone Voltage (V)	Collision Energy (eV)
PEth-16:0/16:0	675.4 > 255.2	35	31
PEth-16:0/18:1	701.4 > 281.2	35	34
PEth-18:1/18:1	727.5 > 281.2	35	32
D <sub>5</sub> -PEth-16:0/18:1 (IS)	706.5 > 281.2	35	32

## Results and Discussion

### Chromatography

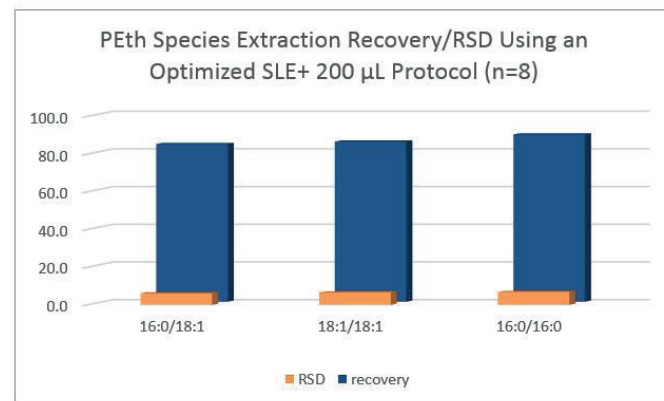
PEth species were chromatographed with a superficially porous C8 column using dilute ammonium acetate (aq) / acetonitrile and propan-2-ol. The overlaid extracted ion chromatogram (EIC) in Figure 2 demonstrates partial separation of the three PEth species was achieved. No inter-species contributions were observed in the EICs, demonstrating the HPLC-MS/MS method is capable of distinguishing between common PEth species.



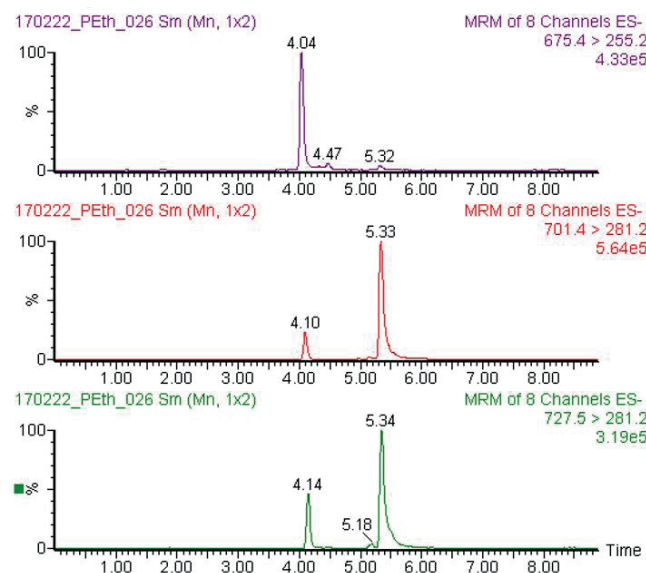
**Figure 2.** PEth Standards in Solvent Equivalent to 200 ng mL<sup>-1</sup> in Whole Blood.

### Recovery and Repeatability

Method performance was assessed by spiking whole blood with three PEth species plus internal standard at 200 ng mL<sup>-1</sup>, equivalent to 1.75 ng when extracting 140  $\mu$ L of pre-treated whole blood. Recovery was determined relative to fortified blanks containing the same amount of extracted matrix. Recoveries for three PEth species were between 84% and 89% using an optimized ISOLUTE® SLE+ 200  $\mu$ L protocol (Figure 3). Extraction RSDs were between 5% and 6% (Figure 3). Representative EICs of 3 PEth species extracted from 20  $\mu$ L whole blood spiked at 200 ng mL<sup>-1</sup> are free from interfering peaks (Figure 4).



**Figure 3.** Percentage Recovery and RSD of PEth Species.



**Figure 4.** Extracted Ion Chromatograms of PEth Species (16:0/16:0, 16:0/18:1, 18:1/18:1) 20  $\mu$ L Whole Blood Spiked at 200 ng mL<sup>-1</sup>

## Calibration Curves

Calibration curves were constructed by spiking whole blood from 20 ng mL<sup>-1</sup> to 20  $\mu$ g mL<sup>-1</sup> for each PEth species prior to extraction; the internal standard was spiked at 2  $\mu$ g mL<sup>-1</sup>. Coefficient of determination ( $r^2$ ) values are demonstrated greater than 0.990 for each PEth species using the optimized extraction protocol. Representative curves are shown in Figure 5.

LOQ was estimated from the calibration curves where the signal/noise ratio was greater than 10:1. S/N values, estimated LOQ, coefficients of determination and precision RSDs are presented in Table 3.

**Table 3.** Lower Limits of Quantitation (LLOQ) using optimized ISOLUTE® SLE+ extraction protocol.

PEth Species	LOQ, ng mL <sup>-1</sup> (S/N)	LOQ, nmol L <sup>-1</sup>	$r^2$	Precision % RSD (n=8)
16:0/16:0	20 (20)	29.5	0.995	6.1
16:0/18:1	20 (12)	28.4	0.994	6.6
18:1/18:1	20 (14)	27.4	0.994	6.8

## Additional Notes

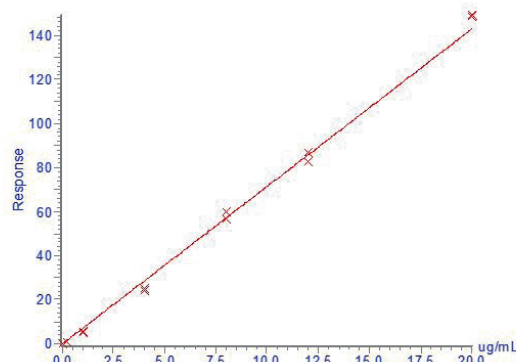
Unless specified, all reagents and solvents are HPLC-grade.

6.25% ammonium hydroxide (aq) / 30% methanol (aq): add 6.25 mL of concentrated ammonium hydroxide (28–30%) and 30 mL of methanol to 63.75 mL 18.2 MΩ cm water.

**Aqueous mobile phase (A):** dissolve 280 mg LC-MS grade ammonium acetate in 200 mL 18.2 MΩ cm water, add to 800 mL LC-MS grade acetonitrile and mix thoroughly.

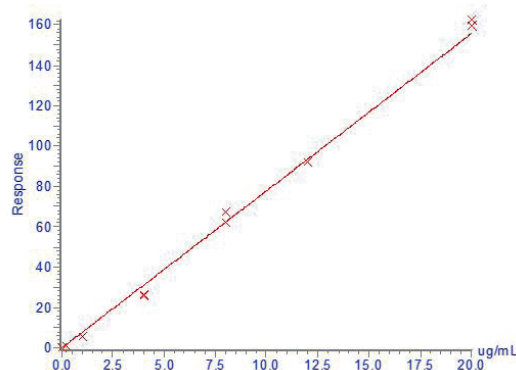
**Organic Mobile Phase (B):** Use an appropriate volume of LC-MS grade propan-2-ol.

Compound name: 16:0/18:1 701.4  
Correlation coefficient:  $r = 0.997657$ ,  $r^2 = 0.995320$   
Calibration curve:  $7.14739 \times x + -0.048429$   
Response type: Internal Std (Ref 1), Area \* (IS Conc. / IS Area)  
Curve type: Linear, Origin: Exclude, Weighting: 1/x Axis trans: None



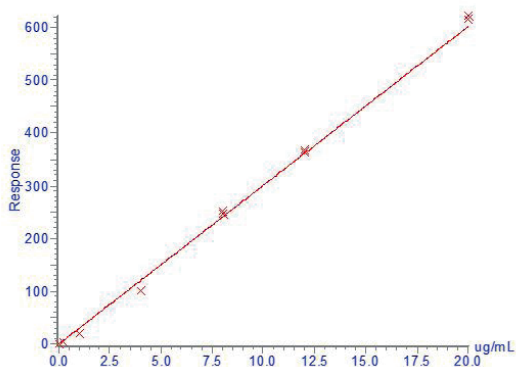
**a) 16:0/18:1**

Compound name: 18:1/18:1 727.5  
Correlation coefficient:  $r = 0.997486$ ,  $r^2 = 0.994978$   
Calibration curve:  $7.78183 \times x + -0.0543766$   
Response type: Internal Std (Ref 1), Area \* (IS Conc. / IS Area)  
Curve type: Linear, Origin: Exclude, Weighting: 1/x Axis trans: None



**b) 18:1/18:1**

Compound name: 16:0/16:0 675.4  
Correlation coefficient:  $r = 0.996908$ ,  $r^2 = 0.993826$   
Calibration curve:  $30.1208 \times x + -0.438999$   
Response type: Internal Std (Ref 1), Area \* (IS Conc. / IS Area)  
Curve type: Linear, Origin: Exclude, Weighting: 1/x Axis trans: None



**c) 16:0/16:0**

**Figure 4.** Representative PEth Species Calibration Curves from 20 ng mL<sup>-1</sup> to 20  $\mu$ g mL<sup>-1</sup>

## Ordering Information

Part Number	Description	Quantity
<b>820-0200-P01</b>	ISOLUTE® SLE+ 200 µL Supported Liquid Extraction Plate	1
<b>For Manual Processing</b>		
<b>PPM-96</b>	Biotage® PRESSURE+ 96 Positive Pressure Manifold	1
<b>For Automated Processing</b>		
<b>414001</b>	Biotage® Extrahera	1
<b>Rack and Reservoir Options</b>		
<b>413991SP</b>	Solvent Rack (25 mL)	1
<b>414045SP</b>	Solvent Reservoir (25 mL)	1
<b>415560SP</b>	Solvent Rack (100 mL)	1
<b>414214SP</b>	Solvent Reservoir (100 mL)	1
<b>Evaporation</b>		
<b>SD-9600-DHS-EU</b>	Biotage® SPE Dry 96 Sample Evaporator 220/240V	1
<b>SD-9600-DHS-NA</b>	Biotage® SPE Dry 96 Sample Evaporator 100/120V	1

# Appendix

## Biotage® Extrahera™ Settings

The method described in this application note was automated on the Biotage® Extrahera™, using ISOLUTE® SLE+ 200 µL plates. Method performance was comparable to manual processing: recovery 73% to 77%, RSD 4.0% to 4.4%,  $r^2$  0.995 to 0.997. This appendix contains the software settings required to configure Extrahera to run this method.

Using this automated procedure, 96 samples can be processed in 22 minutes 33 secs.

An importable electronic copy of this method for Extrahera can be downloaded from [www.biotage.com](http://www.biotage.com)

**Method Name:** TBD  
**Sample Plate/Rack:** 2 mL Sample Plate, 96  
**Extraction Media:** PEth SLE 200



## Settings

### "Sample" Tab


**Sample Type:** PEth Sample  
**Starting Sample Volume (µL):** 20  
**Reuse sample tips?** No  
**Method comment:**

1. If needed, centrifuge the sample plate briefly to collect the sample at the bottom of the well.
2. Prepare internal standard at an appropriate concentration in 6.25% ammonia 30% methanol. We recommend using Solvent Reservoir 6 in Accessory Position 6. An additional Solvent Rack and Solvent Reservoir will be needed\*\*.

Alternatively, use a Solvent Reservoir from position 1 to 5. For this option, a larger volume of solvent + internal standard is needed and the Solvent Inlet Lines will need flushing after use.

\*\* see ordering information on page 4 for suitable solvent rack and reservoir options.

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## Screenshot

**< Cancel Edit SLE Method - PEth Whole Blood Save >**

Method name: PEth Whole Blood Sample plate/rack: 2 mL Sample Plate, 96 Extraction media: PEth SLE 200

**Pre-treatment** **Sample** **Pretreatment** **Load** **Elution**

Pre-treatment: ☒ On

Load: ☒ On

Elution: ☒ On

Number of steps: 1

Solvent: 6.25% NH<sub>4</sub>OH 30% met...

Volume (μL): 300

Mix number of times: 0 Mix volume (μL): 0

Wait time (min): 0

Pause after last step? ☐ No Dispose solvent tips after each step? ☐ No

**< Cancel Edit SLE Method - PEth Whole Blood Save >**

Method name: PEth Whole Blood Sample plate/rack: 2 mL Sample Plate, 96 Extraction media: PEth SLE 200

**Pre-treatment** **Sample** **Pretreatment** **Load** **Elution**

Pre-treatment: ☒ On

Load: ☒ On

Elution: ☒ On

Volume (μL): 140

Air push time (s): 2 Wait time (min): 5

Premix? ☒ Yes Number of times: 3

Pause after each load? ☐ No Collect in position: D (Wa...)

## Settings

Pre-treatment	Activated
No. of steps	1
Pause after last step	No
Dispose tips after last step	No

Solvent
1 6.25% NH <sub>4</sub> OH 30% methanol in water
2
3
4

	1	2	3	4
Volume (μL)	300			
Mix number of times	0			
Mix volume (μL)	0			
Wait time (min)	0			

Load	Activated
Pause after each load	No
Volume (μL)	140
Collect in position	D
Air Push Time (s)	2
Wait time (min)	5
Premix	Yes
Number of times	3

**Edit SLE Method – PEth Whole Blood**

Method name: **PEth Whole Blood** Sample plate/rack: **2 mL Sample Plate, 96** Extraction media: **PEth SLE 200**

**Elution**

Number of steps: **1**

Air push after last elution? **Yes** Air push time (s): **10** Dispose solvent tip after each step? **No**

Solvent: **Ethyl Acetate**

Volume (µL): **750** Collect in position: **A**

Wait time (min): **5** Advanced pressure settings: **Edit...**

Repeat (number of times): **1** Pause after this step? **No**

Elution	Activated
No. of steps	1
Air push after last elution	Yes
Air push time (s)	10
Dispose tips after each step	No

Solvent
1 Ethyl Acetate
2 MTBE
3
4

	1	2	3	4
Volume (µL)	750			
Collect in position	A			
Wait time (min)	5			
Repeat	1			
Pause	No			

#### 'Advanced Settings'

## Solvent Properties

Solvent Description
1 6.25% NH4OH 30% methanol in water
2 Ethyl Acetate
3
4
5
6
7
8
9
10



Solvent	1	2	3	4	5	6	7	8	9	10
<b>Reservoir Type</b>	<b>Refillable</b>		<b>Non Refillable</b>							
Capacity	N/A	N/A								
Aspiration flow rate (mL/min)	10	10								
Dispense flow rate (mL/min)	20	10								
Lower air gap flow rate (mL/min)	20	20								
Lower air gap volume (µL)	5	5								
Upper air gap flow rate (mL/min)	120	120								
Upper air gap volume (µL)	100	100								
Upper air gap dispense pause	300	300								
Conditioning?	Yes	Yes								
Conditioning number of times	3	2								
Conditioning flow rate (mL/min)	20	10								
Conditioning volume (%)	100	100								
Aspirate post dispense	Yes	Yes								
Chlorinated	No	Yes								
Serial dispense	No	No								

**Edit Sample - PEth Sample**

**Sample**

Sample name  
PEth Sample

Sample description

Aspiration flow rate (mL/min)  
10

Dispense flow rate (mL/min)  
5

**Air Gap**

Lower air gap flow rate (mL/min)  
20

Lower air gap volume (µL)  
5

Upper air gap flow rate (mL/min)  
10

Upper air gap volume (µL)  
500

Upper air gap dispense pause (ms)  
0

**Aspirate**

Aspirate post dispense?  
☐ No

**"Sample" Screen**

Sample name	PEth Sample
Sample description	For 1 mL SLE columns
Aspiration flow rate (mL/min)	10
Dispense flow rate (mL/min)	5
Lower air gap flow rate (mL/min)	20
Lower air gap volume (µL)	5
Upper air gap flow rate (mL/min)	100
Upper air gap volume (µL)	500
Upper air gap dispense pause	0
Aspirate post dispense	No

**Edit Extraction Media - PEth SLE 200**

**Extraction Media**

Name  
PEth SLE 200

Manufacturer  
Biotage

Part number  
820-0200-P01

Capacity volume (µL)  
0

Format  
96

Comment

**Pipetting Height**

Solvent dispensation height (mm)  
-128.0

Sample dispensation height (mm)  
-148.0

Aspiration height (mm)  
-145.0

[Tune Pipetting Heights...](#)

**"Extraction Media" Screen**

Name	PEth SLE 200
Manufacturer	Biotage
Part number	820-0200-P01
Capacity volume (µL)	0
Format	96
Solvent dispensation height (mm)	-128.0
Sample dispensation height (mm)	-148.0
Aspiration height (mm)	-145.0
Comment	

**Edit Sample Plate/Rack - 2 mL Sample Plate, 96**

**Sample Plate/Rack**

Name  
2 mL Sample Plate, 96

Capacity volume (µL)  
1800

Format  
96

**Pipetting Height**

Aspiration height (mm)  
-162.0

Pretreatment dispensation height (mm)  
-128.0

[Tune Pipetting Heights...](#)

**"Sample Plate/Rack" Screen**

Name	2 mL Sample Plate, 96
Capacity volume (µL)	1800
Format	96
Aspiration height (mm)	-162.0
Pre-treatment dispensation height (mm)	-128.0

< Cancel	Edit Pipette Tip - 1000 µL Biotage tip	Save >
<div>Pipette Tip</div> <div>Name</div> <div>1000 µL Biotage tip</div> <div>Manufacturer</div> <div>Biotage</div> <div>Part number</div> <div>414141</div> <div>Capacity (µL)</div> <div>1000</div> <div>Length (mm)</div> <div>95</div>		

**"Pipette tip" Screen**

Name	1000 µL Biotage Tip
Manufacturer	Biotage
Part number	414141
Capacity (µL)	1000
Length (mm)	95

**EUROPE**

Main Office: +46 18 565900  
Toll Free: +800 18 565710  
Fax: +46 18 591922  
Order Tel: +46 18 565710  
Order Fax: +46 18 565705  
order@biotage.com  
Support Tel: +46 18 56 59 11  
Support Fax: + 46 18 56 57 11  
eu-1-pointsupport@biotage.com

**NORTH & LATIN AMERICA**

Main Office: +1 704 654 4900  
Toll Free: +1 800 446 4752  
Fax: +1 704 654 4917  
Order Tel: +1 704 654 4900  
Order Fax: +1 434 296 8217  
ordermailbox@biotage.com  
Support Tel: +1 800 446 4752  
Outside US: +1 704 654 4900  
us-1-pointsupport@biotage.com

**JAPAN**

Tel: +81 3 5627 3123  
Fax: +81 3 5627 3121  
jp\_order@biotage.com  
jp-1-pointsupport@biotage.com

**CHINA**

Tel: +86 21 2898 6655  
Fax: +86 21 2898 6153  
cn\_order@biotage.com  
cn-1-pointsupport@biotage.com

**KOREA**

Tel: + 82 31 706 8500  
Fax: + 82 31 706 8510  
korea\_info@biotage.com  
kr-1-pointsupport@biotage.com

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