metanomics health

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Absolute*IDQ*® Kit

User Manual p400 HR

UM-p400-HR-Thermo-3

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Absolute*IDQ*® p400 HR Kit Table of Contents

Absolute*IDQ*® p400 HR Kit for Thermo Scientific™ Q Exactive™

The information in this manual is subject to change without notice and should not be construed as a commitment by BIOCRATES® Life Sciences AG to assume responsibility for any errors that may appear. This manual is believed to be accurate for preparing the Absolute*IDQ*® p400 HR Kit and for using the Met*IDQ*™ Software. **The Absolute*IDQ*® p400 HR Kit is for research use only and not for use in diagnostic procedures.** While every precaution has been taken in the preparation of this manual, BIOCRATES® Life Sciences AG shall not be liable for punitive, incidental, or consequential damage in connection with or arising from the use of this manual.

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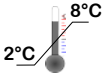
Symbols

Symbol	Description
LOT	LOT No.
PROD	product No.
REF	ordering No.
	expiration date
	store at room temperature
	store in freezer
	store in refrigerator
	note: pay attention to the user manual
	manufacturer
	not for reuse
	attention
	comment

Symbol	*Description*
	important information
	acidic
	irritant
	highly inflammable
	toxic

1 About the Kit

Absolute*IDQ*® p400 HR Kit

The Absolute*IDQ*® p400 HR Kit is based on a combination of several experimental steps. Therefore, **it is required to read this user manual in detail** before proceeding with the analysis of the kit. If you need support, please find the contact details on the last page of this manual. Here you also find links to our video tutorials and FAQ system.

The Absolute*IDQ*® p400 HR Kit has been validated with a Thermo Scientific™ Q Exactive™ Focus mass spectrometer coupled to a Thermo Scientific™ Vanquish™ UHPLC system. Other Q Exactive™ platforms were also tested and can be used with the Kit (see section 2.1).

The Kit combines a flow injection analysis (FIA) and a liquid chromatography (LC) method. The Kit can be used for a variety of application areas, such as biomarker discovery, disease phenotyping, clinical research, pharmaceutical R&D, nutritional or functional food analysis, or to study environmental effects. The assay quantifies up to 408 metabolites from eleven compound classes:

- amino acids
- biogenic amines
- monosaccharides (hexose)
- acylcarnitines
- diglycerides
- triglycerides
- lysophosphatidylcholines
- phosphatidylcholines
- sphingomyelins
- ceramides
- cholesteryl esters

A list of metabolites and details on the analytical performance can be found in the Analytical Specifications document (“AS-p400-HR-#.pdf” on the USB stick). The data acquisition is performed using full MS scan mode and PRM mode for isoleucine. For quantitation, calibration standards in seven concentration levels and stable isotope-labeled internal standards (ISTD) are used. The internal standards are partially integrated in the filters of the Kit plate.

The analytical performance is validated using quality controls (QCs) at three concentration levels. The Biocrates® proprietary Met*IDQ*™ software is an integral part of the Kit and must be installed before starting with the Kit preparation. Please refer to the Met*IDQ*™ manual ("UM-Met*IDQ*-Carbon-#.pdf" on the USB stick).

The Absolute*IDQ*® p400 HR Kit was validated with human EDTA plasma. Both EDTA and heparin are suitable anticoagulants. Due to its biological similarity, human serum can also be used without impairing the analytical performance. For other biological matrices and species, please refer to our provided application notes (USB stick and http://www.biocrates.com). On Biocrates' homepage guidelines for the collection and preparation of biological samples are provided ("Sample Preparation Guidelines").

Up to 82 samples can be analyzed with a full 96-well Kit depending on the number of quality controls (QCs), see info box below. The remaining wells are reserved for one blank, three zero samples, seven calibration standards, and three QCs. The required laboratory equipment is listed in section *2: Required Equipment and Materials (Not Supplied)*.

We recommend analyzing QC level 1 or 2 in replicates of 4 or 5. This guarantees a higher quality of analytical performance when normalizing data within a single plate or across several plates (see Appendix "Data Normalization" in the Met*IDQ* user manual). We also recommend reviewing the EMEA guidelines on bioanalytical method validation (European Medicines Agency, 2011).

Shipping and Storage Information

After receiving an Absolute*IDQ*® p400 HR Kit:

1. Open the Kit box and store the vial box and the Kit plate (sealed in a plastic bag) below -18 °C until use.
2. Store all other contents of the Kit box at room temperature.
3. Keep the Kit plate sealed until use. Mind the expiration date on the Kit plate and vial box.

The Absolute*IDQ*® Kit is shipped in one package, except dry ice shipments (optionally). Keep the Absolute*IDQ*® Kit plate in the plastic bag sealed under nitrogen until use. Biocrates® can only guarantees a proper Kit performance if the plate is stored unopened in its original plastic bag and away from light. Vial box and Kit plate must be stored below -18 °C. All other items can be stored at room temperature. Please find the expiration date on your Kit box. Storage at -80 °C extends the shelf life for additional six month.

1.1 Absolute*IDQ*® p400 HR Kit Contents

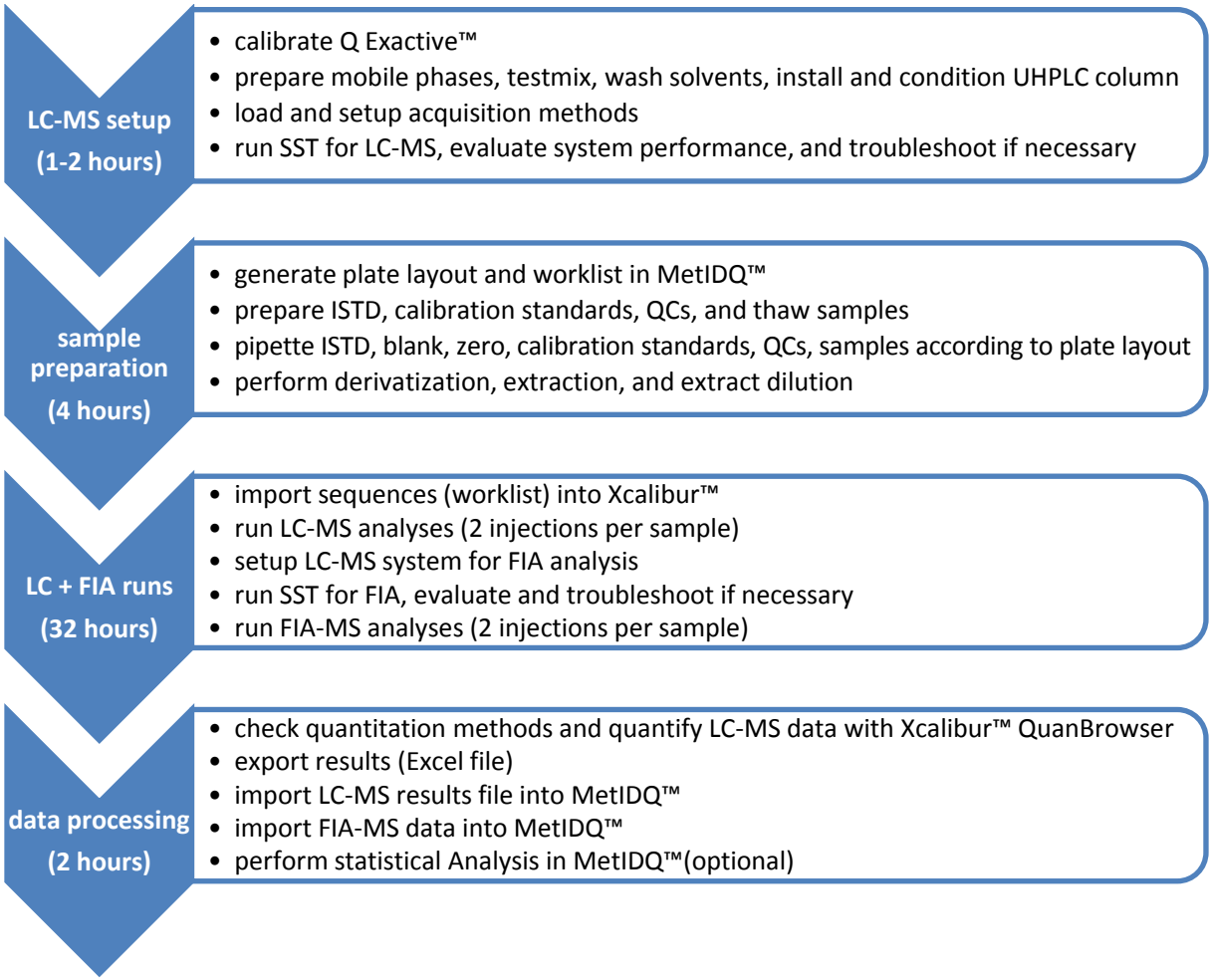
Kit Item	*Description*	*Details*
AbsoluteIDQ® p400 HR Kit plate, 1 item, store below -18 °C!	Plate stack consisting of a filter plate and a capture plate that are attached with tapes	Sealed under nitrogen in a plastic bag. Do not open until starting kit preparation.
96-deep-well plate, 1 item	Empty capture plate	Used to dilute the extracts after Kit preparation.
Silicone mats, 2 items	Silicone coverings for 96-well plates	Used to seal the plate after preparation.
Biocrates FIA Mobile Phase Additive, 2 items (1 spare)	Sealed glass ampules	Component for preparing FIA Solvent. Non-hazardous mixture.
Tube for derivatization solution, 1 item	Empty plastic tube	Used to prepare derivatization solution.
Vial Box: store below -18 °C!		
Testmix LC, 1 glass vial (2 vials in 24-well starter kit)	Biocrates Testmix for LC part (dried)	Standards used for LC system suitability test.
Testmix FIA, 1 plastic vial (2 vials in 24-well starter kit)	Biocrates Testmix for FIA part (dried), purple cap	Standards used for FIA system suitability test.
p400 HR QC, 3 plastic vials	Biocrates Quality Controls (lyophilized plasma): QC1 (green cap), QC2 (blue cap), QC3 (yellow cap)	Spiked human plasma in different concentration levels.
p400 HR Cal, 7 plastic vials	Biocrates Calibration Standards (lyophilized), red caps	Calibration standards used for the LC-MS quantification.
p400 HR ISTD, 1 plastic vial	Biocrates Internal Standard Mix (lyophilized), orange cap	Additional internal standards for Kit plate.

Kit Item	Description	Details
USB memory stick (one USB stick per delivery):		
Met*IDQ*™ Software	Version Carbon	Kit workflow manager.
OracleXE (Express Edition) Database	32-bit and 64-bit versions	Database for MetIDQ™ Software.
User Manual and Quick Start Guide	UM-p400-HR-Thermo-#.pdf Quick Start Guide-p400-HR-Thermo-#.pdf	Read carefully before using the Kit.
User manuals for Met*IDQ*™ software and modules	UM-Met*IDQ*-Carbon-#.pdf UM-StPk-#.pdf	Read carefully before using the Kit.
Analytical Specifications	AS-p400-HR-#.pdf	Read carefully before using the Kit.
Application Notes	pdf documents	Kit application with different sample material and species.
SOPs	pdf documents	Protocols for the analysis of different matrices.
Guidelines for sample collection	pdf documents	Guidelines for collecting plasma, serum and tissue samples.
Quantitation Methods	Xcalibur™ quantitation methods	Methods for data processing.

Instrument specific Files on USB stick

Kit Item	Details	
Quantitation methods for Xcalibur ™ 4.0	Q Exactive™ Focus	KIT2-LC1_XcaliburQuan_1011.pmd KIT2-LC2_XcaliburQuan_1012.pmd
	Q Exactive™	KIT2-LC1_XcaliburQuan_1111.pmd KIT2-LC2_XcaliburQuan_1112.pmd
	Q Exactive™ Plus	KIT2-LC1_XcaliburQuan_1211.pmd KIT2-LC2_XcaliburQuan_1212.pmd
	Q Exactive™ HF	KIT2-LC1_XcaliburQuan_1311.pmd

1.2 Workflow at a glance



Check the LC-MS performance before you start with the Kit preparation. Note that the time designations are approximate.

2 Required Equipment and Materials (Not Supplied)

Instrumentation, laboratory equipment, chemicals, and solvents listed below are required to use the Absolute*IDQ*® p400 HR Kit and are not provided with the Absolute*IDQ*® p400 HR Kit.

2.1 Mass Spectrometer and Laboratory Equipment

Material/Instrument	*Specifications*
Mass Spectrometer	• Thermo Scientific™ Q Exactive™ Focus • Thermo Scientific™ Q Exactive™ • Thermo Scientific™ Q Exactive™ Plus • Thermo Scientific™ Q Exactive™ HF **Ion source: HESI-II**
UHPLC System	• Thermo Scientific™ Vanquish™ UHPLC binary pump • Thermo Scientific™ UltiMate™ 3000 RSLC integrated system (with 10 µL inline filter instead of standard 150 µL mixer) **Injection volumes: 5 µL and 20 µL**
Autosampler	Sample manager for 1 mL deep-well plates, cooled (10 °C)
Column Oven	Column oven, 50 °C
UHPLC Column	UHPLC column for Absolute*IDQ*® p400 HR Kit, available only from Biocrates®
UHPLC Pre-Column	Phenomenex® SecurityGuard™ ULTRA Cartridge C18/XB-C18 for 2.1 mm ID column (Phenomenex® ordering no. AJ0-8782), also available from Biocrates®
UHPLC Pre-Column holder	Phenomenex® SecurityGuard™ ULTRA Holder (Phenomenex® ordering no. AJ0-9000), also available from Biocrates®

Material/Instrument	***Specifications***	
Nitrogen evaporator ***or*** pressure manifold	Nitrogen evaporator for 96 well plates, vendors e.g. Techne, Porvair, VLM or Organomation	Positive Pressure Manifold for 96 well plates, e.g. Waters® Positive Pressure-96 Processor or Biotage® PRESSURE+ 96 Manifold
	Note: **the evaporator or pressure manifold must be located in a fume hood. If a pressure manifold is used, a plate centrifuge is not required.**	
Centrifuge	Must be able to centrifuge 96-well plates of 5 cm height at 500 x *g*	Not required when a pressure manifold is used
Nitrogen supply	Minimum pressure requirement of 4 bar	
Solvent bottles	From 50 to 1000 mL	
Balance	Accuracy < 1 mg	
Plate shaker	e.g. Eppendorf® ThermoMixer® or MixMate®	
Vortexer	Any model	
Pipettes	• Single channel: volume range from 10 µL to 1000 µL • Repeater: using 1.0, 2.5, and 10 mL tips (important for reproducibility), e.g. Eppendorf® Multipette® E3 (Ordering No. 4987000010 or 4987000371) • 8-channel: volume range from 50 µL to 1200 µL, e.g. Eppendorf® Xplorer® plus (Ordering No. 4861000821)	

2.2 Chemicals and Solvents

Solvents/Chemicals	*Specifications*
Ethanol, methanol, acetonitrile, water, and isopropanol	LC-MS grade
Formic acid	LC-MS grade
Phenylisothiocyanate (PITC)	sequencing grade (for example Sigma Aldrich 317861)
Pyridine	p.a. grade or higher
Ammonium acetate	LC-MS grade
Phosphate buffered saline (PBS)	p.a. grade (for example Sigma Aldrich P4417)

2.3 Software

Software	*Required version*	*Details*
Thermo Xcalibur™	Version 4.0	MS data acquisition and quantitation
Microsoft® Excel®	Version 2007 or later	Export of Xcalibur™ quantitation results for Met*IDQ*™
Oracle® database	*Option 1 (recommended):* Full commercial version *Option 2:* Oracle XE, free of charge, provided with Kit USB stick. No support and updates provided by Oracle®.	Required for Met*IDQ*™
Biocrates® Met*IDQ*™	Provided by Biocrates®	Sample registration, Kit validation, data exportation, and statistical analysis (see UM-MetIDQ-Carbon-#.pdf)

It is required that the regional settings on the PC were Xcalibur™ and Met*IDQ*™ are installed to be set to “English US”. In addition, verify that “point” for “decimal symbol” and “comma” for “digit grouping symbol” are defined. Go to *Windows* ***Control Panel > Regional and Language > Formats > Additional Settings > Numbers***.

3 Safety Instructions

3.1 Safety Instruction for Personnel Protection

The mobile phases (solvents) and reagents are chemical substances classified as “hazardous substances”. The sample preparation must be carried out in a fume hood in a laboratory or other location in full compliance with local guidelines. Due to the use of dangerous solvents (e.g. methanol, acetonitrile), the UHPLC-MS system must be properly ventilated. The calibration standards are matrix-free. The quality controls are human plasma that was tested to be free from known pathogens (hepatitis B and C, HIV 1 and 2, syphilis). However, it should still be considered as potentially infectious. For this reason, we recommend treating the quality control samples and your samples with an equal level precaution. The Kit must be processed by trained personnel, such as MTA, CTA, BTA, or higher.

3.2 Proper Disposal of Laboratory Waste

Proper disposal of laboratory waste requires that all waste is collected and separated according to their chemical composition. Unused ampules should be opened and contents disposed as organic halogen-free solvent.

4 Instrumental Setup

It is required that the laboratory staff is familiar and experienced with the mass spectrometer and the operating software.

To avoid peak fronting or splitting of early eluting metabolites in LC-MS analysis, a "post injection mixing chamber" may be required. If a Thermo Scientific™ Vanquish™ without column switching valves is used, install a Viper™ capillary 0.13 x 350 mm (Thermo Scientific™ ordering number 6040.2335) between the autosampler and the UHPLC column.

4.1 Acquisition Methods and Tune Files

Acquisition Methods and Tune Files are not provided with the USB stick due to compatibility reasons with different front-end devices.

→ Create them according to sections 4.3 and 4.4.

Acquisition Methods and Tune Files required for the p400 HR Kit:

MS Instrument	**Q Exactive™ Focus**	**Q Exactive™**	**Q Exactive™ Plus**	**Q Exactive™ HF**
KIT2 LC methods	KIT2-LC1_1011.meth KIT2-LC2_1012.meth	KIT2-LC1_1111.meth KIT2-LC2_1112.meth	KIT2-LC1_1211.meth KIT2-LC2_1212.meth	KIT2-LC1_1311.meth
KIT3 FIA methods	KIT3-FIA1_1011.meth KIT3-FIA2_1012.meth	KIT3-FIA1_1111.meth KIT3-FIA2_1112.meth	KIT3-FIA1_1211.meth KIT3-FIA2_1212.meth	KIT3-FIA1_1311.meth KIT3-FIA2_1312.meth
FIA SST method	KIT3-FIA_SST_1011.meth	KIT3-FIA_SST_1111.meth	KIT3-FIA_SST_1211.meth	KIT3-FIA_SST_1311.meth
Tune files	KIT2-LCtune1_101x.mstune KIT2-LCtune2_101x.mstune KIT3-FIA_101x.mstune	KIT2-LCtune1_111x.mstune KIT2-LCtune2_111x.mstune KIT3-FIA_111x.mstune	KIT2-LCtune1_121x.mstune KIT2-LCtune2_121x.mstune KIT3-FIA_121x.mstune	KIT2-LCtune1_131x.mstune KIT2-LCtune2_131x.mstune KIT3-FIA_131x.mstune

4.2 Quantitation Methods

Quantitation methods for the LC part are provided on the USB stick for each Q Exactive™ platform.

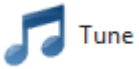
MS Instrument	**Q Exactive™ Focus**	**Q Exactive™**
LC variant	UHPLC	UHPLC
KIT2 LC quantitation methods	KIT2-LC1_XcaliburQuan_1**0**11.pmd KIT2-LC2_XcaliburQuan_1**0**12.pmd	KIT2-LC1_XcaliburQuan_1**1**11.pmd KIT2-LC2_XcaliburQuan_1**1**12.pmd

MS Instrument	**Q Exactive™ Plus**	**Q Exactive™ HF**
LC variant	UHPLC	UHPLC
KIT2 LC quantitation methods	KIT2-LC1_XcaliburQuan_1**2**11.pmd KIT2-LC2_XcaliburQuan_1**2**12.pmd	KIT2-LC1_XcaliburQuan_1**3**11.pmd

4.3 Create Tune Files

Each *Tune File* is specific for one Q Exactive™ instrument and must be created on that PC that is connected to the operated Q Exactive™ instrument.

Create the required *Tune Files* according to the description below.

Step	Instruction	Example
1	Open the “Tune” window.	
2	Create three *Tune Files* for **your** Q Exactive™ platform. The *Tune File* names are shown below.	

MS Instrument	**Q Exactive™ Focus**	**Q Exactive™**	**Q Exactive™ Plus**	**Q Exactive™ HF**
Tune files	KIT2-LCtune1_101x.mstune KIT2-LCtune2_101x.mstune KIT3-FIA_101x.mstune	KIT2-LCtune1_111x.mstune KIT2-LCtune2_111x.mstune KIT3-FIA_111x.mstune	KIT2-LCtune1_121x.mstune KIT2-LCtune2_121x.mstune KIT3-FIA_121x.mstune	KIT2-LCtune1_131x.mstune KIT2-LCtune2_131x.mstune KIT3-FIA_131x.mstune

3	For each *Tune File* use the parameters shown in section 10.3 *MS Settings and Tune Files*.	**10.3 *MS Settings and Tune Files***

4.4 Create Acquisition Methods

Acquistion Methods are specific for a LC-MS instrument combination and must be created on that PC that is connected to the operated Q Exactive™ instrument.

Create the required *Acquisition Methods* according to the description below.

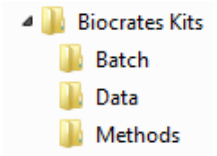
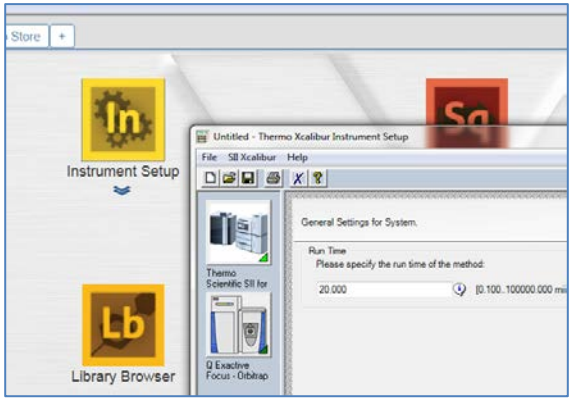
Step	Instruction
1	Open the “Instrument Setup”. 
2	Create all *Acquisition Methods* for **your** Q Exactive™ platform. The *Acquisition Methods* file names are shown below.
!	For Q Exactive™ HF, only one LC method (combined *Full MS* + *PRM*) is used.

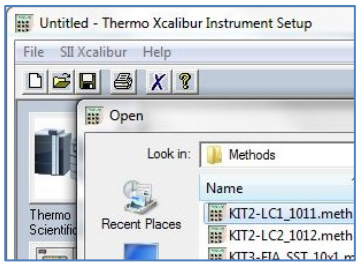
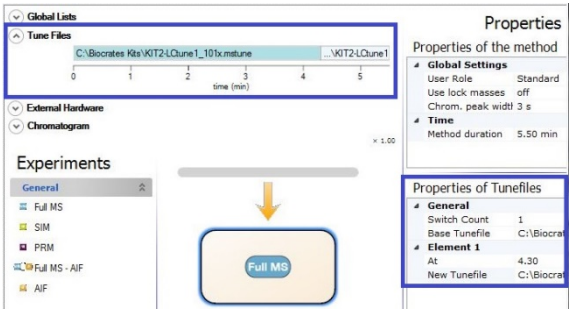
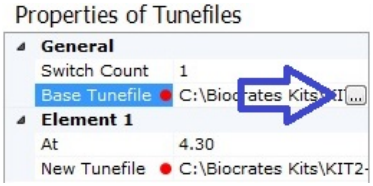
MS Instrument	**Q Exactive™ Focus**	**Q Exactive™**	**Q Exactive™ Plus**	**Q Exactive™ HF**
LC variant	UHPLC	UHPLC	UHPLC	UHPLC
KIT2 LC methods	KIT2-LC1_1011.meth KIT2-LC2_1012.meth	KIT2-LC1_1111.meth KIT2-LC2_1112.meth	KIT2-LC1_1211.meth KIT2-LC2_1212.meth	KIT2-LC1_1311.meth
KIT3 FIA methods	KIT3-FIA1_1011.meth KIT3-FIA2_1012.meth	KIT3-FIA1_1111.meth KIT3-FIA2_1112.meth	KIT3-FIA1_1211.meth KIT3-FIA2_1212.meth	KIT3-FIA1_1311.meth KIT3-FIA2_1312.meth
FIA SST method	KIT3-FIA_SST_1011.meth	KIT3-FIA_SST_1111.meth	KIT3-FIA_SST_1211.meth	KIT3-FIA_SST_1311.meth

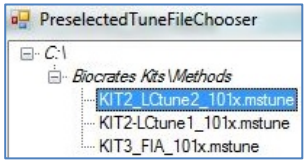
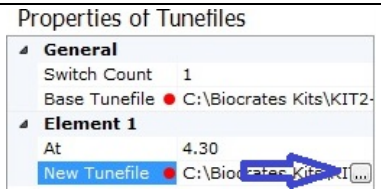
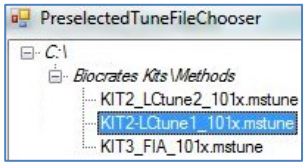
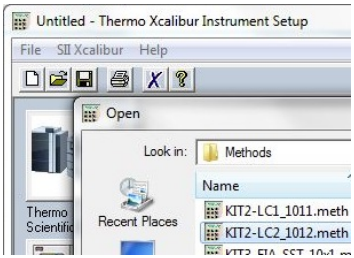
Note: **no acquisition methods are provided with the USB stick.**

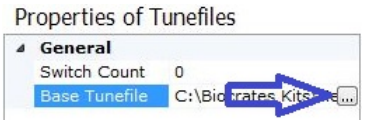
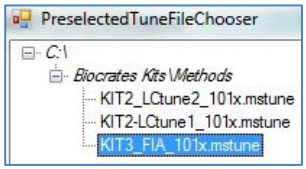
3	For each *Acquisition Methods* use the parameters shown in section 10.3 *MS Settings and Tune Files*.	☞ **10.3 *MS Settings and Tune Files***

4.5 Link Tune Files to Acquisition Methods

Step	Instruction	Example
4	On the PC that operates the Q Exactive™ instrument (hereinafter *MS-PC*), create a new folder structure **on the C: drive**. *Information:* these described folder names will be used in the following instructions.	e.g. 
⚠	To be able to link a *Tune File* to an Xcalibur™ acquisition method, **all methods have to be located on the C: drive**.	
5	Copy all required - acquisition methods - quantitation methods - tune files into the folder “Methods” on the *MS-PC*, according to section 4.1 and 4.2.	
6	To view or edit an acquisition method, start ***Xcalibur™*** and open the ***Instrument Setup***.	

Step	Instruction	Example
	1st LC Method (all Q Exactive™ types)	
	Two tune files are linked to each LC acquisition method.	
7	Open the 1st KIT2-LC acquisition method: - KIT2-LC1_1x11.meth	
8	Activate the MS part of the method by clicking on the "Q Exactive - Orbitrap" icon.	
9	Expand the "Tune Files" section.	
10	<u>Link the 1st tune file:</u> In the dialogue "Properties of Tunefiles" define the "Base Tunefile" under **General** by clicking on the … button.	

Step	Instruction	Example
11	Select the tune file - KIT2-LCtune1_1x1x.mstune The tune file is located in the Methods folder, e.g. “Biocrates Kits/Methods”.	
12	Link the 2nd tune file: In the dialogue “Properties of Tunefiles” define the “New Tunefile” under **Element 1** by clicking on the … button.	
13	Select and link the tune file - KIT2-LCtune2_1x1x.mstune Click “Save” in the acquisition method.	
	2nd LC Method (not applicable to Q Exactive™ HF)	
14	Open the 2nd KIT2-LC acquisition method: - KIT2-LC2_1x12.meth	
15	Link the 1st and 2nd tune files in the acquisition method “KIT2-LC2_1x12.meth”: - KIT2-LCtune1_1x1x.mstune - KIT2-LCtune2_1x1x.mstune For this, **repeat steps 8 – 13.**	

Step	Instruction	Example
	FIA Methods	
	One tune file is linked to each FIA acquisition method.	
16	To each FIA acquisition method: - KIT3-FIA1_1x11.meth - KIT3-FIA2_1x12.meth - KIT3-FIA_SST_1x11.meth link the tune file - KIT3-FIA_1x11.mstune	
17	Open the FIA acquisition methods one by one and link the FIA tune file "KIT3-FIA_1x11.mstune" as described above for the LC methods. ***Note:*** Only one tune file per FIA method is used.	

4.6 Autosampler and pump settings in the acquisition methods

For pump and autosampler settings refer to the appendix 10.1 *Pump Settings* and 10.2 *Autosampler and Column Oven settings*.

The following plate types must be selected for the corresponding autosampler:

- Vanquish™: "*WellPlate96*"
- UltiMate™ 3000: "*96-Deepwells*"

See section 10.2 *Autosampler and Column Oven settings*.

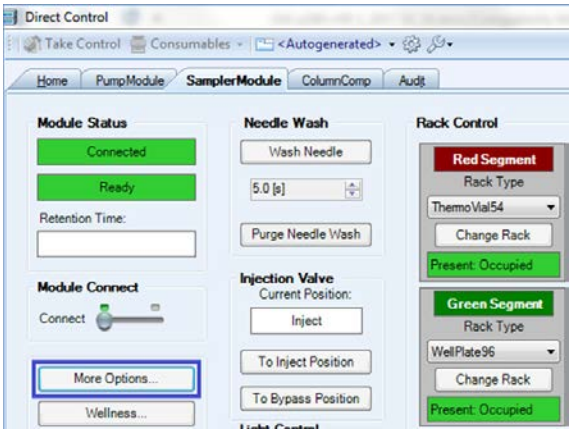
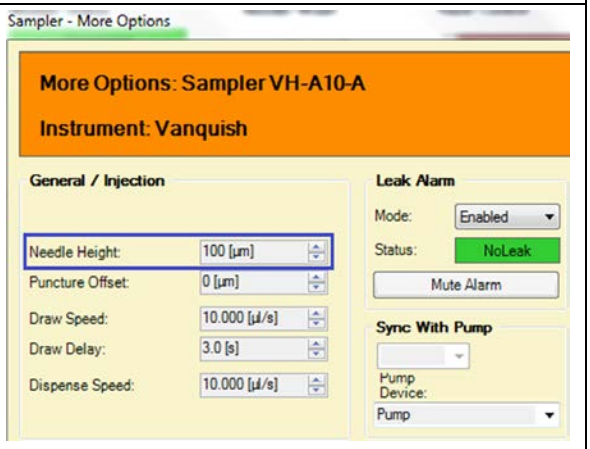
Kit installation only: to adjust the settings of your autosampler for the p400 HR Kit, use the 1 mL 96 deep-well plate with a silicone mat that you received with the Kit *Setup Box*.

- Check the needle penetration of the selected plate type "WellPlate96" (for Vanquish™) or "*96-Deepwells*" (for UltiMate™ 3000). A good value is approximately 0.1 mm above the bottom of the well.

Integrate rinsing steps to avoid cross contamination.

Wash solvents see 5.4 *Autosampler Wash Solvents*.

Vanquish™ Autosampler settings

Step	Instruction	Example
1	Open the “Direct Control” of the *SII interface*. In the “SamplerModule” tab, select “More Options…”.	
2	Define “100 [µm]” for “Needle Height”. Press the *Enter* key on the keyboard to confirm this setting and close the “More Options” window.	

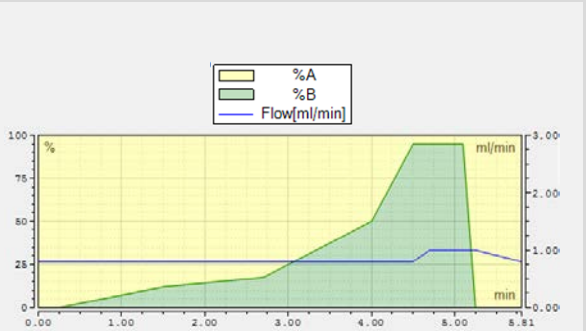
4.7 UHPLC System

Solvents: Solvent A and Solvent B (*see* 5.2)

UHPLC gradient – **LC part**

Methods: *KIT2-LC1_1x11.meth* and *KIT2-LC2_1x12.meth*

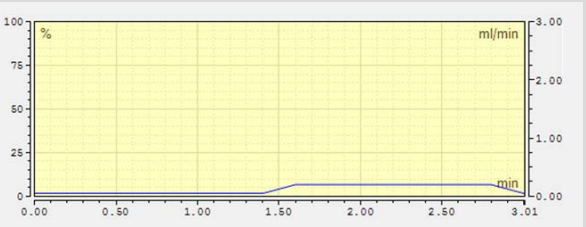
No	*Time(min)*	*Flow (mL/min)*	*%B*	*Curve*
1	0.00	Run		
2	0.00	0.8	0.0	5
3	0.25	0.8	0.0	5
4	1.50	0.8	12.0	5
5	2.70	0.8	17.5	5
6	4.00	0.8	50.0	5
7	4.50	0.8	95.0	5
8	4.70	1.0	95.0	5
9	5.10	1.0	95.0	5
10	5.25	1.0	0.0	5
11	5.80	0.8	0.0	5
12	5.81	Stop Run		



Gradient – **FIA part**

Methods: *KIT3-FIA1_1x11.meth*, *KIT3-FIA2_1x12.meth* and *KIT3-FIA_SST_1x11.meth*

No	*Time(min)*	*Flow (mL/min)*	*%B*	*Curve*
1	0.00	Run		
2	0.00	0.05	0.0	5
3	1.40	0.05	0.0	5
4	1.60	0.20	0.0	5
5	2.80	0.20	0.0	5
6	3.00	0.05	0.0	5
7	3.01	Stop Run		



Column cleaning: It is recommended to clean the column after each plate for at least 30 min using the Wash Solvent (see section 5.4, page 37). Change the pre-column at least after the analyses of every three Kits.

Column storage: 100% acetonitrile.

5 Preparing Solvents

Follow lab safety procedures.
Use fume hood and gloves.
Dispose organic solvents properly.

Testmix vials are provided with each Kit. Perform a system suitability test (SST) for the LC and FIA parts with the testmixes in order to check the instrument performance **before** starting with the Kit preparation. See section *6 System Suitability Test (SST)* for instructions.
If the SST fails, do not start with the Kit preparation! Otherwise you will not be able to analyze your samples reliably and may lose sample information. Perform troubleshooting and contact Biocrates® Customer Support if necessary. If you need our technical support, the testmix data files are required.

5.1 Preparing Mobile Phases and Solvents

5.2 LC Part – Solvent A and B

Mobile Phase	*Description*
Solvent A (1000 mL)	1000 mL water + 2 mL formic acid
Solvent B (500 mL)	500 mL acetonitrile + 1 mL formic acid

5.3 FIA Part – FIA Solvent

Follow the instructions below to open the glass ampule (FIA Mobile Phase Additive) and to prepare the FIA Solvent. Use freshly prepared solvents only. You will find two ampules in the Kit box, one is sufficient to make enough FIA Solvent for one Kit. The second is a spare one, in case of a mistake or if you want to run a Kit plate again.

Step	*Instruction*	*Example*
1	Find the white dot above the neck of the ampule.	
2	Use glass-handling safety gloves when breaking the ampule open. Hold the ampule upright in one hand, then grip the top of the ampule firmly between the thumb and forefinger, placing your thumb on the white dot. Snap off the top of the ampule by bending it sharply backwards.	
3	Mix the contents of the ampules with 290 mL of methanol to make the FIA Solvent. **FIA Solvent (300 mL) = 290 mL methanol + 1 ampule *FIA Mobile Phase Additive***	
	The empty ampule can be handled as common laboratory waste. Do not recycle empty ampules.	

5.4 Autosampler Wash Solvents

Prepare 500 mL of each solvent for one Kit.

Solvent	*Description*
Wash Solvent	*25% acetonitrile, 25% methanol, 25% isopropanol, 25% water* 125 mL acetonitrile + 125 mL methanol + 125 mL isopropanol + 125 mL water
Seal Wash	Thermo Vanquish ™: 75% isopropanol, 25 % water and 0.1 % FA Thermo UltiMate™ 3000 RS: 10% methanol, 90 % water Other systems: as recommended by the manufacturer

6 System Suitability Test (SST) and Instrument Calibration

The System Suitability Test (SST) is used to check the UHPLC-MS system performance **before** starting with the preparation of the Absolute*IDQ*® p400 HR Kit. Use the system settings described in this section. All required instrument method parameters are shown in sections 10.1, 10.2 and 10.3. Follow the instructions for your LC-MS instrumental setup.

LC-MS instrument method parameters:

- 10.1 Pump Settings
- 10.2 Autosampler and Column Oven settings
- 10.3 MS Settings and Tune Files

If the SST fails, do not start with the Kit preparation. Perform troubleshooting or contact Biocrates® Customer Support. The system may not be sensitive enough to detect all metabolites, especially in the concentration range of lower calibration standard levels.

6.1 Cleaning of LC-MS/MS System

Clean the entire LC-MS system before using the Absolute*IDQ*® p400 HR Kit.

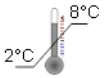
Step	*Instruction*
1	Clean the *Ion Sweep Cone* of the MS instrument.
2	Clean the *Ion Transfer Tube*.
3	Install all Wash Solvents and prime.
4	Install all solvents (Solvent A, Solvent B, FIA Solvent) and purge the lines. Flush all LC capillaries using all three solvents.

6.2 Prepare Blank and Testmix

Two testmix vials are provided with each Kit, one for the LC part and one for FIA part. The testmix vials are matrix-free and do not contain internal standards. The Testmix LC contains all amino acids and biogenic amines that are measured in the LC part. The Testmix FIA contains exemplary metabolites of the FIA part. The SST must be passed successfully before using the Kit.

Testmix LC vial ***Testmix FIA vial***

Item	***Preparation***
Blank **LC**	Add 1000 µL of 50% methanol (in water) to an empty vial.
Testmix **LC**	Add 600 µL of 50% methanol (in water) to the vial “Testmix LC”.
Blank **FIA**	Add 1000 µL of FIA Solvent (see 5.3, page 36) to an empty vial.
Testmix **FIA Stock**	Add 200 µL of 90% methanol (in water) to the vial “Testmix FIA”.
Testmix **FIA**	1. Add 2000 µL of FIA Solvent (see 5.3, page 36) to an empty vial. 2. Add 10 µL of Testmix FIA **Stock**.
All vials	Vortex the testmix vials for 15 sec. Store at +4 °C until use.



Dissolve testmix vials shortly before use. The reconstituted testmix can be stored for 1 week at +4 °C.

6.3 Mass Calibration of the Q Exactive™

Always perform a mass calibration before starting with the Kit. Perform a "Calmix Evaluation" with "Positive ion evaluation".

→ For a mass calibration or evaluation refer to the Q Exactive™ user manual.

The p400 HR Kit analyzes metabolites with a mass below 100 Da. For this a "Customized Calibration" in positive ion mode is required. Follow the manufacturer's instructions for calibrating the MS system.

The following procedure is described for a Q Exactive™ Focus and may differ slightly when another Q Exactive™ type is used.

6.3.1 Calibration Solution for Customized Mass Calibration

To ensure high mass accuracies for the Absolute*IDQ*® p400 HR Kit, perform a customized mass calibration using the following Calibration Solution:

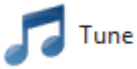
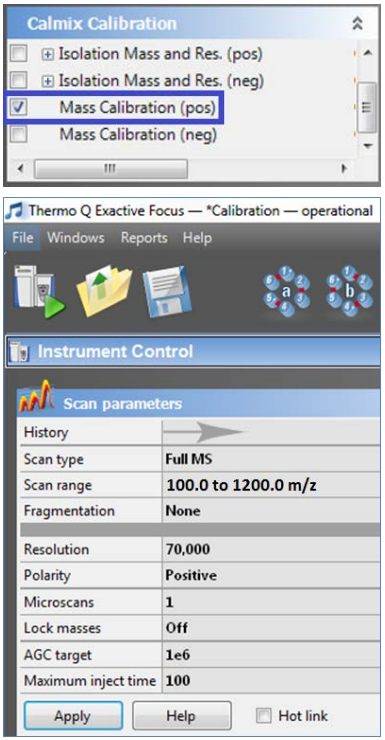
Item	***Preparation***
Customized Calibration Solution	1. Add 1 mL of "Thermo Scientific™ Pierce LTQ Velos ESI Positive Ion Calibration Solution" to an empty vial. 2. Add 100 µL of Testmix FIA **Stock** (see 6.2).

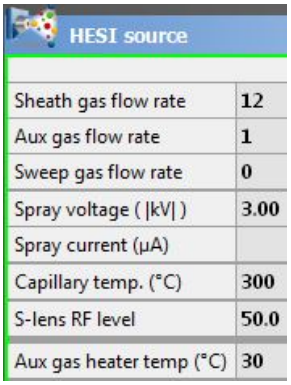
Ordering information:

Thermo Scientific™ Pierce LTQ Velos ESI Positive Ion Calibration Solution

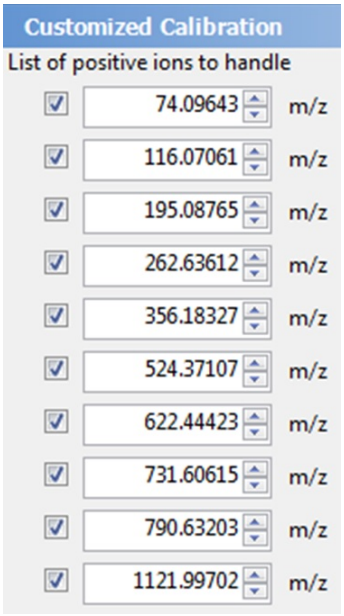
- Thermo Scientific™ ordering number PI-88323

6.3.2 Instrument Calibration

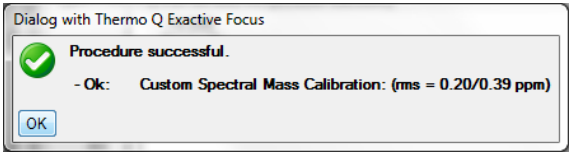
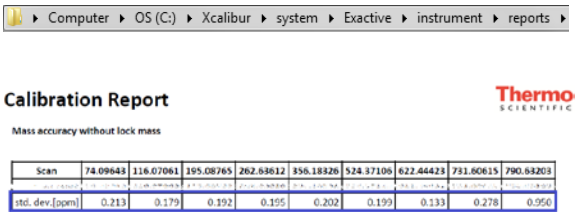
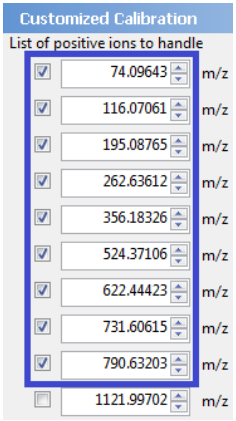
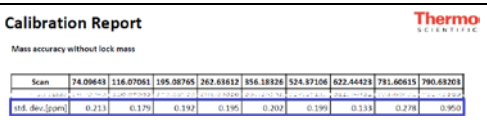
Step	Instruction	Example
1	Open the “Tune” window.	
2	Turn on the MS.	
3	Load that *Tune File* that is used for instrument calibration.	
4	In the “Instrument Control” panel go to “Scan parameters”. Define the values as shown on the right.	

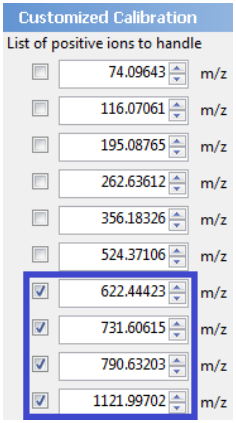
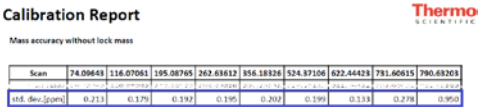
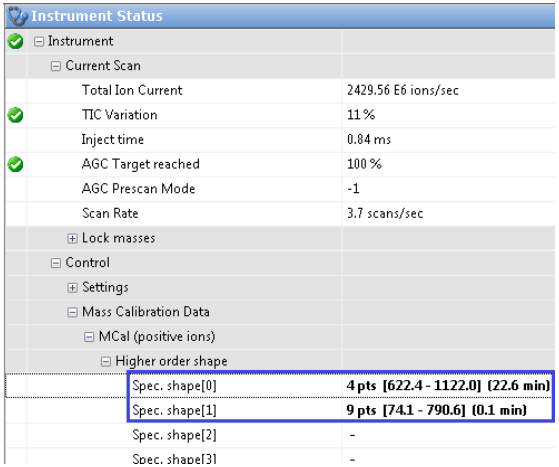
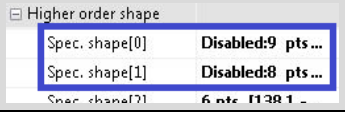
Step	Instruction	Example
5	Inject the *Customized Calibration Solution*: - Fill the syringe for direct infusion with ***Customized Calibration Solution*** (see 6.3.1, page 41) - Install the syringe, flush the lines and apply a flow of 10 µL/min - Wait for a stable TIC signal If the signal is not stable: 1. Vary flow rate between 10-20 µL/min 2. Vary gas flow parameters *Sheath gas*: approx. 7 - 15 *Aux gas*: approx. 1 - 3	

<table>
<tr><th>Step</th><th>Instruction</th><th>Example</th></tr>
<tr><td>6</td><td>Perform a Customized Calibration:
- Go to the “Calmix Calibration” tab and select “Mass Calibration (pos)”
- Select “Customized Calibration”
- 10 components are used for calibration, use the masses shown in the screenshot on the right.

<u>Masses for “Customized Calibration”:</u>
<table>
<tr><td>No</td><td>m/z</td></tr>
<tr><td>1</td><td>74.09643</td></tr>
<tr><td>2</td><td>116.07061</td></tr>
<tr><td>3</td><td>195.08765</td></tr>
<tr><td>4</td><td>262.63612</td></tr>
<tr><td>5</td><td>356.18327</td></tr>
<tr><td>6</td><td>524.37107</td></tr>
<tr><td>7</td><td>622.44423</td></tr>
<tr><td>8</td><td>731.60615</td></tr>
<tr><td>9</td><td>790.63203</td></tr>
<tr><td>10</td><td>1121.99702</td></tr>
</table>
<u>This table is available on the USB stick:</u>
- Folder: System Suitability Test\Customized Calibration
- File: Masses for Customized Calibration.xlsx</td><td> 

Keep this window open during the calibration process.</td></tr>
<tr><td>7</td><td>To start the calibration click “Calibrate”</td><td>Calibrate</td></tr>
</table>

Step	Instruction	Example
8	Wait until the calibration was performed.	
9	Open the Thermo ***Calibration Report***, saved by default in this folder: *"c:\xcalibur\system\exactive\instrument\reports"* Verify that all masses on page 1 **and** 2 were detected in all scans and there are no zero values. ➢ If not, repeat the Customized Calibration using a higher flow rate.	
	Only if Customized Calibration fails repeatedly, split calibration. See steps 10 – 14.	
10	**1st Customized Calibration:** • Select the tab *Customized Calibration* • Activate the first 9 masses • Click Calibrate	
11	Verify the Thermo ***Calibration Report*** according to step 9.	

Step	Instruction	Example
12	**2nd Customized Calibration:** • Select the tab *Customized Calibration* • Activate the last 4 masses • Click Calibrate	
13	Verify the Thermo ***Calibration Report*** according to step 9.	
14	Go to *Instrument Status* > *Control* > *Mass Calibration Data* > *MCal (positive ions)* > *Higher order shape*. Check that both calibrations from steps 10 and 12 are linked. ➢ If not, repeat steps 10 and 12.	
	If required, deactivate other calibrations.	

6.4 Conditioning the UHPLC Column

Step	Instruction
1	Install the analytical column together with the pre-column (incl. pre-column holder) and flush the column for 20 min with 95% Solvent B at a flow rate of 0.5 mL/min.
2	Before the first injection, equilibrate the system at starting condition (100% Solvent A, flow rate 0.8 mL/min, column oven temperature 50 °C).
3	Perform the SST (see next section).
i	Only if the SST fails due to insufficient cleaning, e.g. contaminations in blank, do the following steps.
4	Change all tubings, if possible.
5	Install a new ESI electrode, if required.
6	Clean all switching valves.
7	Repeat all instructions described in section 6.1 Cleaning of LC-MS/MS System.

6.5 Perform the System Suitability Test

The System Suitability Test (SST) is used to check the UHPLC-MS system performance before the Absolute*IDQ*® p400 HR Kit is prepared. Use each method with the parameters as described in Appendix 10.1, 10.2 and 10.3.

Use **ESI probe position ring B**.

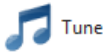
Perform the SST before starting with the Kit. If the SST fails, do not start with the Kit preparation and perform troubleshooting! If required, contact Thermo or Biocrates® Customer Support.

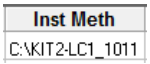
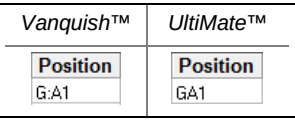
6.5.1 SST – LC part

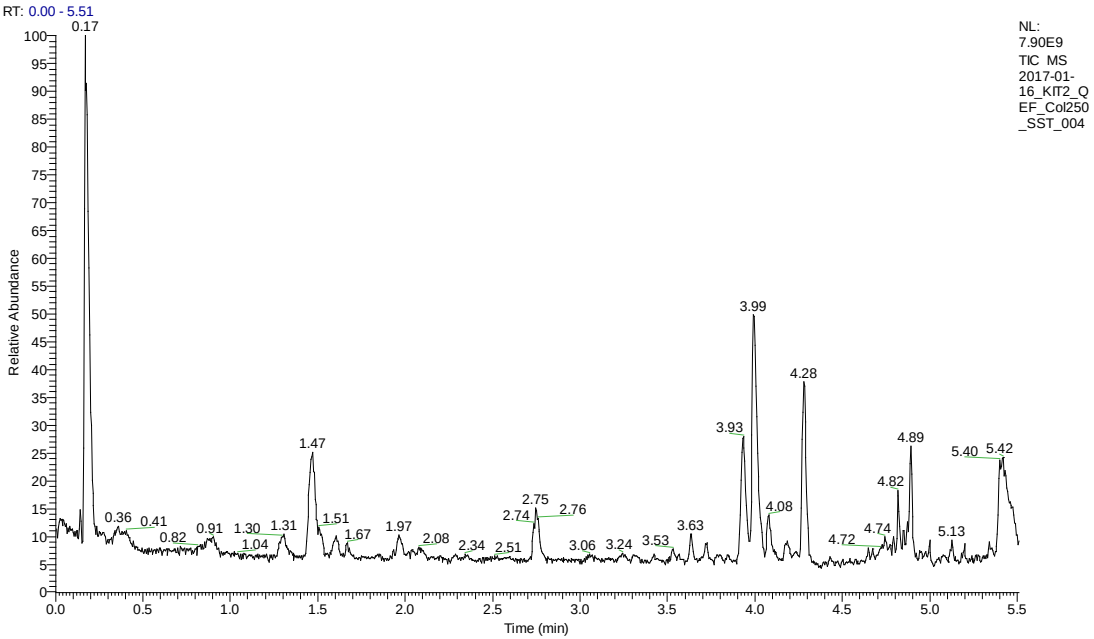
Use the appropriate acquisition method "KIT2_LC1_1x11.meth" for your Q Exactive™ instrument.

MS Instrument	**Q Exactive™ Focus**	**Q Exactive™**	**Q Exactive™ Plus**	**Q Exactive™ HF**
LC variant	UHPLC	UHPLC	UHPLC	UHPLC
KIT2 LC methods	KIT2-LC1_1011.meth	KIT2-LC1_1111.meth	KIT2-LC1_1211.meth	KIT2-LC1_1311.meth
Tune files	KIT2-LCtune1_101x.mstune KIT2-LCtune2_101x.mstune	KIT2-LCtune1_111x.mstune KIT2-LCtune2_111x.mstune	KIT2-LCtune1_121x.mstune KIT2-LCtune2_121x.mstune	KIT2-LCtune1_131x.mstune KIT2-LCtune2_131x.mstune

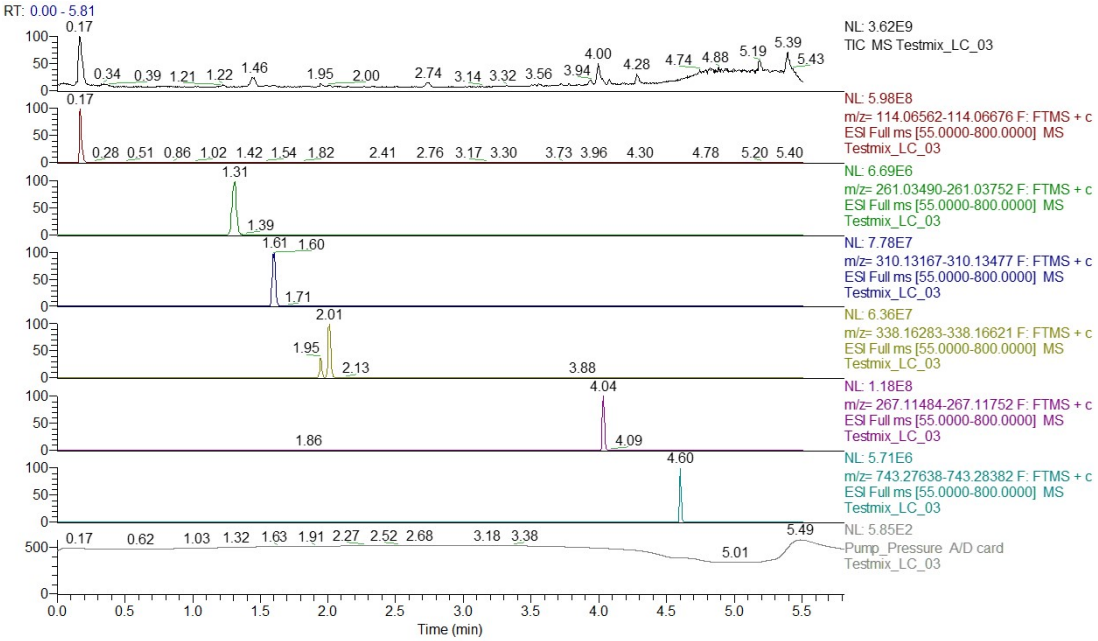
Use the blank LC and Testmix LC vials prepared in section 6.2 for the SST.

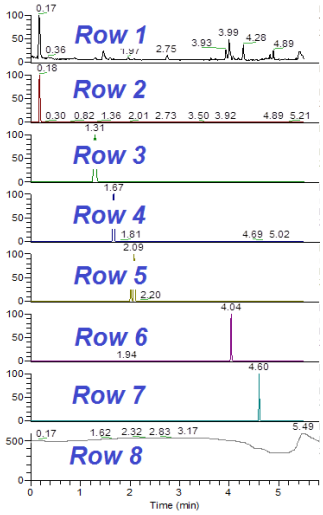
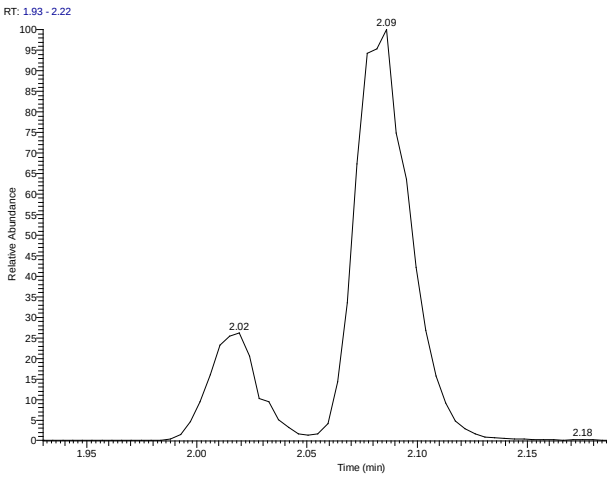
Step	***Instruction***
1	Use the acquisition method *KIT2-LC1_1x11.meth* for the LC SST for the used Q Exactive™ platform according to the table shown above.
	An overview of all methods is given in section 4.1. **Do not rename any method file!**
2	Double-check all parameters in the acquisition method for all instrument parts (MS, pump, autosampler, column oven). If necessary, type in the correct parameters according to Appendix 10.1, 10.2 and 10.3.
3	Open the "Tune" window and turn on the instrument.
4	Load the Tune File *KIT2-LCtune1_1x1x.mstune* 
	Before starting an injection, wait until all LC-MS parameters are stable.
5	Place the blank LC vial and Testmix LC vial into the cooled autosampler tray.

Step	Instruction
6	Create a sample sequence: - 3x blank LC - 3x testmix LC - 2x blank LC by loading the Xcalibur™ sequence "**Sequence_p400_LC-SST.sld**", provided with the USB stick in the folder "System Suitability Test\Secquences". **Injection volume: 5 µL**
7	Load the acquisition method *KIT2-LC1_1x11.meth* in the Xcalibur™ sequence, e.g. 
8	Define the correct well positions of the blank LC and Testmix LC vials, e.g. 
9	Submit the sequence. 
	An example testmix file (.raw) is located on the USB stick in the folder "Testmix Files". Use these data to check the testmix performance of your LC-MS system.

Step	Instruction
10	Figure 1 shows the TIC of an example chromatogram of a Testmix LC injection. MS instrument: Thermo Scientific™ Q Exactive™ Focus Pump system: Thermo Scientific™ Vanquish™ Acquisition method: KIT2-LC1_1011.meth ***Note:*** The testmix does not contain internal standards.  ***Figure 1:*** *Example Testmix LC chromatogram measured with Thermo Vanquish™ + Q Exactive™ Focus.*

Step	Instruction
11	To evaluate the Testmix LC performance use the *Qual Browser* layout "KIT2_SST.lyt" provided with the USB stick in the folder "System Suitability Test\Layouts". To apply a layout go to ***File > Layout > Apply…*** and select "KIT2_SST.lyt". An exemplary Testmix LC chromatogram is also provided with the USB stick. See folder "Testmix Files".
12	The retention time (RT) of each analyte must be stable over all three Testmix LC injections. If not, perform troubleshooting according to Biocrates® online FAQ, section LC Troubleshooting.
	**Biocrates® FAQ:** https://support.biocrates.com/tiki-index.php

Step	Instruction
13	Figure 2 shows an example chromatogram of a Testmix LC injection using the following setup: MS instrument: Thermo Scientific™ Q Exactive™ Focus Pump system: Thermo Scientific™ Vanquish™ Acquisition method: KIT2-LC1_1011.meth *Qual Browser* layout: KIT2_SST.lyt  ***Figure 2:*** *Example Testmix LC chromatogram with layout "KIT2_SST.lyt" applied. Acquired with a Thermo Vanquish™ + Q Exactive™ Focus.*

Step	Instruction
14	To evaluate the Testmix LC performance, use these SST criteria. **SST criteria:** • Rows 3-7: blank chromatograms should not show any peaks • Rows 2-7: stable RTs over three Testmix LC injections, maximum tolerance ±0.02 min • Rows 2-4: early eluting peaks do not show significant broadening, fronting, tailing or splitting • Row 5: good separation of ADMA and SDMA peaks (see chromatogram below) • Row 7: peak height of at least 2.0E6 cps • Row 8: the backpressure profile should not show any ripples (not shown for all pump systems)  Row 5 – ADMA and SDMA peak separation: 
	No internal standards are included in the testmix.

Step	Instruction
15	**<u>Check autosampler settings for injections from 96 deep well plates (first Kit use only):</u>** • Transfer 100 µL from the reconstituted vial "Testmix LC" into well positons A1 and H12 of an empty 1 mL deep-well plate, provided with the first Kit order. • Make one injection from both well positions A1 and H12 using the acquisition method "KIT2-LC1_1011.meth". • Apply the *Qual Browser* layout "KIT2_SST.lyt". • Compare the obtained chromatograms with the ones acquired during the LC SST. • All peak intensity in rows 2-7 should be comparable with the ones from the LC SST. ➢ If the peak intensities are reduced, it is possible that less than the specified sample volume was injected. Check the autosampler settings described in section 10.2 *Autosampler and Column Oven settings*.
✓	If the SST meets the required criteria <u>continue</u> with the SST for the FIA part.
✗	If the SST fails, the system may not be sensitive enough to detect all metabolites. Double-check the LC-MS configuration, instrument method parameters, and clean the entire LC-MS system according to the chapters 4 and 5. Perform the SST again. **Please feel free to contact the Customer Support whenever you have questions.**
⚠	**If the SST fails, do not start with the Kit preparation! Otherwise you may not be able to analyze your samples reliably and may lose sample information. Perform trouble-shooting and contact Biocrates® Customer Support.**

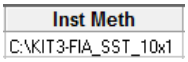
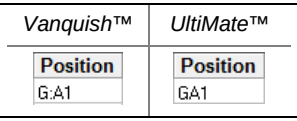
6.5.2 SST – FIA part

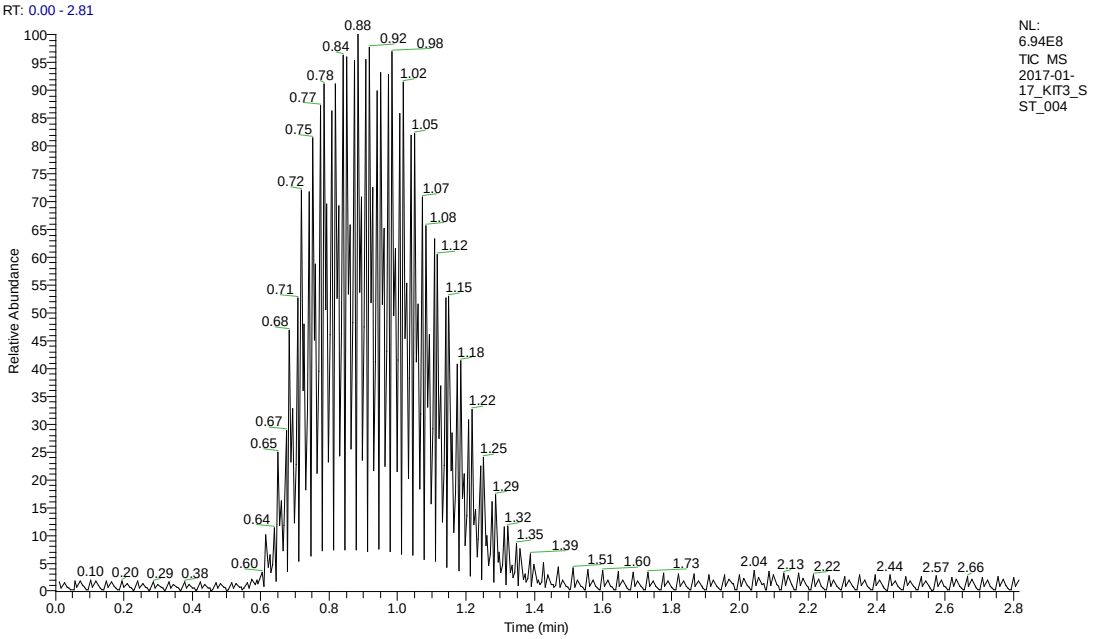
Use the appropriate acquisition method "KIT3_FIA_SST_1x11.meth" for your Q Exactive™ instrument.

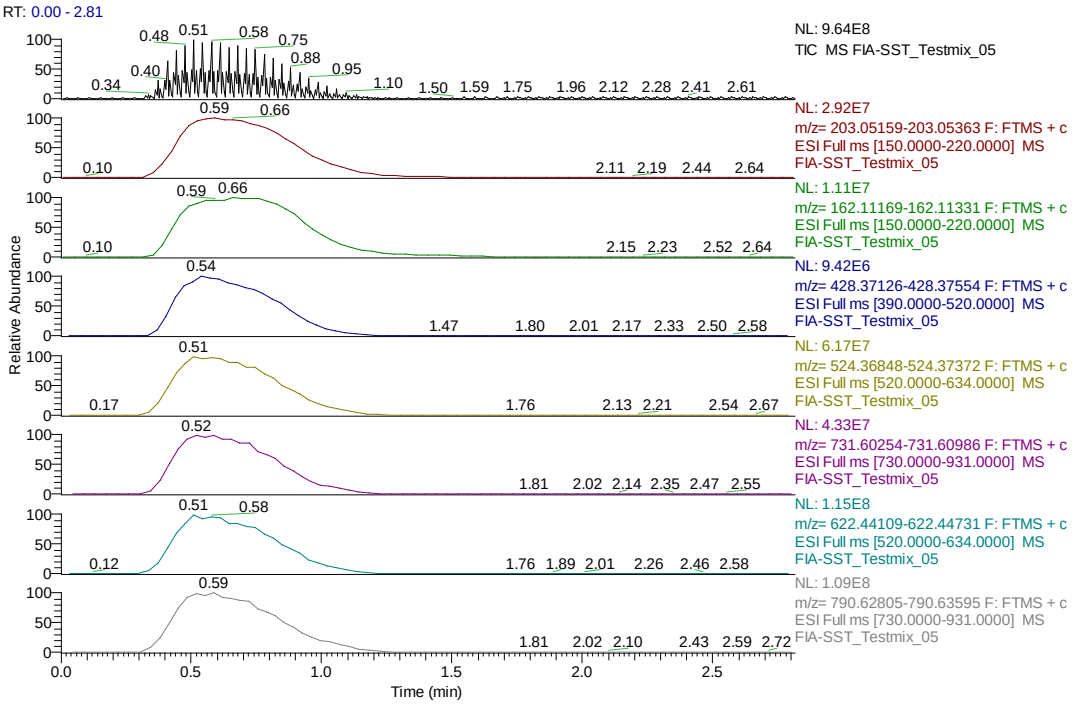
MS Instrument	**Q Exactive™ Focus**	**Q Exactive™**	**Q Exactive™ Plus**	**Q Exactive™ HF**
FIA SST method	KIT3-FIA_SST_1011.meth	KIT3-FIA_SST_1111.meth	KIT3-FIA_SST_1211.meth	KIT3-FIA_SST_1311.meth
Tune files	KIT3-FIA_1011. mstune	KIT3-FIA_1111. mstune	KIT3-FIA_1211. mstune	KIT3-FIA_1311. mstune

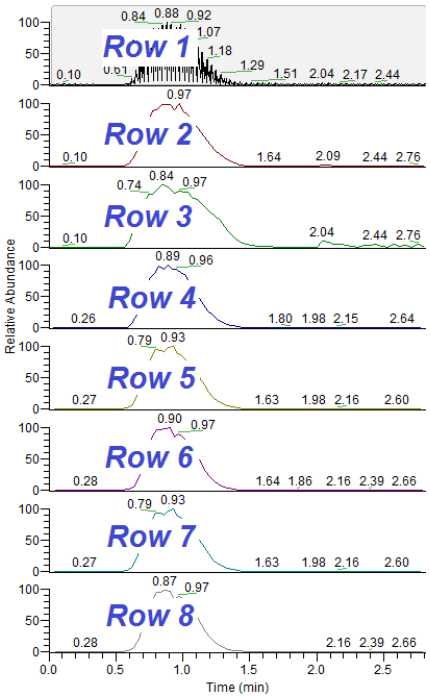
Use the blank FIA and Testmix FIA vials prepared in section 6.2 for the SST.

Step	*Instruction*
1	Use the acquisition method *KIT3-FIA_SST_1x11.meth* for the FIA SST for the used Q Exactive™ platform according to the table shown above.
	An overview of all methods is given in section 4.1. **Do not rename any method file!**
2	Double-check all parameters in the acquisition method for all instrument parts (MS, pump, autosampler, column oven). If necessary, type in the correct parameters according to Appendix 10.1, 10.2 and 10.3.
3	Prepare the autosampler for FIA by one of the following options: - Remove the column and, if possible, connect the injector directly with the ion source. *or* - Use a bypass or another column line (without a column installed).
	For good FIA peaks minimize dead volumes by using as few connecting parts as possible.
4	Flush the system with the *FIA Solvent* at a flow rate of 0.2 mL/min for 10 min.

Step	Instruction
5	Open the “Tune” window and turn on the instrument. 
6	Load the Tune File *KIT3-FIA_1x11.mstune*
	Before starting an injection, wait until all LC-MS parameters are stable.
7	Open the “Direct Control” window and lower the lower pressure limit to 0 bar. 
8	Place the blank FIA vial and Testmix FIA vial in the cooled autosampler tray.
9	Create a sample sequence: - 3x blank FIA - 3x testmix FIA - 2x blank FIA by loading the Xcalibur™ sequence “**Sequence_p400_FIA-SST.sld**”, provided with the USB stick in the folder “System Suitability Test\Secquences”. **Injection volume: 20 µL**
10	Load the acquisition method *KIT3-FIA_SST_1x11.meth* in the Xcalibur™ sequence, e.g. 
11	Define the correct well positions of the blank FIA and Testmix FIA vials, e.g. 
12	Submit the sequence. 
	An example testmix file (.raw) is located on the USB stick in the folder “Testmix Files”. Use these data to check the testmix performance of your LC-MS system.

Step	Instruction
13	Figure 3 shows the TIC of an example chromatogram of a p400 Kit Testmix FIA injection using the following setup: MS instrument: Thermo Scientific™ Q Exactive™ Focus Pump system: Thermo Scientific™ Vanquish™ Acquisition method: KIT3-FIA_SST_1011.meth  ***Figure 3:*** *Example Testmix FIA chromatogram measured with Thermo Vanquish™ + Q Exactive™ Focus.*
14	To evaluate the Testmix FIA performance, use the *Qual Browser* layout “KIT3_SST.lyt”. This layout is provided with the USB stick in the folder “System Suitability Test\Layouts”. - An exemplary Testmix FIA chromatogram is provided with the USB stick. See folder “Testmix Files”.

Step	Instruction
15	Figure 4 shows an example chromatogram of a Testmix FIA injection using the following setup: MS instrument: Thermo Scientific™ Q Exactive™ Focus Pump system: Thermo Scientific™ Vanquish™ Acquisition method: KIT3-FIA_SST_1011.meth *Qual Browser* layout: KIT3_SST.lyt  ***Figure 4:*** *Example Testmix FIA chromatogram with layout "KIT3_SST.lyt" applied. Acquired with a Thermo Vanquish™ + Q Exactive™ Focus.*

Step	Instruction
16	To evaluate the Testmix FIA performance, use the following SST criteria: **SST criteria:** • Rows 2-8: no major peak tailing or splitting • Rows 1-2 + 5-8: peak intensities of at least 1.0E7 cps • Rows 3-4: peak intensities of at least 1.0E6 cps 
	No internal standards are included in the testmix.

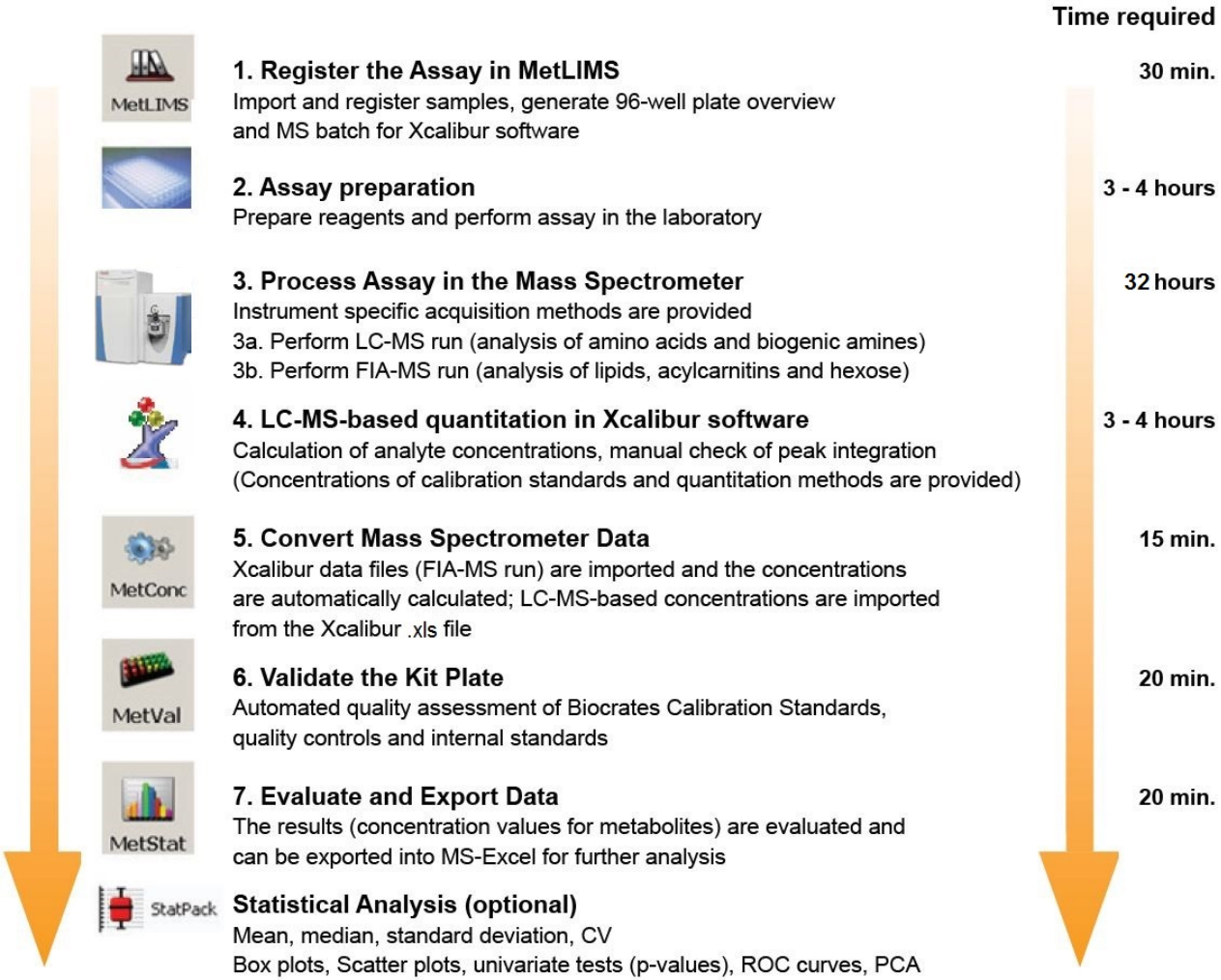
Step	Instruction
✓	If the SSTs of LC and FIA parts meet the required criteria you may start with the Kit preparation.
✗	If either the SST of the LC or FIA part fails, the system may not be sensitive enough to detect all metabolites. Double-check the LC-MS configuration, instrument method parameters, and clean the entire LC-MS system according to the sections 4 and 5. Perform the SST again. If one or more rows do not show any intensities ("FIA peaks"), check the mass accuracy of the MS system. If needed, perform a mass calibration and an eFT parameter calibration. ***Note:*** There may be signals in Row 1 (TIC), even if the there is an issue with the MS mass accuracy. **Please feel free to contact Biocrates® Customer Support whenever you have questions.**
⚠	**If the SST fails, do not start with the Kit preparation! Otherwise, you may not be able to analyze your samples reliably and may lose sample information. Perform troubleshooting and contact Biocrates® Customer Support.**

7 Kit Preparation

The Met*IDQ*™ software is an integral part of the Absolute*IDQ*® p400 HR Kit. Please read the Met*IDQ*™ Carbon manual carefully (*UM-MetIDQ-Carbon-#.pdf*) and install Oracle® and Met*IDQ*™ before you start with the Kit.

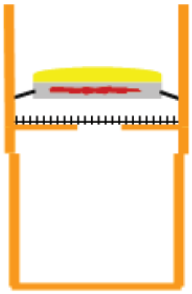
7.1 Overview Kit Workflow

The Kit workflow is described in detail in the following sections. For a proper Kit performance, do not combine the Kit with components from other manufacturers.

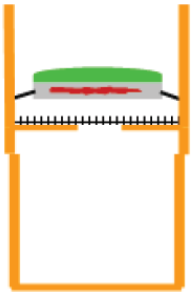


7.2 Overview Lab Workflow

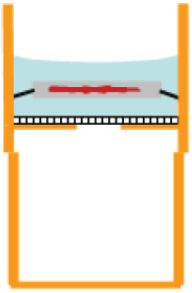
1. Add 10 µL of internal standard mix, add 10 µL of samples, dry 30 min under nitrogen flow.



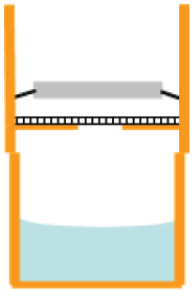
2. Derivatization using 5% PITC, incubate 25 min, dry 60 min under nitrogen flow.



3. Add 300 µL extraction solvent, shake 30 min at 450 rpm.



4. Filtration by centrifugation, 2 min at 500 x g.



5. Remove 150 µL and transfer to a second 96-deep-well plate, dilute with 150 µL water for subsequent LC-MS/MS assay.
6. Dilute all wells in the original plate with 250 µL of FIA mobile phase.

7.3 Prepare Kit Components and Samples

Dissolve all vials shortly before preparing the Kit.

7.3.1 Phosphate Buffered Saline (PBS)

PBS is used as Zero Sample, because the salt content is similar to plasma, resulting in comparable ion suppression and background noise. PBS can also be used when other sample material is analyzed. The limit of detection (LOD) for each metabolite is calculated by Met*IDQ*™. The LOD is defined as three times the background noise level.

While PBS is recommended for blood based samples (e.g. plasma), alternatively the Zero Sample could be whatever the sample matrix. For example, when tissue extract samples are used, use this extraction solvent as Zero Sample.

Zero Sample	*Instruction*
PBS Solution	Prepare according to manufacturer‘s information.

7.3.2 Internal Standard Mix (ISTD)

Do not dissolve until use. **Centrifuge the vial before opening at 10 000 x *g* for 2 min.**

Standard	*Instruction*
Internal Standard	1. Add 1200 µL of water to the lyophilized ISTD. 2. Shake for 15 min at 1200 rpm and vortex several times.

7.3.3 Calibration Standards (Cal1 – Cal7)

Do not dissolve until use. **Centrifuge the vials before opening at 10 000 x *g* for 2 min.** The seven standards contain the amino acids and biogenic amines and are used to generate the calibration curves for the LC-MS part.

Standard	*Instruction*
Biocrates® Standards	1. Add 100 µL of water to each of the 7 Calibration Standards. 2. Shake for 15 min at 1200 rpm and vortex several times. 3. Gently tap the tubes on the table or use a centrifuge to make sure that the solution is at the bottom of the tube.

7.3.4 Quality Control Samples (QC1 – QC3)

Do not dissolve until use. **Centrifuge the vials before opening at 10 000 x *g* for 2 min.** The QC samples are human plasma based samples with analytes added in defined concentrations. QCs in three levels are provided.

Note: **We recommend pipetting QC2 after every 20th samples**

Sample	*Instruction*
QC Samples	1. Add 100 µL water to each QC vial and shake for 15 min at 1200 rpm. 2. Vortex the reconstituted QCs and centrifuge at 4 °C for 5 min at 2750 × *g* before loading onto the Kit plate.

7.3.5 Plasma Samples

Sample	*Instruction*
Plasma Samples	If you analyze plasma samples with the Kit, vortex the plasma samples after thawing. Centrifuge at 4 °C for 5 min at 2750 × *g* (as the QCs) before loading onto the Kit plate. Make sure that the centrifuge has reached a temperature below 20 °C before use. If the plasma samples are of high viscosity (i.e. plasma from small animals or of a certain diseased group), you should apply higher centrifugation speed (e.g. 5000 × *g*).

7.4 Preparing Solvents and Reagents

7.4.1 Pre-Mix for Derivatization

Solution	*Instruction*
1900 µL ethanol 1900 µL water 1900 µL pyridine	1. Pipette 1900 µL (or 2 x 950 µL) of each solvent into the empty plastic tube that you find in the Kit box. 2. Vortex for 10 sec.

7.4.2 Phenylisothiocyanate (PITC) Derivatization Solution

Prepare the solution directly before the derivatization!

Note: The stability of the solution is reduced after adding PITC to the pre-mix.

Solution	*Instruction*
Derivatization Solution	1. Remove the PITC from the freezer and allow it to equilibrate to room temperature. 2. Add 300 µL of fresh PITC to the pre-mix. 3. Vortex rigorously until the solution is clear.

7.4.3 Extraction Solvent

This solution is stable for approx. three months after preparation.

Solution	*Instruction*
5 mM ammonium acetate in **methanol**	Dissolve 19 mg ammonium acetate in 50 mL **methanol**.

7.4.4 Mobile Phases

For more details see sections

- 5.2 *LC Part – Solvent A and B*
- 5.3 *FIA Part – FIA Solvent*.

LC part:

Mobile Phase	*Description*
Solvent A (1000 mL)	1000 mL water + 2 mL formic acid
Solvent B (500 mL)	500 mL acetonitrile + 1 mL formic acid

FIA part:

Mobile Phase	*Description*
FIA Solvent (300 mL)	290 mL methanol + 1 ampule *FIA Mobile Phase Additive*

7.4.5 Register the Kit Plate in MetIDQ

Step	Instruction	Example
1	To register the Kit plate in MetIDQ™ follow the instructions of the MetIDQ™ Carbon user manual (*UM-MetIDQ-Carbon-#.pdf*) in section *4.1.4 Generate Plate Layout and Worklist for MS run*.	MetIDQ™ User Manual
2	When generating the worklist in ***MetIDQ > MetLIMS > Projects***, use the appropriate MetIDQ™ OPs shown in the table below.	Generate Worklist

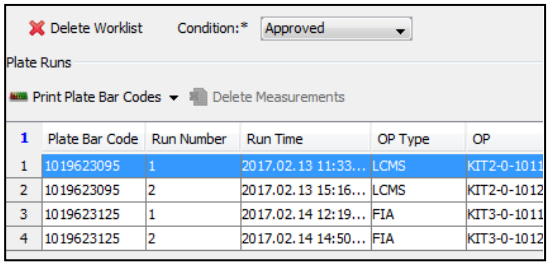
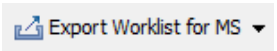
!	For Q Exactive™ HF, only one LC method (combined *Full MS* + *PRM*) is used. ☞ Create only one LC *Plate Run* using OP **KIT2-0-1311**.

Instrument	Q Exactive™ Focus	Q Exactive™	Q Exactive™ Plus	Q Exactive™ HF
LC OPs	**KIT2-0-1011**	**KIT2-0-1111**	**KIT2-0-1211**	**KIT2-0-1311**
	KIT2-0-1012	**KIT2-0-1112**	**KIT2-0-1212**	
FIA OPs	**KIT3-0-1011**	**KIT3-0-1111**	**KIT3-0-1211**	**KIT3-0-1311**
	KIT3-0-1012	**KIT3-0-1112**	**KIT3-0-1212**	

i	Two injections are performed in both LC and FIA parts. For each injection, a separate MetIDQ™ OP and Xcalibur™ acquistion method is used. *An example for a Q Exactive™ Focus is given below.*

Example for a Q Exactive™ Focus:

	Injection	OP code	Acquisition method	Quantitation method
LC	1	KIT2-0-1011	KIT2-LC1_1011.meth	KIT2-LC1_XcaliburQuan_1011.pmd
	2	KIT2-0-1012	KIT2-LC2_1011.meth	KIT2-LC2_XcaliburQuan_1011.pmd
FIA	1	KIT3-0-1011	KIT3-FIA1_1011.meth	no quantitation method *quantified by MetIDQ™*
	2	KIT3-0-1012	KIT3-FIA2_1012.meth	

3	1. Register one LC Kit plate (*Plate Run*) using the OP "KIT2-0-1x11". 2. Once the LC plate layout is satisfactory, make three more copies of this *Plate Run*. 3. Right-click on the plate entry and select "Copy". 4. Right-click again and "Paste derived plate" three times. 5. Change the OPs of each newly created plate according to the table above according to your MS instrument.	 1 Plate Bar Code Run Number Run Time OP Type OP 1 1019623095 1 2017.02.13 11:33... LCMS KIT2-0-1011 2 1019623095 2 2017.02.13 15:16... LCMS KIT2-0-1012 3 1019623125 1 2017.02.14 12:19... FIA KIT3-0-1011 4 1019623125 2 2017.02.14 14:50... FIA KIT3-0-1012 *Example for a Q Exactive™ Focus*
4	Generate the pipetting layout and autosampler worklist by selecting "Export Worklist for MS" in Met*IDQ*™.	

7.4.6 Prepare the Kit Plate

1. Make sure that all samples and the Kit plate layouts are registered in MetIDQ™ and that the MetIDQ™ acquisition batch file (.csv) for the Xcalibur™ software was generated.
2. Find the AbsoluteIDQ® Kit plate in the Kit box. Remove the plastic bag and follow the step-by-step instructions below to prepare the assay.
3. Follow lab safety protocol while preparing the Kit. Use a fume hood and gloves.

QCs are used to ensure the accuracy and reproducibility of the analysis. It is recommended that QC2 is measured in replicates of 5 (after every 20 samples).

QC2 replicates are required for inter-plate normalization (see Appendix "Data Normalization" in the MetIDQ™ Carbon user manual) in order to guarantee the best quality of your results. You may also refer to the EMEA guidelines on bioanalytical method validation (European Medicines Agency, 2011).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Cal5										
B	Zero	Cal6			QC2					QC2		
C	Zero	Cal7										
D	Zero	QC1										
E	Cal1	QC2										
F	Cal2	QC3					QC2					
G	Cal3											
H	Cal4											QC2

<table>
<tr><th>Step</th><th>Instructions</th></tr>
<tr><td>1</td><td>Remove the plastic lid of the AbsoluteIDQ® Kit plate.</td></tr>
<tr><td>2</td><td>Add 10 µL of the ISTD to all wells of the Kit plate with exception of the blank well position A1. Pipette directly onto the filter in the center of each well. Do not pipette on the wall of the wells. We recommend using an Eppendorf Multipette® (repeater) adjusted to maximum dispensing speed.
Do <u>not</u> use an 8-channel pipette!</td></tr>
<tr><td>3</td><td><u>Pipette 10 µL of each sample (Zero, Calibration Standards, QCs and experimental samples):</u>
Use a single-channel pipette to pipette 10 µL onto the center of each filter. Gently touch the filter inserts with the pipette tip while pipetting the samples. Do not pipette on the wall of the wells and avoid cross-contamination. Use a fresh tip for each sample.
<table>
<tr><td>Well A1 → Blank:</td><td>do <u>not</u> pipette anything</td><td>Blank</td></tr>
<tr><td>Well B1 → Zero:
Well C1 → Zero:
Well D1 → Zero:</td><td>pipette 10 µL of PBS
pipette 10 µL of PBS
pipette 10 µL of PBS</td><td>Zero</td></tr>
<tr><td>Well E1 → Cal1:
Well F1 → Cal2:
Well G1 → Cal3:
Well H1 → Cal4:
Well A2 → Cal5:
Well B2 → Cal6:
Well C2 → Cal7:</td><td>pipette 10 µL of Calibration Standard level 1
pipette 10 µL of Calibration Standard level 2
pipette 10 µL of Calibration Standard level 3
pipette 10 µL of Calibration Standard level 4
pipette 10 µL of Calibration Standard level 5
pipette 10 µL of Calibration Standard level 6
pipette 10 µL of Calibration Standard level 7</td><td>Cal1-7</td></tr>
<tr><td>Well D2 → QC1:
Well E2 → QC2:
Well F2 → QC3:</td><td>pipette 10 µL of Quality Control level 1
pipette 10 µL of Quality Control level 2
pipette 10 µL of Quality Control level 3</td><td>QC1
QC2
QC3</td></tr>
<tr><td>Well G2 – H12 →</td><td>pipette 10 µL according to the MetIDQ plate layout</td><td>Samples
QC2</td></tr>
</table>
</td></tr>
</table>

Step	Instructions
4	Dry down the samples for 30 min at room temperature under nitrogen, according to the following info box.
	Dry the samples: Use a Nitrogen Evaporator ***or*** Pressure Manifold. **Nitrogen Evaporator:** • Make sure the evaporator needles are at least 5 mm above the filter inserts. • Adjust the pressure at the nitrogen pressure reduction valve to about 3-4 bar and check that there is a nitrogen flow at the end of the evaporator needles. **Pressure Manifold:** • Refer to the “Instructions for Using a Pressure Manifold for p180, p400 and p150 Kit” on the USB stick located in the folder “Instructions”.
5	Prepare the *PITC Derivatization Solution*, see section 7.4.2 (page 66).
	**Use the *PITC Derivatization Solution* shortly after adding PITC to the *Pre-Mix.***
6	Pipette 50 µL of the *PITC Derivatization Solution* (see 7.4.2) to each well (incl. the *blank*, well A1). Pipette directly on the center of the filter. Do not pipette on the wall of the wells. We recommend the use of an Eppendorf Multipette® adjusted to medium dispensing speed.
7	Cover the Kit plate with the plastic lid and incubate at room temperature for 25 min.
8	Remove the plastic lid. Dry the Kit plate with a Nitrogen Evaporator ***or*** Pressure Manifold (see info box above) for 60 min.
	Make sure the Kit plate is completely dried. Ineffective evaporation of PITC and pyridine will impair the Kit performance.
9	Add 300 µL of *Extraction Solvent* (see 7.4.3) to each well. Use an Eppendorf Multipette® (repeater) adjusted to low dispensing speed or an 8-channel pipette

Step	Instructions
10	Cover the Kit plate with the plastic lid. Shake the Kit plate at room temperature using an Eppendorf® ThermoMixer® or MixMate® at 450 rpm for 30 min.
⚠	Be careful when shaking the Kit plate with another shaker as specified in step 10. Due to the risk of cross-contamination, adjust the speed of the mixer carefully. Make sure there is no spill over.
11	Centrifuge the Kit plate for 2 min at 500 × *g* (make sure the centrifuge is balanced) ***or*** use a Pressure Manifold (see "Instructions for Using a Pressure Manifold for p180, p400 and p150 Kit" on the USB stick located in the folder "Instructions").
i	After the elution, check that the fill level is the same in all wells in the capture plate. If not, repeat the elution process and apply higher *g* or higher pressure, respectively.
12	Carefully remove the tape from the sides of the Kit plate. Separate the lower capture plate (containing the samples extracts, labeled "Use for FIA") from the upper filter plate. Take care that nothing spills or splashes over during this process.
⚠	**In case of any delays, seal the capture plate with one of the silicon mats that you find in the Kit box and store it at 4 °C before continuing with the next steps. The plate can be stored up to two days at 4 °C. Do not store the plate below 0 °C!**
i	The following preparation steps 13 – 16 should be performed immediately.

Step	Instructions
Q Exactive™ Focus / Q Exactive™ Plus / Q Exactive™ HF / Q Exactive™	
	For this step you need: • Capture plate labeled “Use for FIA“ from step 12 • Empty 96-deepwell plate labeled “Use for LC“ (provided with the Kit box) • 2 silicone mats (provided with the Kit box)
	Make sure you transfer every extract exactly to the same well position of the empty deep well plate. We recommend the use of a multichannel pipette. Condition pipette tips: aspirate and dispense 3 times before transferring!
	It is strongly recommended to run the LC plate (labeled “Use for LC“) first, since biogenic amines show limited stability over a longer period after adding water. Run the LC plate latest the next day after preparation.
13	The LC and FIA analyses are performed using two separate plates at different concentrations.
14	**Dilute extracts for LC:** Remove 150 µL from each well of the capture plate (labeled “Use for FIA“) and transfer it to the empty 96 deep well plate labeled “Use for LC“. Add 150 µL water to each well and seal the plate with a silicone mat. Make sure that the silicone mat is sealed completely on the plate. Firmly press the mat’s naps into the wells. Shake for 5 min at 500 rpm.
15	**Dilute extracts for FIA:** Use the plate labeled “Use for FIA”. Add 250 µL of *FIA Solvent* (see 7.4.4) to each well and seal the plate with a silicone mat. Make sure that the silicone mat is seated correctly on the plate. Firmly press the mat’s naps into the wells. Shake for 5 min at 500 rpm.
16	Both plates, labeled “Use for LC“ and “Use for FIA“, are now ready for LC-MS and FIA-MS analysis, respectively. Place the sealed plates into the autosampler at 10 °C or store at 4 °C.
	In case of any delays store the plates at +4 °C. The autosampler stability is: • LC part: 2 days • FIA part: 7 days. *In case of extract evaporation, fill up the wells to the original volume with methanol and shake the plate.* • **Never store the LC plate or the FIA plate below 0 °C!**
17	Continue with section 7.

8 Processing the Kit Plate with the Mass Spectrometer

Ensure that the mass spectrometer meets all manufacturer specifications and has been properly calibrated. Take care that the deep well capture plate (sealed with the silicone mat) is in the correct autosampler rack. For additional information, refer to the Biocrates® Video Tutorial:

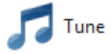
YouTube 9B MS Measurement: Batch File/Acquisition Method Import for Thermo MS Instruments

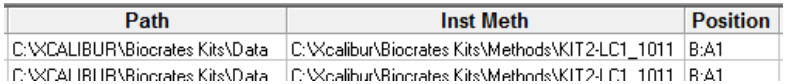
8.1 LC part

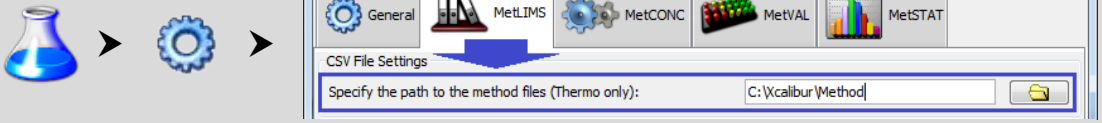
We recommend running the LC-MS assay first. The autosampler stability of some biogenic amines is shorter compared to the analytes of the FIA part. For the LC-MS assay, the data processing and concentration calculation is performed using the Xcalibur™ software. For first time users, we recommend beginning with a 24-well Starter Kit before attempting a full plate.

! Use **ESI probe position ring B**.

Step	Instructions
1	Place the LC Kit plate in the autosampler.
2	Open the “Tune” window and turn on the instrument.
3	Load the Tune File *KIT2-LCtune1_1x1x.mstune*
i	Before starting an injection, wait until all LC-MS parameters are stable.



Step	Instructions
⚠	After each injection, MS parameters (e.g. *Capillary Temperature*) from the active *Tune File* are applied. To reduce batch run times and to guarantee reliable Kit performance, load the Tune File *KIT2-LCtune1_1x1x.mstune* before starting the Kit run. ***Note:*** If another Tune File is active, MS parameters (e.g. *Capillary Temperature*) may not be stable during a Kit run.
ℹ	Only continue with the next steps if the system suitability test passed. If not done before the Kit preparation perform a system suitability test according to section 6.5.1 *SST – LC part* and 6.5.2 *SST – FIA part*
4	Before the first injection, equilibrate the system at starting conditions (100% Solvent A, flow rate 0.8 mL/min, column oven temperature of 50 °C).
5	Import the LC sequence file (.csv file, created by Met*IDQ*™ in section 7.4.5) into Xcalibur™ before starting with the mass spectrometer processing. Go to the Thermo Xcalibur™ *Sequence Setup*. 1. From the menu bar, select ***File > Import Sequence***. 2. Click *Browse*. 3. Successively import both .csv sequence file for the LC part. *Note:* In the “Import Sequence” Window keep all check-boxes selected. 4. Click Open to import each file.
6	In the Xcalibur™ sequences check the columns *Path, Inst Meth* and *Position*. - *Path*: define folder where .raw files are written e.g. C:\Xcalibur\Biocrates Kits\Data - *Inst Meth: selct folder where acquisition method is located,* e.g. *C:\Xcalibur\Biocrates Kits\Methods* - *Position: place the kit plate in the defined tray* e.g. blue 

Step	Instructions
	The *Path* of the acquisitions methods in the Xcalibur™ sequence can be defined in Met*IDQ*™. Refere to the Met*IDQ*™ user manual (*UM-MetIDQ-Carbon-#.pdf*), section 4.1.5 *Export Worklist for Kit measurement*. 
7	Before starting the Kit analysis, double-check each Xcalibur™ sequence. The *Blank* sample will be injected three times to condition the system.
	Do not rename or alter the samples, data files, or acquisition methods. Otherwise the data files will not be compatible with Met*IDQ*™.
8	Submit the sequence and monitor the data from the first injections to ensure that the assay is running properly.

8.2 FIA part

!	Use **ESI probe position ring B**.

⚠	The FIA part is more sensitive to autosampler contaminations than the LC part, as hundreds of lipids are analyzed. In our experience, autosamplers are often contaminated with lipids. Make sure that all system parts are cleaned and the background noise level is low. If necessary, clean the autosampler parts such as needle, needle seat, sample loop, or switching valves.

Step	Instructions
1	Remove the column and connect the autosampler directly to the ion source. Alternatively, a bypass or separate column line (without a column installed) can be used. In this case, make sure to have as few connecting parts as possible to minimize dead volume and obtain the best FIA "peaks". Flush the system with the *FIA Solvent*.
2	Place the FIA Kit plate in the autosampler.
3	Open the "Tune" window and turn on the instrument.
4	Load the Tune File *KIT3-FIA_1x1x.mstune*
i	Before starting an injection, wait until all LC-MS parameters are stable.
⚠	After each injection, MS parameters (e.g. *Capillary Temperature*) from the active *Tune File* are applied. To reduce batch run times and to guarantee a good Kit performance load the Tune File *KIT3-FIA_1x1x.mstune* before starting the Kit run. ***Note:*** If another Tune File is active, MS parameters (e.g. *Capillary Temperature*) may not be stable during a Kit run.
i	Only continue with the next steps if the system suitability test (SST, see 6.5.2) passed.
5	Before the first injection flush all lines with *FIA Solvent* (100% *FIA Solvent*, flow rate 0.2 mL/min for 10 min).

<table>
<tr><th>Step</th><th>Instructions</th></tr>
<tr><td>6</td><td>Import the FIA sequence file (.csv file, created by MetIDQ in section 7.4.5) into Xcalibur™ before starting with the mass spectrometer processing.
Go to the Thermo Xcalibur™ Sequence Setup.
1. From the menu bar, select File > Import Sequence.
2. Click Browse.
3. Successively import both .csv sequence file for the FIA part.
<u>Note:</u> In the “Import Sequence” Window keep all check-boxes selected.
4. Click Open to import each file.</td></tr>
<tr><td>7</td><td>In the Xcalibur™ sequences check the columns Path, Inst Meth and Position.
- Path: define folder where .raw files are written
e.g. C:\Xcalibur\Biocrates Kits\Data
- Inst Meth: selct folder where acquisition method is located,
e.g. C:\Xcalibur\Biocrates Kits\Methods
- Position: place the kit plate in the defined tray
e.g. blue
<table><tr><th>Path</th><th>Inst Meth</th><th>Position</th></tr><tr><td>C:\XCALIBUR\Biocrates Kits\Data</td><td>C:\Xcalibur\Biocrates Kits\Methods\KIT2-LC1_1011</td><td>B:A1</td></tr><tr><td>C:\XCALIBUR\Biocrates Kits\Data</td><td>C:\Xcalibur\Biocrates Kits\Methods\KIT2-LC1_1011</td><td>B:A1</td></tr></table></td></tr>
<tr><td>8</td><td>Before starting the Kit analysis, double-check each Xcalibur™ sequence.
The Blank sample will be injected three times to condition the system.</td></tr>
<tr><td>  </td><td>Do not rename the samples, data files, or acquisition methods. Otherwise the data files will not be compatible with MetIDQ™.</td></tr>
<tr><td>9</td><td>Submit the sequence and monitor the data from the first injections to ensure that the assay is running properly.</td></tr>
</table>

Recommended cleaning of tubing and ESI electrode

The FIA method operates at relatively low flow rates using biological samples and matrix, therefore, deposits can remain in the tubing or ESI electrode. It is recommended to integrate routine washing steps to ensure a robust performance of the Kit. We suggest the following or similar washing steps.

1. Wash tubing and ESI probe for 60 min using water at a flow rate of 0.5 mL/min.
2. Wash tubing and ESI probe for 60 min using isopropanol at a flow rate of 0.5 mL/min.
3. Wash tubing and ESI probe for 20 min using *FIA Solvent* at a flow rate of 0.5 mL/min.

Troubleshooting: One of the most likely reasons for instabilities in the FIA profile is that there are deposits in the ESI electrode or tubing. It is recommended to monitor the backpressure pressure profile during Kit runs (when the flow rate is at 0.2 mL/min). A significant increase in pressure is an indication for the need to clean or replace the ESI electrode or tubing.

9 Data Processing – LC Part

The quantitation of the FIA part is performed automatically by the Met*IDQ*™ software and is described in the software manual. The LC data quantitation is carried out in the Xcalibur™ software. The quantitation process is based on a seven point calibration and internal standard normalization. Relevant calibration parameters such as calibration standard concentrations can be found in the Analytical Specifications ("AS-p400-HR-#.pdf" on the USB stick). Follow the instructions in this section or review the following Biocrates® Video Tutorial:

10C Thermo MS Measurement: Quantitation, Result File Import, Validation in MetIDQ

9.1 Quantitation Method

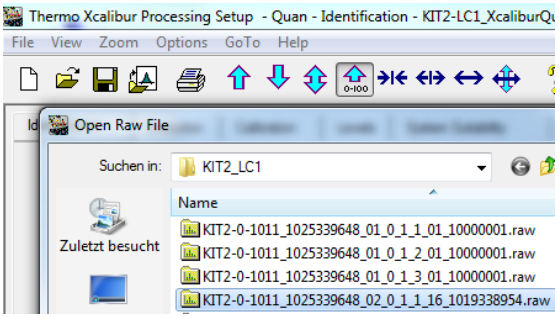
The required quantitation methods are provided with the Kit USB stick. Use the appropirate quantitation methods for your Q Exactive™ and update the retention times (see next section).

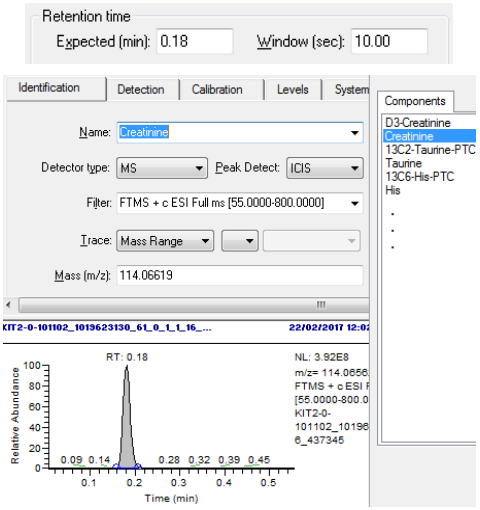
MS Instrument	**Q Exactive™ Focus**	**Q Exactive™**
LC variant	UHPLC	UHPLC
KIT2 LC quantitation methods	KIT2-LC1_XcaliburQuan_1011.pmd KIT2-LC2_XcaliburQuan_1012.pmd	KIT2-LC1_XcaliburQuan_1111.pmd KIT2-LC2_XcaliburQuan_1112.pmd

MS Instrument	**Q Exactive™ Plus**	**Q Exactive™ HF**
LC variant	UHPLC	UHPLC
KIT2 LC quantitation methods	KIT2-LC1_XcaliburQuan_1211.pmd KIT2-LC2_XcaliburQuan_1212.pmd	KIT2-LC1_XcaliburQuan_1311.pmd

9.2 Adjust Retention Times

Before starting with the quantitation process, adjust the retention times in the quantitation methods.

Step	Instructions	Examples
	LC injection 1 – Quantitation Method	
1	Open the quantitation method *KIT2-LC1_XcaliburQuan_1x11.pmd* ***or*** *KIT2-LC1_XcaliburQuan_1311.pmd* – Q Exactive™ HF only – with the ***Xcalibur™ > Processing Setup***.	
2	Open the .raw file of *Calibration Standard* level 5 (Cal 5) acquired with the method *KIT2-LC1_1x11.meth*. Cal 5 has the barcode 1019338954 and is injected from well position 02 by default. 1006062609 Example file name of Cal 5: KIT2-0-1011_1025339648_02_0_1_1_16_1019338954.raw	

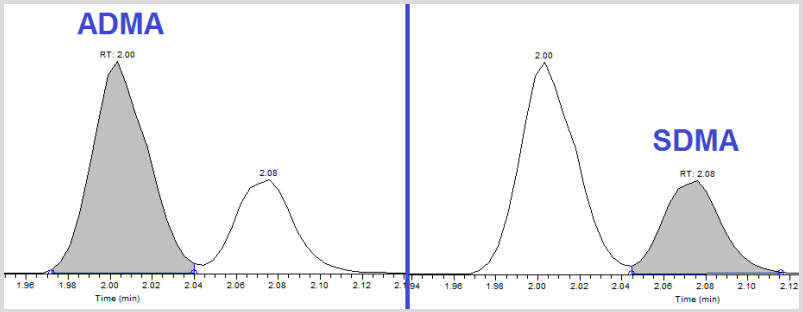
3	Check the integration (under the “detection tab”) and RT (under the “Identification” tab) of every analyte and internal standard. If required, adjust the - Retention time (RT) - Peak integration parameter To apply changes click “OK”. Finally save all changes.	

Peak integration:

ADMA and SDMA:

They have the same mass and are separated by chromatography.

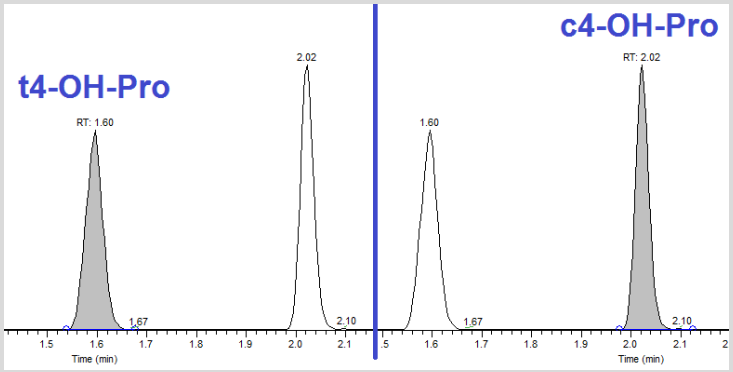
- 1st peak: ADMA
- 2nd peak: SDMA



t4-OH-Pro and c4-OH-Pro:

They have the same mass and are separated by chromatography.

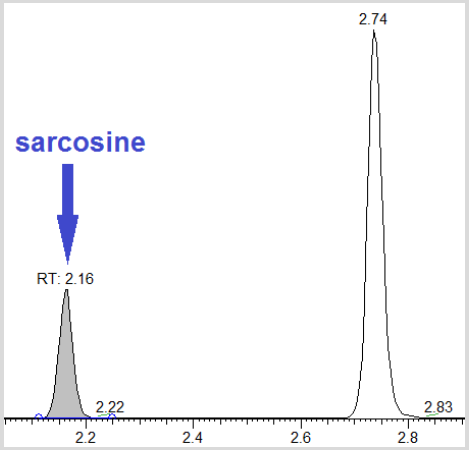
- 1st peak: t4-OH-Pro
- 2nd peak: c4-OH-Pro



Sarcosine:

It is always the first peak.

- 1st peak: sarcosine

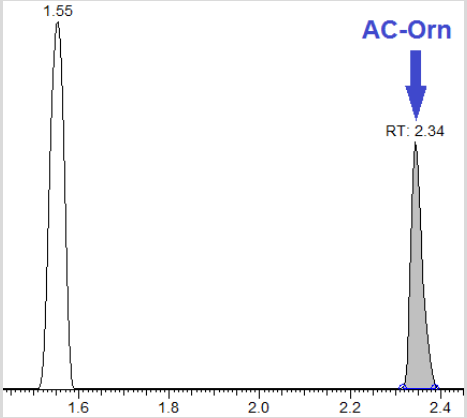


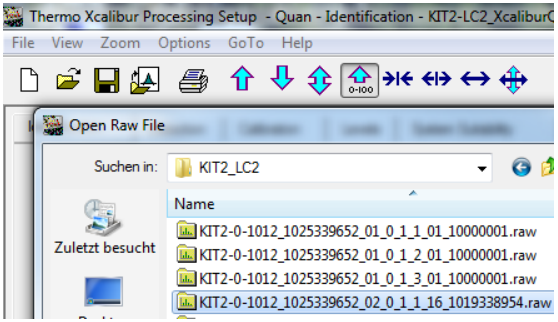
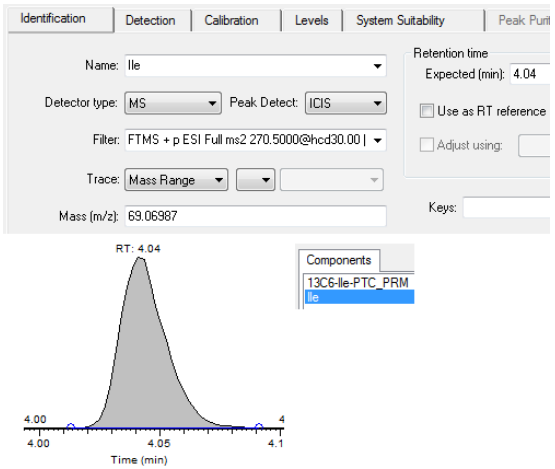
Ac-Orn:

It is always the second peak.

Note: Using the Testmix LC there will only be one peak that is Ac-Orn.

- Tesmtix LC: only 1 peak
- Kit: 2nd peak



	LC injection 2 – Quantitation Method (not applicable to Q Exactive™ HF)	
4	Open the quantitation method *KIT2-LC2_XcaliburQuan_1x12.pmd* with the ***Xcalibur™ > Processing Setup***.	
5	Open the .raw file of Cal 5 acquired with the method *KIT2-LC1_1x12.meth*, e.g. KIT2-0-1012_1025339652_ 02_0_1_1_16_1019338954.raw	
6	Check the integration of *Ile* and its internal standard (under the “detection tab”) and RT (under the “Identification” tab). If required, adjust the - Retention time (RT) - Peak integration parameter To apply changes click “OK”. Finally save all changes.	

9.3 Quantitation Procedure

It is recommended to have an identical folder structure on both the MS-PC (PC that operates the instrument) and PC used for MS data quantitation.

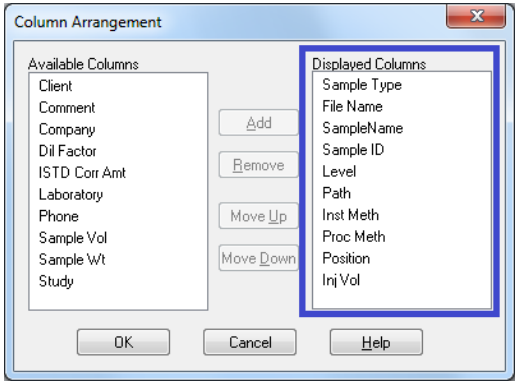
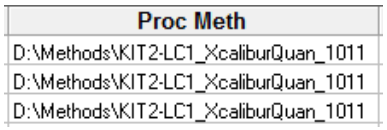
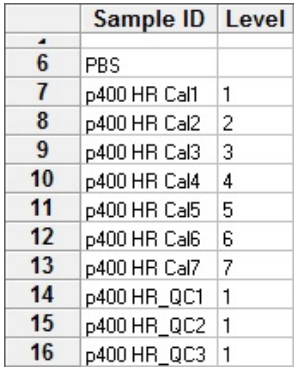
File name example:

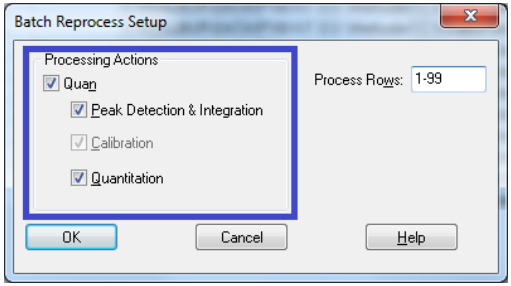
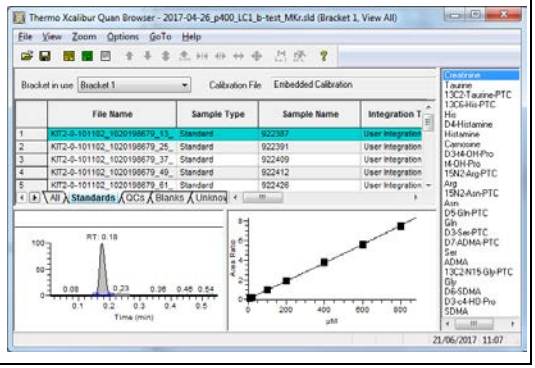
KIT2-0-1011_1013396222_01_0_1_1_01_10000001.raw

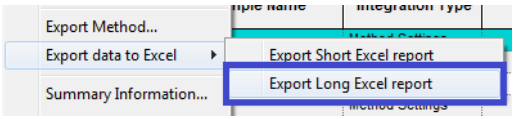
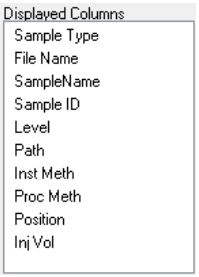
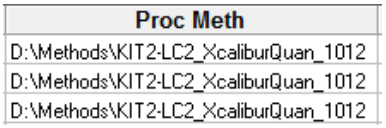
OP-Name_plate barcode_well position_acquisition method_

run number_injection_number_Sample type ID_sample barcode

Step	Instructions	Examples
	We recommend performing the quantitation on another PC, e.g. office PC. Copy all data (MS data, sequence list, quantitation methods) from the data acquisition PC (MS-PC) to the quantitation PC.	
	Sequence lists for quantitation: Except for Q Exactive™ HF, **two sequence lists** with separate quantitation methods are required according to the 1st (LC1) and 2nd (LC2) injections. For Q Exactive™, Q Exactive™ Focus and Q Exactive™ Plus use: - *KIT2-LC1_XcaliburQuan_1x11.pmd* and - *KIT2-LC2_XcaliburQuan_1x12.pmd* For Q Exactive™ HF use: - *KIT2-LC1_XcaliburQuan_1311.pmd* The required sample lists were generated by Met*IDQ*, see section 7.4.5.	

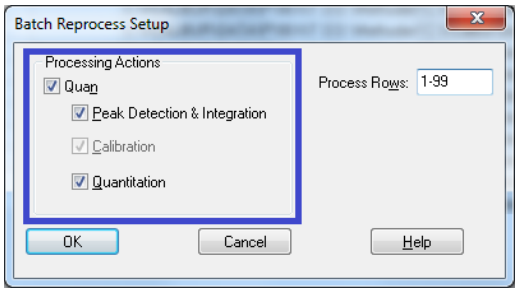
Step	Instructions	Examples
	LC injection 1 – Quantitation (all Q Exactive™ types)	
1	Open the sequence of LC injection 1 (acquisition method *KIT2-LC1_1x11.pmd*) in the Xcalibur™ *Sequence Setup* window. Make sure that the columns “Level” and “Proc Meth” are shown. They can be added via ***Change > Column Arrangement...***. Also add the other columns which are shown in the list “Displayed Column” according to the screenshot (order not important). Click “Ok”.	
2	Select the quantitation method: Double-click in the first cell of the column “Proc Meth” and select - *KIT2-LC**1**_1x1**1**.meth* with updated retention times according to section 9.2.	
3	In the column “Level“, check the levels for calibration standards and QCs. - Calibration standards: e.g. Cal 3 > Level 3 - QCs: all QCs levels > Level 1 ***Note:*** For all QCs, Level1 is used, as the accuracies are checked in Met*IDQ*™.	

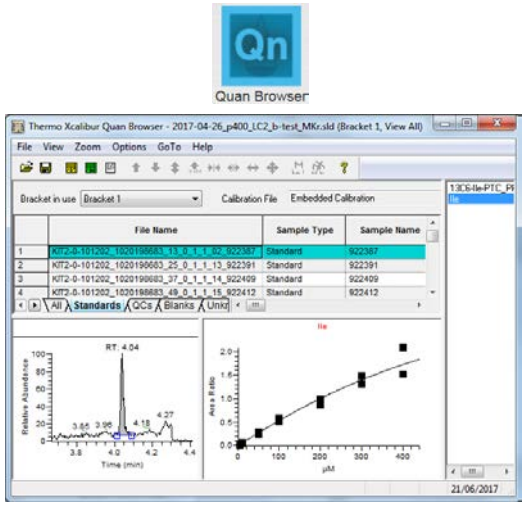
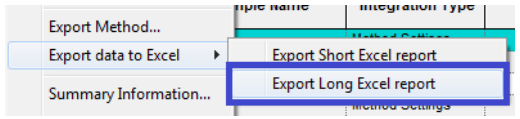
Step	Instructions	Examples
4	Start the quantitation process: 1. Click on the button “Batch Reprocess Setup” 2. Select ☑**Quan** ☑**Peak Detection & Integration** ☑Calibration ☑**Quantitation** 3. Click “OK”. The quantitation process can take several minutes. Wait until the processing window disappears. ℹ During this process .rst files are created.	  
5	- Open *Quan Browser.* - Open the sequence list file (.sld) used for data acquisition and quantitation. - Select “Show All sample types” in the pop-up window. - Evaluate the calibration curves and the peak integration. - Page through all analytes and ISTD, if necessary adjust the peak integration.	 

Step	Instructions	Examples
6	Export the quantitation results: From the menu bar, select ***File > Export data to Excel > Export long Excel report*** The results are saved as Excel file (.xls) in the "Data" folder.	
LC injection 2 – Quantitation (not applicable to Q Exactive™ HF)		
7	Open the sequence of LC injection 2 (acquisition method *KIT2-LC2_1x12.pmd*) in the Xcalibur™ Sequence Setup window. Make sure that the columns shown in the screenshot are visible.	
8	Select the quantitation method: Double-click in the first cell of the column "Proc Meth" and select - *KIT2-LC2_1x12.pmd* with updated RTs, according to section 9.2.	

<table>
<tr><th>Step</th><th>Instructions</th><th>Examples</th></tr>
<tr><td>9</td><td>In the column “Level“, check the levels for calibration standards and QCs.
- calibration standards: e.g. Cal 3 > Level 3
- QCs: all QCs levels > Level 1

<u>Note:</u> For all QCs Level1 is used, as the accuracies are checked in MetIDQ™.</td><td><table><tr><th></th><th>Sample ID</th><th>Level</th></tr><tr><td>6</td><td>PBS</td><td></td></tr><tr><td>7</td><td>p400 HR Cal1</td><td>1</td></tr><tr><td>8</td><td>p400 HR Cal2</td><td>2</td></tr><tr><td>9</td><td>p400 HR Cal3</td><td>3</td></tr><tr><td>10</td><td>p400 HR Cal4</td><td>4</td></tr><tr><td>11</td><td>p400 HR Cal5</td><td>5</td></tr><tr><td>12</td><td>p400 HR Cal6</td><td>6</td></tr><tr><td>13</td><td>p400 HR Cal7</td><td>7</td></tr><tr><td>14</td><td>p400 HR_QC1</td><td>1</td></tr><tr><td>15</td><td>p400 HR_QC2</td><td>1</td></tr><tr><td>16</td><td>p400 HR_QC3</td><td>1</td></tr></table></td></tr>
<tr><td>10</td><td>Start the quantitation process:
1. Click on the button “Batch Reprocess Set-up”.
2. Select ☑Quan
☑Peak Detection & Integration
☑Calibration
☑Quantitation
3. Click “OK”.

The quantitation process can take several minutes. Wait until the processing window disappears.</td><td> 
  </td></tr>
</table>

Step	Instructions	Examples
11	- Open *Quan Browser.* - Open the sequence list file (.sld) used for data acquisition and quantitation. - Select “Show All sample types” in the pop-up window or the “All” tab below the sample list. - Evaluate the calibration curves and the peak integration. - Page through all analytes and ISTD, if necessary adjust the peak integration.	
12	Export the quantitation results: From the menu bar, select ***File > Export data to Excel > Export long Excel report*** The results are saved as Excel file (.xls) in the folder “Data”.	
13	**Import the kit data into Met*IDQ*™:** • Quantitation results (.xls files) from LC injection 1 and 2 • MS data (.raw files) from FIA injections 1 and 2	
	The accuracy of the calculated QC concentrations is checked in Met*IDQ*™. The accuracies are calculated by Met*IDQ*™ and visualized in ***MetVAL*** after importing the result files (.xls).	

Please refer to the Met*IDQ*™ Carbon user manual section **5. Converting and Importing Mass Spectrometer Data**. To evaluate the results and perform statistics, you can use the Met*IDQ*™ tool **StatPack,** as well as other tools such as **MetaboAnalyst**.

10 Appendix

10.1 Pump Settings

UHPLC Gradient for methods *KIT2-LC1_1x11.meth* and *KIT2-LC2_1x12.meth*

No	Time (min)	Flow (mL/min)	B (%)	Curve
1	0.00	Run		
2	0.00	0.8	0.0	5
3	0.25	0.8	0.0	5
4	1.50	0.8	12.0	5
5	2.70	0.8	17.5	5
6	4.00	0.8	50.0	5
7	4.50	0.8	95.0	5
8	4.70	1.0	95.0	5
9	5.10	1.0	95.0	5
10	5.25	1.0	0.0	5
11	5.80	0.8	0.0	5
12	5.81	Stop Run		

FIA Gradient for methods *KIT3-FIA1_1x11.meth*, *KIT3-FIA2_1x12.meth* and *KIT3-FIA_SST_1x11.meth*

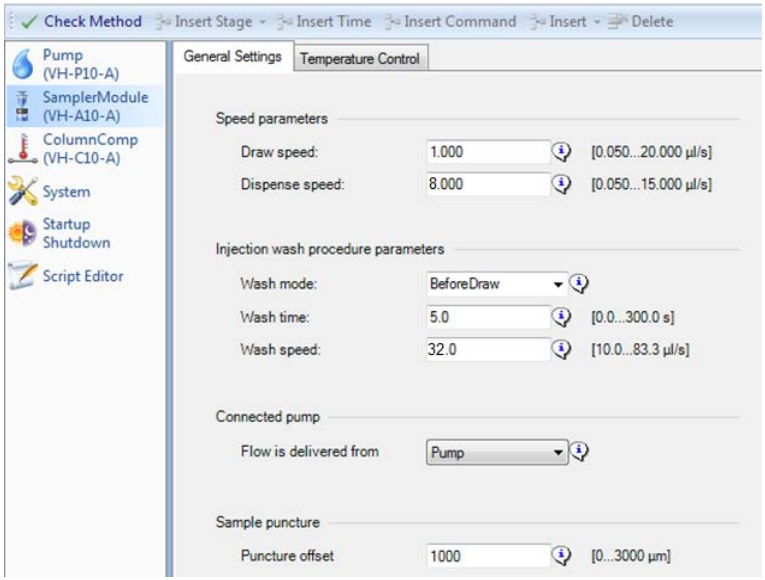
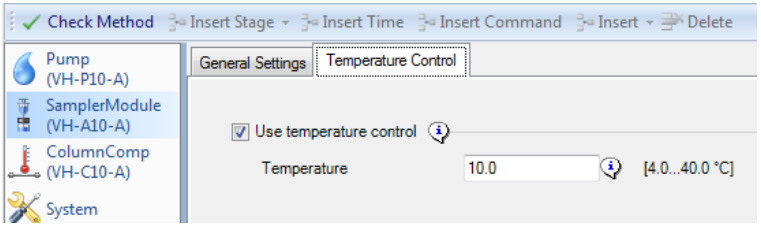
No	Time (min)	Flow (mL/min)	B (%)	Curve
1	0.00	Run		
2	0.00	0.05	0.0	5
3	1.40	0.05	0.0	5
4	1.60	0.20	0.0	5
5	2.80	0.20	0.0	5
6	3.00	0.05	0.0	5
7	3.01	Stop Run		

10.2 Autosampler and Column Oven settings

10.2.1 Thermo Vanquish™

Autosampler

Parameter	*Value*
Wash Solvent	25% acetonitrile, 25% methanol, 25% isopropanol, 25% water
Seal Wash	75% isopropanol, 25% water, 0.1% formic acid
Needle Height	100 µm
Plate Type	ThermoVial54 (for testmix vials) WellPlate96 (for Kit plate)
Post injection mixer	Viper 0.13 x 35 mm capillary ***or*** 7-port column selection valve
Speed parameters (of syringe)	Draw speed LC: 1 µL/s FIA: 2 µL/s Dispense speed 8 µL/s
Wash procedure	Wash mode Before Draw Wash time 5 sec Wash speed 32 µL/s
Sample temperature	10 °C

Parameter	*Value*
Sampler Module	 

Column Compartment

Parameter	*Value*
Column Oven	Temperature: 50 °C
Column Pre-Heater	Temperature: 50 °C
Post Column Cooler	Temperature: 40 °C

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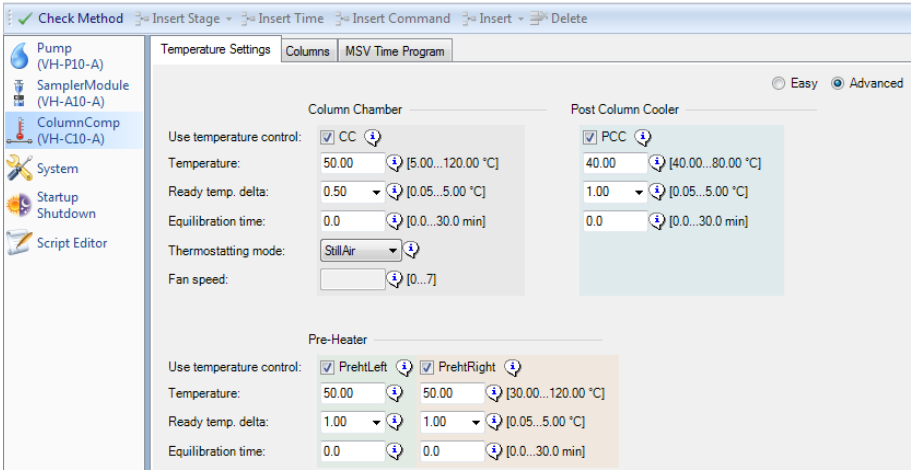
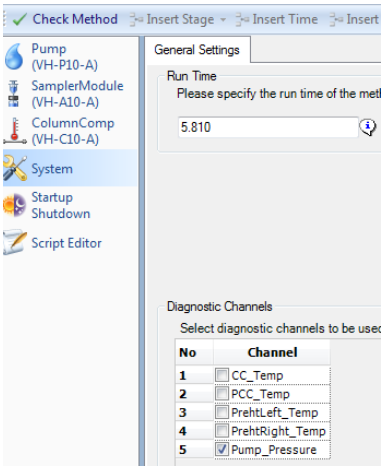
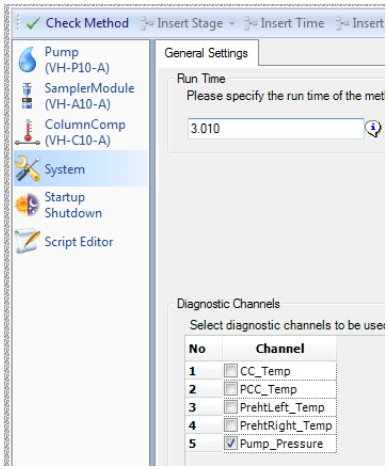
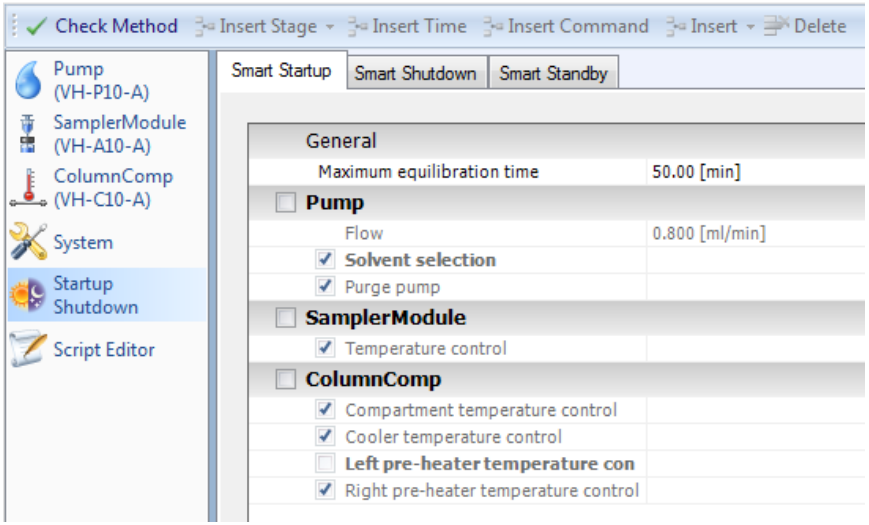
Parameter	Value
Column Compartment	

General Settings

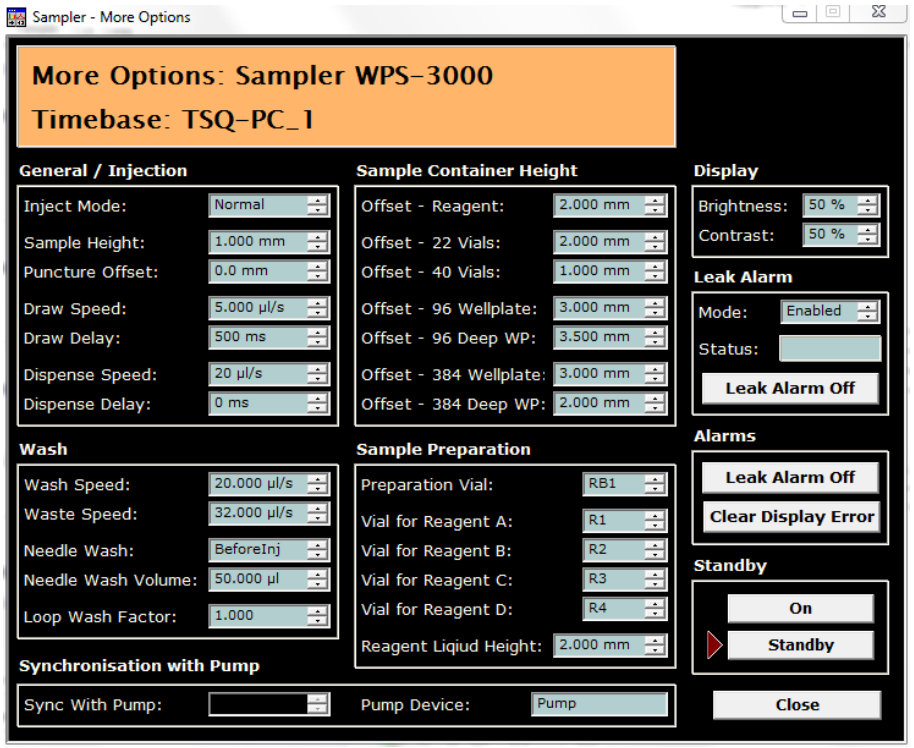
Parameter	Value	
	LC	FIA
System		

Parameter	*Value*
Startup Shutdown	

10.2.2 Thermo UltiMate™ 3000 RS

Autosampler

Parameter	*Value*
Wash Solvent	25% acetonitrile, 25% methanol, 25% isopropanol, 25% water
Seal Wash	10% methanol, 90% water
Plate Type	40 Vials (for testmix vials) 96-Deepwells (for Kit plate)
General/Injection	Injection mode Normal Sample height 1 mm Puncture offset 0.0 mm Draw speed 5 µL/s Draw delay 500 ms Dispense speed 20 µL/s Dispense delay 0 ms
Wash procedure	Wash Speed 20 µL/s Waste speed 32 µL/s Needle wash BeforeInj Needle wash volume 50 µL Loop wash factor 1
Sample temperature	10 °C

Parameter	Value
Sampler	

10.2.3 Injection Volume

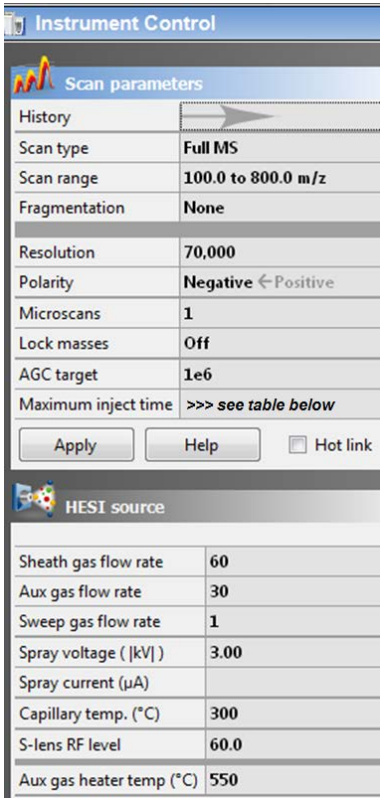
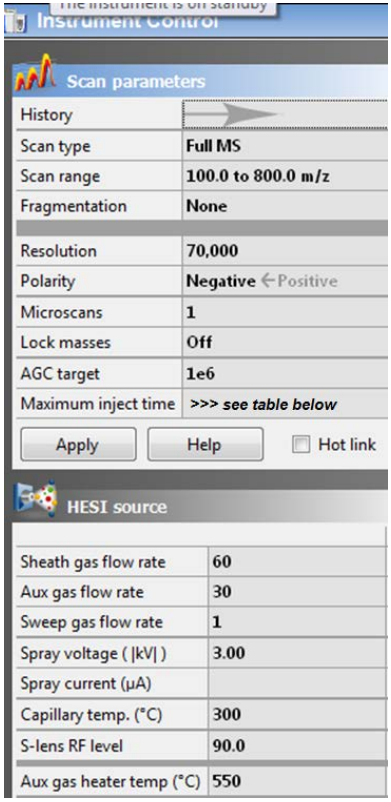
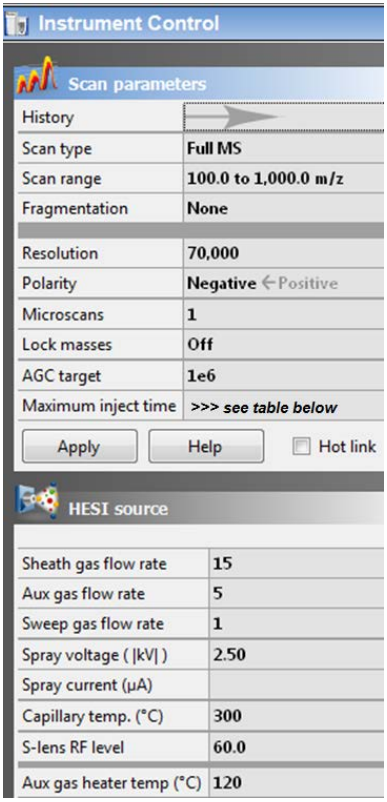
Parameter	Method	Value
Injection Volume	LC	5 µL
	FIA	20 µL

10.3 MS Settings and Tune Files

Tune file	*File name*
LC tune 1	*KIT2-LCtune1_1x1x.mstune*
LC tune 2	*KIT2-LCtune2_1x1x.mstune*
FIA tune	*KIT3-FIA_1x11.mstune*

⚠ **Apply ALL values to positive AND negative polarity mode!**

	tune file →	***LC tune 1***	***LC tune 2***	***FIA tune***
Option	***Parameter***	***Value***	***Value***	***Value***
Scan parameters	Scan type	Full MS	Full MS	Full MS
	Scan range [m/z]	100.0 to 800.0	100.0 to 800.0	100.0 to 1 000.0
	Fragementation	None	None	None
	Resolution	*see page 110*	*see page 110*	*see page 110*
	Polarity	Positive/Negative	Positive/Negative	Positive/Negative
	Microscans	1	1	1
	Lock masses	Off	Off	Off
	AGC target	1e6	1e6	1e6
	Maximum injection time (IT)	*see page 110*	*see page 110*	*see page 110*
HESI source	Sheath gas flow rate	60	60	15
	Aux gas flow rate	30	30	5
	Sweep gas flow rate	1	1	1
	Spray voltage [kV]	3.00	3.00	2.50
	Spray current [µA]	---	---	---
	Capillary temp. [°C]	300	300	300
	S-lens RF level	60	90	60
	Aux gas heater temp [°C]	550	550	120
	ESI probe position	ring B	ring B	ring B

LC tune 1	*LC tune 2*	*FIA tune*
		

Shown *Resolution* and *Maximum IT* for Q Exactive™ Focus.

➔ For other Q Exactive™ platforms refer to page 110.

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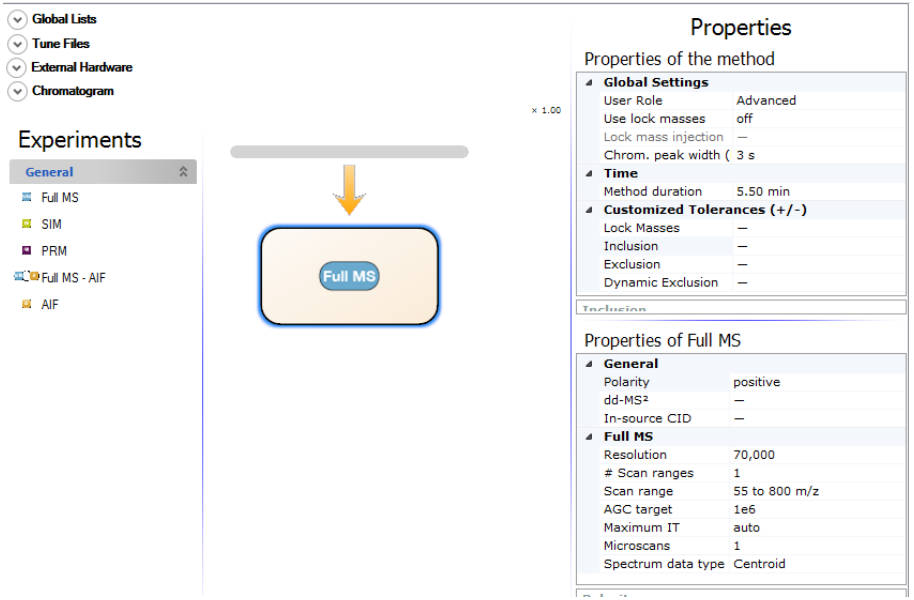
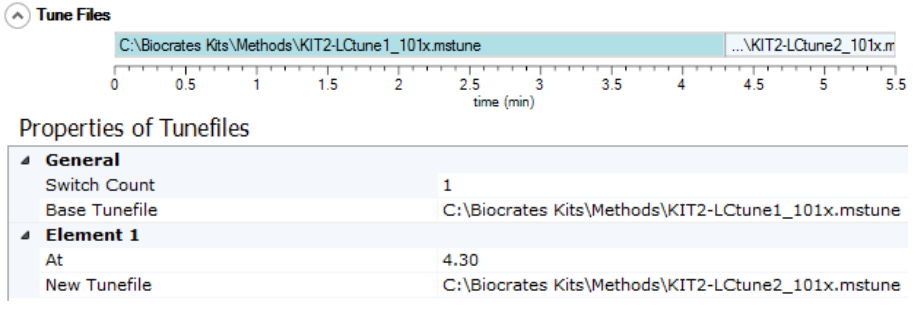
LC1 Method for Q Exactive™, Q Exactive™ Focus and Q Exactive™ Plus

KIT2-LC1_1x11.meth

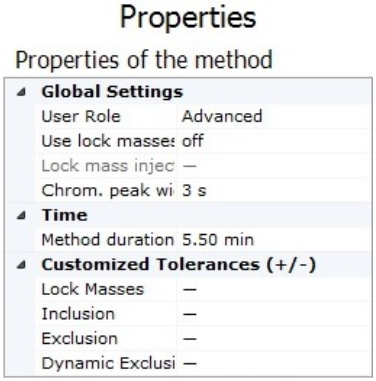
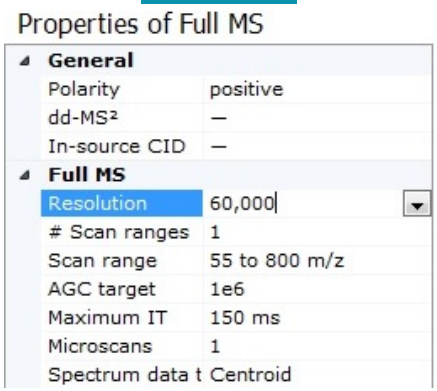
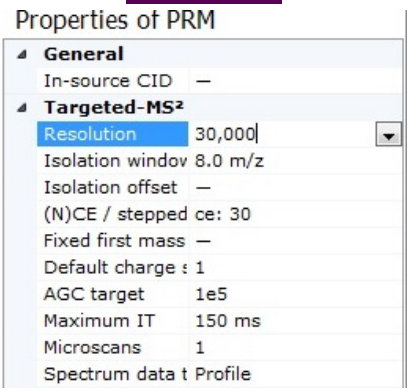
Parameter	Value
General Settings *Q Exactive™ platform*	
Tune Files *Q Exactive™ platform*	

Shown *Resolution* and *Maximum IT* for Q Exactive™ Focus.

➔ For other Q Exactive™ platforms refer to page 110.

LC Method for **Q Exactive™ HF** (*KIT2-LC1_1311.meth*)

Method parameter **only** for **Q Exactive™ HF (combined *Full MS* + *PRM*)**.

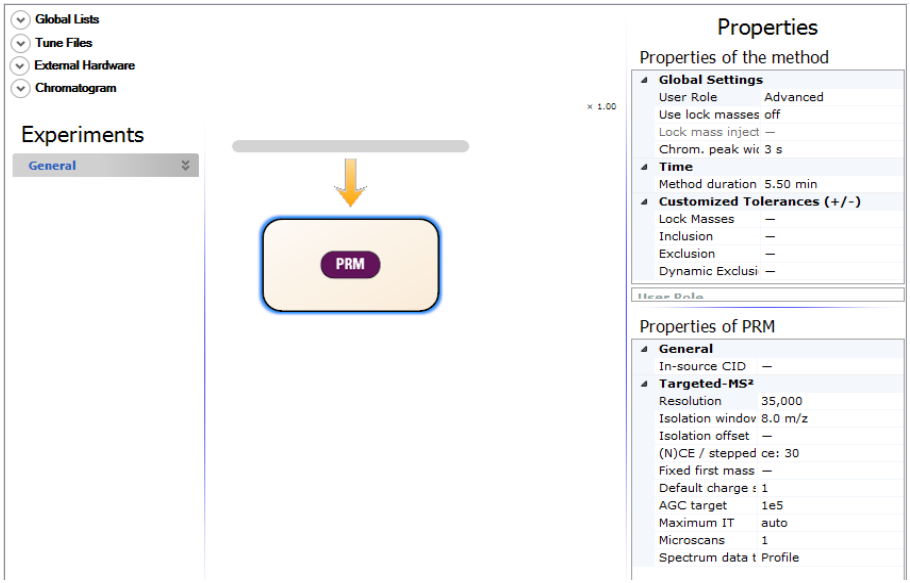
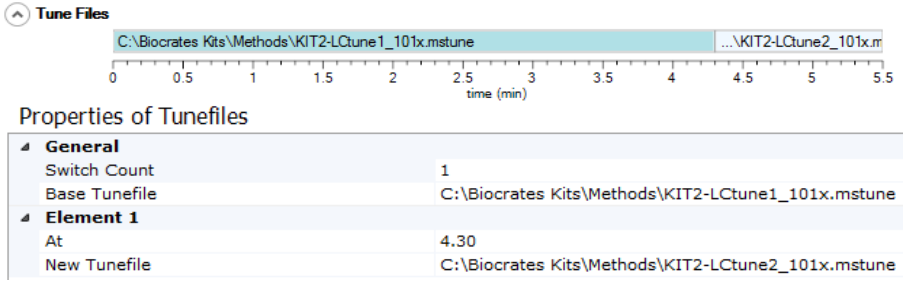
Parameter	*Value*
General Settings *Q Exactive™ platform*	 ➢ add *Full MS* and *PRM* to one method  **Full MS**  **PRM** 
Inclusion List **PRM**	

Parameter	Value
Tune Files *Q Exactive™ platform*	

Using a Q Exactive™ HF, the data acquisition is fast enough to combine *Full MS* and *PRM* in one method without loss of analysis quality.

LC2 Method for Q Exactive™, Q Exactive™ Focus and Q Exactive™ Plus

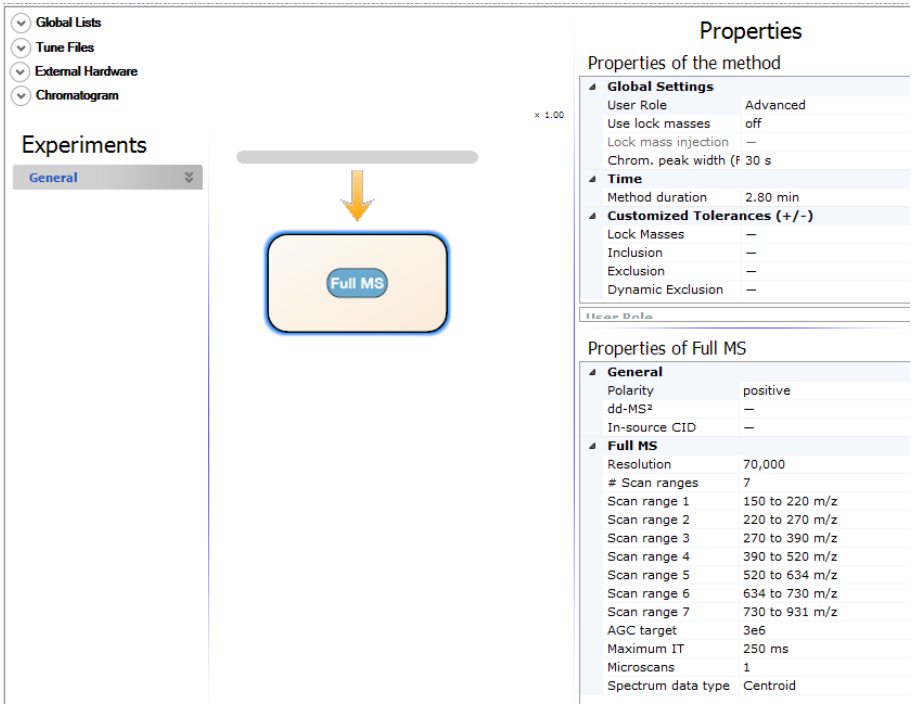
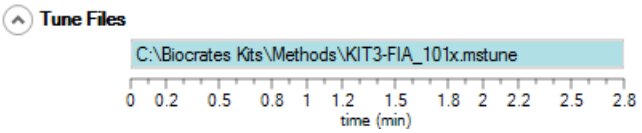
KIT2-LC2_1x12.meth

Parameter	*Value*
General Settings *Q Exactive™ platform*	
Inclusion List *Q Exactive™ platform*	
Tune Files *Q Exactive™ platform*	

Shown *Resolution* and *Maximum IT* for Q Exactive™ Focus.
➔ For other Q Exactive™ platforms refer to page 110.

FIA_SST Method (all Q Exactive™ platforms)

KIT3-SST_1x11.meth

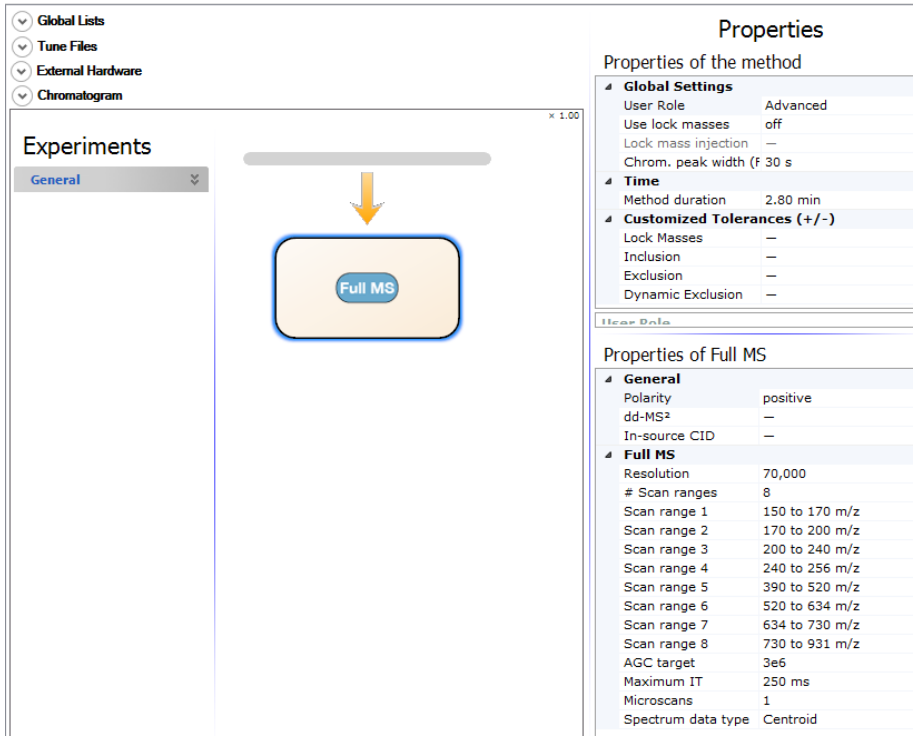
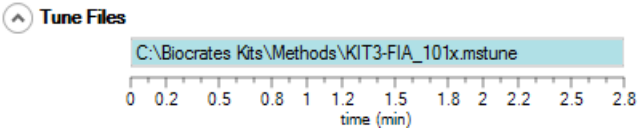
Parameter	Value
General Settings *Q Exactive™ platform*	
Tune Files *Q Exactive™ platform*	

Shown *Resolution* and *Maximum IT* for Q Exactive™ Focus.

➔ For other Q Exactive™ platforms refer to page 110.

FIA1 Method (all Q Exactive™ platforms)

KIT3-FIA1_1x11.meth

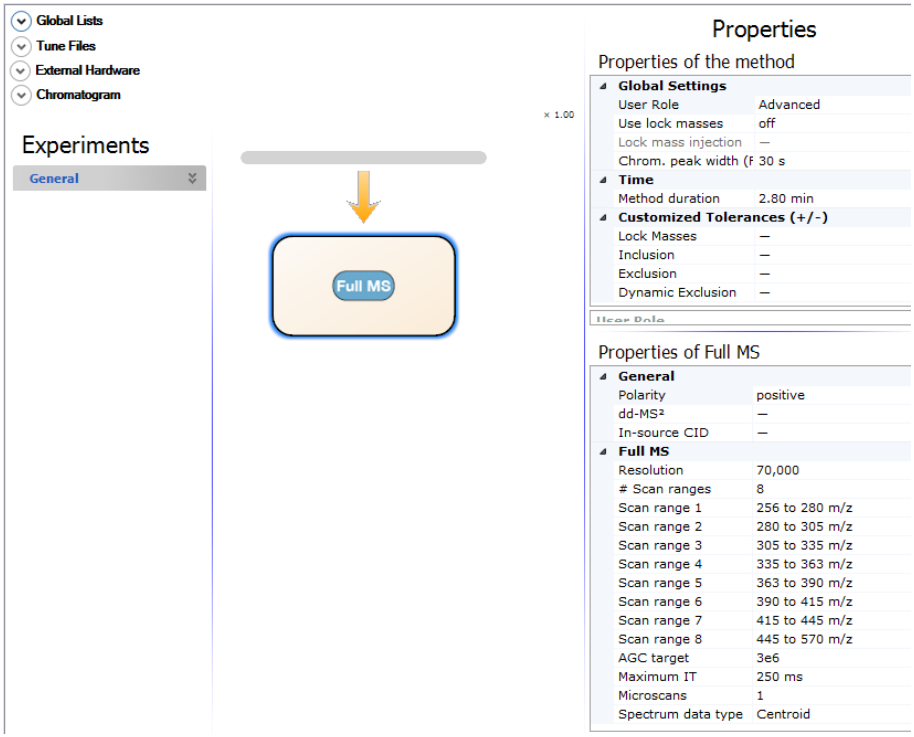
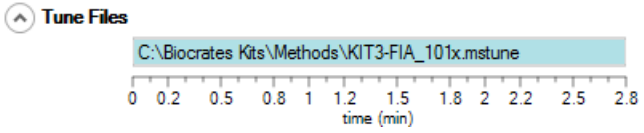
Parameter	*Value*
General Settings *Q Exactive™ platform*	
Tune Files *Q Exactive™ platform*	

Shown *Resolution* and *Maximum IT* for Q Exactive™ Focus.

➔ For other Q Exactive™ platforms refer to page 110.

FIA2 Method (all Q Exactive™ platforms)

KIT3-FIA2_1x12.meth

Parameter	Value
General Settings *Q Exactive™ platform*	
Tune Files *Q Exactive™ platform*	

Shown *Resolution* and *Maximum IT* for Q Exactive™ Focus.

➔ For other Q Exactive™ platforms refer to page 110.

MS resolution for Tune Files and Acquisition Methods

	Q Exactive™ Focus	*Q Exactive™*	*Q Exactive™ Plus*	*Q Exactive™ HF*
LC tune 1	70 000	70 000	70 000	60 000
LC tune 2				
FIA tune				120 000
LC1 Method *KIT2-LC1_1x11.meth*	70 000	70 000	70 000	60 000
LC2 Method *KIT2-LC2_1x12.meth*	35 000	35 000	35 000	30 000
FIA_SST Method *KIT3-SST_1x11.meth*	70 000	70 000	70 000	120 000
FIA1 Method *KIT3-FIA1_1x11.meth*				
FIA2 Method *KIT3-FIA2_1x12.meth*				

Maximum IT for Tune Files and Acquisition Methods

	Q Exactive™ Focus	*Q Exactive™*	*Q Exactive™ Plus*	*Q Exactive™ HF*
LC1, LC2	250 ms	250 ms	250 ms	150 ms
FIA_SST, FIA1, FIA2	250 ms	250 ms	250 ms	250 ms

10.4 Abbreviations

µL	microliter
bar	bar
Cal	calibration standard
Da	Dalton
ESI	electrospray ionization
ISTD	internal standard
LC-MS	instrument combination of UHPLC pump and Q Exactive™
min	minutes
mL	milliliter
MS-PC	PC that controls the Q Exactive™ instrument
PRM	parallel reaction monitoring
QC	quality control
RT	retention time
sec	seconds
UHPLC	ultra high performance liquid chromatography

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