

WORK INSTRUCTIONS FOR LCMS DATA ACQUISITION FOR UNTARGETED METABOLOMICS ANALYSIS

MPMF-WIN-0501-V2

1. PURPOSE

This document describes steps required to prepare QExactive Classic LCMS system for data acquisition for metabolomics analysis.

2. RESPONSIBILITIES

- 2.1. It is the responsibility of the Platform manager to oversee the implementation of this procedure and to ensure that any staff member authorised to use this WIN has achieved an acceptable level of understanding and competence in the procedure and use of the equipment.
- 2.2. Approved operators are responsible for ensuring that all instructions covered in this WIN are performed correctly and with care for the equipment. Operators will ensure any deviations from the document will not have a negative effect on sample analysis.

3. HEALTH AND SAFETY CONSIDERATIONS

3.1 General

Appropriate PPE including safety glasses, gloves, laboratory coat, enclosed shoes must be worn. All non-sharps material waste should be disposed of in biohazard bins, Sharps waste including pipette tips must be disposed of in sharps containers and chemical waste disposed of in appropriate class waste container. Lab coat, gloves and safety glasses must be worn during the process.

3.2 Chemical Hazard

Treat all liquid, solid and gaseous reagents and waste as potentially hazardous which can cause injury, illness or death and handle appropriately. Preparation of buffers containing organic acids, organic solvents or toxic class chemicals should be handled in the fume hood, care should be taken to avoid inhalation and skin contact. Dispose of waste properly and carefully following the laboratory waste disposal SOP.

3.3 Burns Hazard

Take precautions when handling equipment – surfaces may be hot and can cause burns. Liquid Nitrogen and dry Ice- can cause burns always wear PPE including cryo gloves and use the fume hood where practical. Liquid Nitrogen is also an Asphyxiant- so there is no warning if the atmosphere has become oxygen deficient.

3.4 Electrical hazard

Take precautions when handling wires or assembling or disassembling and connecting equipment.

3.5 Sharp objects hazard.

Care must be taken when handling a syringe, blade or other sharp objects.

3.6 Further information

Refer <https://www.monash.edu/ohs> for detailed procedural information

Refer to risk assessment 15263 (MPMF: Operation of mass spectrometers); 38195 (MPMF: High Pressure Liquid Chromatography (HPLC) use) in SARAH.

4 PROCEDURE

4.1 Equipment and materials

- 4.1.1 QE mass spectrometer coupled to Dionex Ultimate 3000 RS chromatographic system, 6-port valve and direct infusion setup
- 4.1.2 Water bath sonicator
- 4.1.3 Balances
- 4.1.4 Solution filtration system with vacuum

4.2 Materials

- 4.2.1 Formic acid for LCMS
- 4.2.2 Acetonitrile for LCMS
- 4.2.3 Methanol for LCMS
- 4.2.4 MilliQ water
- 4.2.5 Calmix for positive mode – Thermo Scientific
- 4.2.6 Calmix for negative mode - Thermo Scientific
- 4.2.7 Metabolomics standards – MSMLS standards, Iroa Technologies
- 4.2.8 Metabolomics QC sample – in house
- 4.2.9 Vials, caps and inserts
- 4.2.10 Nylon filter membrane 0.2 μ m
- 4.2.11 Analytical column– pZIC-HILIC, 5 μ m, 4.2 mm x 100mm, Merck, Cat.No.1.50461.0001
- 4.2.12 Guard column– pZIC-HILIC, 5 μ m, 20 x 2.1 mm, Merck, Cat.No.1.50438.0001

4.3 Solution preparation for use in LCMS

Always measure out volatile substances in the fume hood. If dissolving a solid in a solvent, the resulting solution must be filtered. All solutions must be degassed (sonicated for 10 mins) before use in the LCMS system.

4.3.1 Rear wash fluid - 10% (v/v) MeOH

To prepare 1 L of rear wash fluid mix 900 ml of MilliQ water and 100 ml of methanol in a clean Schott bottle. Degas the solution in water bath sonicator for 10 min. Solution can be used within 1 year from the day of preparation.

4.3.2 Syringe wash fluid – 50% (v/v) IPA

To prepare 1 L of 50% (v/v) IPA mix 500 ml of IPA and 500 ml of MilliQ water. Degas the solution in water bath sonicator for 10 min. Use the solution within 1 year from the day of preparation.

4.3.3 Aqueous mobile phase

For metabolomics method use 20 mM ammonium carbonate solution. To prepare 1 L of this solution weigh out 1.9218 g of ammonium carbonate and put directly into Schott bottle, add 1 L of MilliQ water. It should dissolve with gentle swirling. Filter into clean Schott bottle using 0.2 μ m nylon filter. Degas the solution in water bath sonicator for 10 min. Use the solution within 1 month from the day of preparation.

4.3.4 Organic mobile phase

For metabolomics method use acetonitrile. Transfer acetonitrile into a Schott bottle and degas the solution in water bath sonicator for 10 min. Use the solution for unlimited time.

4.3.5 pHILIC storage solution

80 % acetonitrile 5 mM ammonium formate storage solution is recommended by manufacturer. Store the column in this solution for periods longer than few days. To prepare 1

L of this solution dissolve 315 mg of ammonium formate in 200 ml of MilliQ water and add 800 ml of acetonitrile. Degas the solution in water bath sonicator for 10 min. Use the solution within 1 year.

4.3.6 Blank solution

Blank solution is 100% IPA and is always kept at vial position B2 in the autosampler.

4.4 MS preparation

Preparation of MS includes cleaning ion transfer capillary and calibration. Both procedures should be performed weekly or before every long batch.

Cleaning MS:

4.4.1 Ensure instrument is in Standby mode (*XCalibur* program and *Tune* program) on the computer

4.4.2 Ensure the spray shield (conical piece) and capillary temperature is cool enough to handle. This can be speeded up by and changing ion-source capillary temperature (*Tune* program) from 320 °C to 0 °C and turning up sheath gas flow.

Note: Ensure you have one hour to complete cleaning procedure and replace HESI-source chamber. If the source is open for longer than 60 min, electronics will switch off and after turning back on it will require 180 min to equilibrate. Therefore, if cleaning procedure is interrupted, please mount HESI source back.

4.4.3 Remove waste pipe (bottom outlet) from HESI source

4.4.4 Disconnect tubing that is coming from valve to HESI source

4.4.5 Unclip HESI source and gently place on its side on the benchtop

4.4.6 Slide off spray shield and put into a clean 1 L beaker

4.4.7 Use the special spanner (and small Allan key) to unscrew capillary and slide it out. Put capillary into the same beaker as spray shield

4.4.8 Fill beaker with just enough 100% hypergrade MeOH. Sonicate both items in the beaker for 10 mins

4.4.9 Remove items and allow to air dry

4.4.10 Use yellow fine "sandpaper" to gently clean any grime off the shield or capillary

4.4.11 Place capillary and spray shield back onto MS (fits into notch) and fit HESI source back on and clip into place

4.4.12 Connect waste pipe and solvent inlet. Set ion-source (capillary) temperature back to required setting

4.4.13 For calibration set ion source probe at position A (all the way down), for analysis lift up the ion source probe to position C

4.4.14 Calibration can be performed before or after ion capillary clean

Calibrating MS:

4.4.15 Disconnect post-valve tubing if necessary and connect syringe tubing to HESI source inlet and place HESI source probe is at the position A

4.4.16 Go to *Tune* program, load positive calibration tune file:

C:\Xcalibur\methods\CURRENT_METHODS\HESI_Pos_Calmix_Tune_060414.mstune

4.4.17 Load syringe with ~100 µL positive calmix calibration fluid, ensure there are no air bubbles in the syringe and place it into the syringe pump. Syringe pump is controlled by *Tune* software

4.4.18 Start infusion by clicking on the red syringe button, check flow rate is 3 µL/min

Note: be careful not to set flow rate higher than 10 µL/min, because it can crush the glass syringe

- 4.4.19 Switch on MS scanning by clicking on the yellow *Standby* button and choose green *On* button in the top left corner. In the spectrum window you should see detected masses in real time
- 4.4.20 Allow flow to run for 1 min until stable spray is observed (identified by low TIC variation < 10%) check total ion current is ~109 and AGC target is at 100% (recommended)
- 4.4.21 If the spray is not stable under the current tune parameter, try to increase voltage or set sheat gas at 2 units and see if the spray is stabilized. Also, auxiliary gas temperature and auxiliary gas pressure can be slightly increased
- 4.4.22 In the *Tune* program on the left side go to *Calibrate* tab and choose *Calmix Calibration*. Tick the positive calibration box and untick other boxes.
- 4.4.23 Check for the presence of the peaks you wish to calibrate with and click *Calibrate* under the *Calibration* tab
- 4.4.24 In the pop-up message calibration result is displayed. If mass error without lock masses is under 0.3 ppm then the calibration is fine to use (i.e. calibration will be shown as successful). In the pop-up message the first number is mass error in ppm with lock masses, the second number is the mass error without lock masses.
- 4.4.25 Take a screen shot (press *pmtScrn* button) on keyboard, and paste it to most recent logbook (found on desktop) for that year, for example C:\Xcalibur\QExactive Logbook Jan 20xx. Alternatively, all successful calibration reports are automatically saved and stored at C:\Xcalibur\system\Exactive\instrument\reports
- 4.4.26 For metabolomics analysis it is necessary to additionally calibrate low mass range for positive and negative mode using customized calibration. Use the same Calmix solutions as for normal mass range calibration. After calibrating normal range for positive mode, load tune file for positive mode low mass range:
C:\Xcalibur\methods\CURRENT METHODS\HESI_Pos_Calmix_Low_Mass_Tune.mstune
- 4.4.27 Wait until spray is stable under new tune conditions. If the spray is not stable try adjusting CID energy by increasing/decreasing by 5 or 10 eV or adjusting aux gas temperature or adding aux gas (2 units)
- 4.4.28 Under *Calibrate* tab, select *Customized Calibration* and enter the following m/z values for positive mode: 138.06619, 195.08765, 524.26496, 74.09643. After these values addedn once they will be saved
- 4.4.29 Click *Calibrate*;
- 4.4.30 If mass error is <0.3 ppm (the second number), calibration is successful. If the error is higher than 0.3 ppm, wait until spray is stable and click *Calibrate* again.
- 4.4.31 To calibrate negative mode bring mass spectrometer to *Standby* mode by right clicking on the green *On* button and choosing yellow *Standby* button (infusion will be stopped automatically)
- 4.4.32 Load negative calibration file: C:\Xcalibur\methods\HESI_Neg_Calmix_Tune.mstune
- 4.4.33 Clean syringe with 50% methanol solution and load with ~100 µL of negative *Calmix* calibration fluid ensure there are no bubbles and place the syringe to syringe pump
- 4.4.34 Start infusing with flow rate of 3 µl/min
- 4.4.35 Repeat steps 4.4.19 – 4.4.25 for negative mode calibration
- 4.4.36 For negative low mass calibration load tune file
C:\Xcalibur\methods\CURRENT_METHODS\HESI_Neg_Calmix_Low_Mass_Tune.mstune
- 4.4.37 Under *Calibrate* tab select *Customized Calibration* and enter the following m/z values for negative mode: 265.14790, 514.28440, 68.99576, 96.96010;
- 4.4.38 Check if the spray is stable (TIC variation <10%) and the necessary ions are detected, then click *Calibrate*;
- 4.4.39 If mass error is <0.3 ppm (the second number), calibration is successful. If the error is higher than 0.3 ppm, wait until spray is stable and click *Calibrate* again.
- 4.4.40 Stop scanning by clicking on yellow *Standby* button
- 4.4.41 Bring HESI source probe back to position C and connect the tubing from the valve. MS is ready to use.

If necessary valve position can be changed by clicking on the left valve icon in Tune software (Parkville setup: position 1 – to MS, position 2- to waste). Valve position setting is included in the acquisition method, therefore there is no need to switch on/off the valve manually before starting the batch. Other valve setups are possible.

4.5 Preparing LC pump module

- 4.5.1 Click valve icon on Tune program to bring valve into waste position
- 4.5.2 To take control from XCalibur to Chromeleon, click Take Control on the Chromeleon homepage and stop the flow if necessary
- 4.5.3 Replace old mobile phase solutions with fresh solutions
- 4.5.4 Top up/replace with fresh rear wash fluid (10% methanol) and syringe wash fluid (50% IPA), if required
- 4.5.5 Ensure that there is enough solvent for the next batch to avoid topping up. If mobile phase solutions will require topping up during batch run, ensure that these solutions are from the same preparation and are homogenous and degassed. Avoid topping up a running batch with solutions from different preparations

Note: If buffered mobile phases were used previously, first purge with solvent high in water content to prevent clogging in purging unit. Same is valid after purging highly buffered phases: it is recommended to quickly purge with a solvent of high water content.

- 4.5.6 Open the valve and purge the lines which solvents were changed. Lines A&B and C&D can be purged at once by setting the composition to 50% each. Purging should be continued until 20 ml of solvent is passed through every line (for example 400 s at 5 ml/min for 2 lines). On completion of purge, close valve
- 4.5.7 Disconnect column and place pre-column tubing into glass beaker
- 4.5.8 Set flow rate manually (Chromeleon program → open HPG-3400 pump tab) to 1 mL/min or similar and allow system to be flushed of any prior mobile phases for 2-3 mins
- 4.5.9 Empty beaker waste into solvent waste carboy
- 4.5.10 Install the column and set the flow 0.3 ml/min and starting gradient composition 80% B, let flow for a few minutes and check the pressure (~ 40 – 140 bar). LC is ready.

4.6 Sample list preparation

This list tells the LCMS system in which the order to run your samples.

- 4.6.1 Open an Excel file containing sample order
- 4.6.2 In the next column add vial positions for every sample and place the samples into the sample holder according to these positions
- 4.6.3 In the following column use randomisation function “=RAND()” to generate random numbers

Note: Do not include your standards, QC samples or blanks in the randomisation. The standards will be placed at the beginning of the sequence and QC and blanks will be placed every 5-10 experimental samples

- 4.6.4 Do not use any symbols or spaces for sample names except underscore, otherwise the sample list will not be imported into XCalibur
- 4.6.5 Use custom sort to randomise by replicate number first, then by randomisation
- 4.6.6 Open sequence template as .csv file “Xcalibur_Sequence.csv” and Save As with a new file name into a relevant folder.
- 4.6.7 Copy randomised File Name, Vial and Sample ID, modify the sequence by inserting blanks, QCs and standards
- 4.6.8 Always place at least two conditioning blanks at the beginning of the sequence. This is always injected from vial position B2 (a vial containing 100% isopropanol). It is not the “extraction buffer blanks” that are made during an extraction
- 4.6.9 Place the standards you wish to run after the conditioning blanks
- 4.6.10 Place a washing blank (vial position B2)
- 4.6.11 Place two QCs that will undergo MSMS (pos and neg mode)
- 4.6.12 Place a QC between every 5-10 experimental samples throughout the list after that. Ensure a QC is placed before the first, and after the last, of the experimental samples
- 4.6.13 Place two washing blanks (vial position B2) after the last QC

- 4.6.14 Place another washing blank (vial position B2) that will be used for the shutdown procedure
- 4.6.15 Ensure sample type field is filled in with either: Unknown, Blank, QC or Std Bracket.

Note: Spell these correctly or the file will not be imported to *Xcalibur*

- 4.6.16 Save .csv file
- 4.6.17 Open Xcalibur sequence editor and import the sample list: File -> Import -> browse for your .csv file -> click OK
- 4.6.18 Fill in fields Path, Instrument Method and Processing Method
- 4.6.19 Make a folder in local disc C for the Raw files to go. Select this folder when filling in the Path field
- 4.6.20 Fill in Instrument Method field for experimental samples:

C:\Xcalibur\methods\CURRENT_METHODS\pHILIC\
Metabolomics_pHILIC_Parkville_v1.meth or Metabolomics_pHILIC_Clayton_v1.meth

- 4.6.21 Fill in Instrument Method field for MSMS samples (QC sample):

C:\Xcalibur\methods\CURRENT_METHODS\pHILIC\
Metabolomics_pHILIC_MS2pos_Parkville_v1.meth and C:\Xcalibur\methods\
CURRENT_METHODS\pHILIC\Metabolomics_pHILIC_MS2neg_Parkville_v1.meth or
Metabolomics_pHILIC_MS2pos_Clayton_v1.meth and
Metabolomics_pHILIC_MS2neg_Clayton_v1.meth

- 4.6.22 Fill in instrument method field for shutdown sample (vial position B2):

C:\Xcalibur\methods\CURRENT_METHODS\pHILIC\pHILIC_shutdown_Parkville_v1.meth
or pHILIC_shutdown_Clayton_v1.meth

- 4.6.23 Fill in processing method for system suitability test sample:

C:\Xcalibur\methods\CURRENT_METHODS\pHILIC\
Metabolomics_QC_pHILIC_proc.proc

- 4.6.24 Save As the sequence into the same folder where raw files will be saved

4.7 Sample analysis

- 4.7.1 Check that there is enough space on the C: drive of the computer (> 100Gb)
- 4.7.2 Select all the lines in the XCalibur sequence editor, navigate to Actions and choose Run This Sequence or click the corresponding icon
- 4.7.3 In the pop-up window untick box for Process if no processing method is specified, then click OK. The samples will appear in the queue on the left hand panel and the log messages will be displayed at the bottom. Click OK in pop-up windows
- 4.7.4 Wait until the first injection is started to ensure there are no errors
- 4.7.5 During the run check:
 - 4.7.6 No errors
 - 4.7.7 TIC is consistent and <20%
 - 4.7.8 LC pump pressure range is normal (40 -200 bar):
 - 4.7.9 No leaks
 - 4.7.10 Chromatogram shows expected profile
 - 4.7.11 No droplets are forming from the ESI spray
 - 4.7.12 System suitability test sample pass the requirements

Acquired data is saved as .raw files in your specified folder.

5. REFERENCES

5.1. Internal

5.2. External

PQMS-MPMF-EXT-0024-exactive-series-manbre0012255

PQMS-MBPF-EXT-0025 DCMSLink 2.12 for Xcalibur - Quick Start Guide.pdf

6. APPENDICES