HILIC UPLC-QTOF Analysis of Small Molecules in Human Plasma and Serum

NPC.SOP.MS004 Version 2.1

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Table of contents

1. Purpose

The purpose of this standard operating procedure (SOP) is to provide step-by-step instructions for the sample preparation and hydrophilic interaction liquid chromatographic (HILIC) analysis of human blood samples. This includes: details of sample preparation, mobile phase preparation and UPLC-MS analysis of the samples.

The aim of this SOP is to reduce the risk from handling biological material and to ensure accurate, reproducible results through efficient and consistent sample handling. This SOP must be used alongside proforma NPC.PRO.MS004.

2. Scope

Prior to following this SOP samples will have been sorted and formatted as detailed in NPC.SOP.CC004 and NPC.SOP.CC005.

This SOP details the methodology for the preparation of human blood (serum and plasma) for HILIC analysis. After formatting there will be a 96-well plate labeled for HILIC analysis, containing 50 µL of each sample in columns 1-10, rows A-H (columns 11 and 12 are empty and are reserved for quality control (QC) samples). This SOP defines how to remove protein from the samples and include both internal standards to each sample and each QC sample. This SOP details the procedure and methodology required to then analyse the prepared samples by HILIC UPLC-MS.

3. Analysis Notes

In addition to sample analysis, the analytical run consists of: blanks, conditioning runs, 5 start study reference (SR), two dilution series, 5 end SR, and quality control samples: SR and long term reference (LTR) samples. The SR is also used to acquire MSMS data using the data independent/dependent acquisition (DIDA) technique of choice (MS^E, DDA, SONAR or a combination of all three modes depending upon availability) for future annotation if needed.

The rationale behind the above analytical set is as follows:

The first 40 injections (10 blanks and 30 SR) are the conditioning runs to ensure that the instrument is stable and ready for sample analysis. This set of injections help to maintain consistency in the run, thus reducing the level of pre-processing sample normalisation required.

Blanks help identify any system peaks present.

The quality control samples (SR/LTR) are run throughout the analysis to monitor the analytical session and allow for normalisation of the sample set post-analysis.

The dilution series acts as a data filtering tool that gives insight into which features respond to dilution, and thus, which are the features which are monitored and measured post acquisition. Features which do not change are considered noise and removed. A project batch should not normally consist of more than a 1000 customer samples. A dilution series will be run at the beginning and end of each batch. The dilutions series are prepared with undiluted SR, allowing to the highly concentrated compounds in diluted samples to be encompassed.

DIDA will only be performed upon the SR and gives additional information in terms of fragmentation data/spectra, thus assisting in feature identification.

Please note that this procedure involves the handling of a Class II rated biological hazard. Please ensure you have undergone all appropriate training and read all relevant risk assessments prior to any sample handling.

4. Materials

Consumables

- Formatted HILIC blood sample plate
- HILIC study reference (plasma/serum)
- Plasma/serum long term reference
- Eppendorf 0.5 mL purple combitip
- Eppendorf 1 mL yellow combitip
- Eppendorf 2.5 mL green combitip
- Eppendorf 10 mL orange combitip
- Eppendorf tubes (1.5 / 2 mL)
- Analytical plate barcode labels
- Box of 300 µL Eppendorf orange tips
- Sample plate jig
- Eppendorf microplate 96/U-PP
- UPLC vials
- Analytical plate barcode labels
- Heat seal foil
- LCMS 0.1% formic acid in water
- LCMS 0.1% formic acid in acetonitrile
- LCMS ammonium formate
- LCMS grade isopropanol
- LCMS grade acetonitrile (stored at -20°C)
- Ultrapure water (18 MΩ)
- Leucine enkephalin

Equipment

- Heat sealer
- Plate shaker
- Eppendorf Multipette Xstream
- Eppendorf Xplorer 20-300 µL 12 channel pipette
- Eppendorf automatic pipettes
- Centrifuge equipped with 96-well plate rotor
- Balance (precision 0.0001 g)
- Ultrasonic bath
- Calibrated pH meter
- -20 ºC and -80 ºC freezers
- Fridge
- Waters Xevo G2-S QTOF Mass Spectrometer
- Waters Acquity binary solvent manager
- Waters Acquity column manager
- Waters 2777 sample manager
- UPLC column: Waters Acquity UPLC BEH HILIC 1.7 µm, 2.1 X 150 mm, P/N: 186003462

Personal protective equipment

- White laboratory coats
- Nitrile gloves
- Eye protection
- Class II biosafety cabinet

Before commencement of any activities described in this document, personnel must be adequately trained and must adhere to all local health and safety procedures.

5. Procedure - Samples

5.1. HILIC Internal Standard (IStd)

PART A of Proforma NPC.PRO.MS004

5.1.1. Of the two internal standards outlined in the table below (Table 1), N-Benzoyl-d_5-glycine is required to be at a concentration of 100 µM in each blood sample and Adenosine-2-d-1 at a concentration of 8 µM. 10 µL of internal standard solution is added to each well. The final volume in each well after sample preparation will be 480 µL, therefore the internal standard solution mixture must be prepared at a X 48 concentration for each compound; 4.8 mM and 0.384 mM respectively.

5.1.2. Individually weigh Adenosine-2-d-1 and N-Benzoyl-d_5-glycine into pre-weighed glass weigh boats, and transfer to a volumetric flask.

5.1.3. Re-weigh the weigh boats to ascertain the exact mass of compound added to the volumetric flask.

5.1.4. Make the volumetric flask up to volume with LCMS grade water and sonicate until visibly dissolved.

5.1.5. The quantity of the internal standard solution can be scaled up or down depending on the volumes required for the project (approx. 15 mL for a batch of 1000 samples).

5.1.6. Pipette the internal standard solution into 1 mL aliquots and store in the freezer at -80 °C until required.

Table 1. Internal standards

Compound	Molecular weight	Stock concentration (µM)	Final concentration (µM)
N-Benzoyl-d_5-glycine	184.2	4800	100
Adenosine-2-d-1	268.25	384	8

5.2. HILIC method reference (MR) solution

PART B of Proforma NPC.PRO.MS004

5.2.1. Prior to sample preparation, a method reference solution will be added to the SR and LTR. The MR solution will be prepared as outlined below.

5.2.2. Each of the compounds included in the method reference and the individual required concentrations are outlined in Table 2.

5.2.3. To prepare a 50 mL MR solution, individually weight the required quantity of each standard into a 2 mL plastic microcentrifuge tube (excluding Uracil) and add 1 mL of LCMS grade water using an automatic pipette. Sonicate each compound until it dissolves.

5.2.4. Weigh the required quantity of Uracil-2-^{13}C,$^{15}N_2$ into a glass weigh boat and transfer into an empty 50 mL volumetric flask.

5.2.5. Transfer the contents of each of the plastic tubes to the 50 mL volumetric flask. Rinse out each tube with an additional 1 mL of LCMS grade water, washing into the 50 mL volumetric flask. If some of the standards have not fully dissolved, wash the tube several times with 1 mL water until all contents have been transferred into the 50 mL volumetric flask.

5.2.6. Once all compounds have been transferred to the volumetric flask, fill the remaining volume with LCMS grade water and mix until the Uracil has dissolved and the solution is homogenous.

5.2.7. Dispense the MR solution into 1 mL aliquots and store in the freezer at -80 °C until required.

5.2.8. The quantity of the MR solution can be scaled up or down depending in the volumes required for the project (approx. 3 mL for a batch of 1000 samples).

Table 2. Method reference standards

Compound	Molecular weight	Final conc. in sample (µM)	Conc. in MR solution (µM)
L-Phenylalanine-^{13}C,$^{15}N_9$	175.12	50	2400
Adenine-2d1	135.15	4	192
Taurine-^{15}N	126.14	50	2400
Creatine-d3 H_2O	152.17	5	240
L-Arginine-$^{13}C_6$	216.62	50	2400
L-Tryptophan-d5	209.26	50	2400
Uracil-2-^{13}C,$^{15}N_2$	115.07	50	2400

PART C of Proforma NPC.PRO.MS004 - *On the Day of MS-SR preparation*

5.3. Analytical long term reference (analytical LTR)

5.3.1. Defrost plasma/serum stock LTR at 2-8 °C (number of aliquots depends upon study size, with 1x 11.5 mL aliquot sufficient for 1000 samples).

5.3.2. Combine the plasma/serum LTR, LCMS grade water, and MR together in a 5:5:1 ratio v/v (*e.g. 7.5 mL LTR, 7.5 mL LCMS grade water, and 1.5 mL MR*). This mixture will be called analytical LTR.

5.3.3. Vortex mix and briefly spin to collect all volume at the bottom.

5.3.4. Dispense 1 mL aliquots of the analytical LTR solution into 1.5 mL Eppendorf tubes, at least one for each plate.

5.3.5. Store at -80 °C until required.

5.4. Analytical study reference (analytical SR)

5.4.1. Combine HILIC plasma/serum stock MS- SR (see NPC.SOP.CC005, section 6.2) with LCMS grade water and the MR together in a 5:5:1 ratio v/v (*e.g. 7.5 mL SR, 7.5 mL LCMS grade water, and 1.5 mL MR*). This mixture will be called analytical SR.

5.4.2. Vortex mix and briefly spin to collect all volume at the bottom.

5.4.3. Dispense 1 mL aliquots of the analytical SR solution into 1.5 mL Eppendorf tubes, at least one for each plate.

5.4.4. Prepare at least two 165 µL aliquots of analytical SR solution in separate 2 mL Eppendorf tubes for Start/End SR using an automatic pipette. *N.B. It is recommended to prepare more aliquots in case of restart SR is required after instrument failure.*

5.4.5. Store all aliquots at -80°C until required.

5.5. SR containing MR (SR+MR) for instrument conditioning, dilutions series, and DIDA samples

5.5.1. To prepare SR+MR, combine HILIC plasma/serum stock MS- SR with MR in a ratio of 10:1 v/v. Per 1000 sample batch, recommended volumes are 1 mL SR and 100 µL MR.

5.5.2. Mix for 5 min at 1400 rpm at 2-8 °C, and briefly spin to collect all volume at the bottom.

5.5.3. For conditioning and DIDA runs directly prepare 2x 165 µL aliquots of SR+MR solution in separate 2 mL Eppendorf tubes for instrument conditioning (1x) and DIDA (1x) analysis using an automatic pipette. *N.B. Use LTR for instrument conditioning, if SR volume is limited. Rescale accordingly.*

5.5.4 Store all aliquots at -80°C until required.

5.6. Blanks

5.6.1. Prepare at least 2x blanks by adding 15 µL MR to 150 µL of LCMS grade water in 2 mL Eppendorf tubes.

5.6.2. Vortex mix.

5.6.3. Store at -80°C until required.

More blanks can be made when needed as follows.

- Prepare blanks by adding 100 µL of LCMS grade water, 10 µL IStd-Soln, 10 µL of MR-Soln and 360 µL of LCMS grade acetonitrile for each blank.
- Vortex mix.
- Aliquot into appropriate UPLC vial.

5.7. SR dilution preparation and Start/End series

5.7.1. Prior to start of analysis, preferable on the day of MS-SR preparation

PART D of Proforma NPC.PRO.MS004

5.7.1.1. See Section 5.5 for details on how to prepare SR+MR.

5.7.1.2. A dilution series of the SR and MR will be performed and run at the beginning and end of every batch of 1000 samples.

5.7.1.3. Prepare the master dilution sequence (following Table 3) of the SR+MR with water and IStd in 2 mL Eppendorf tubes using appropriately sized automatic pipettes.

5.7.1.4. The acetonitrile addition [see far right column in table 3] will be performed on the day of analysis [section 5.7.2.].

5.7.1.5. Vortex mix the contents of the tube for each master dilution point.

5.7.1.6. Dispense the contents of each master dilution point into 3 separate sets of aliquots (two sets for the beginning and end of each batch and a set as a spare) as per the volumes in Table 3. Dispense into 2 mL Eppendorf tubes and store at -80 °C until required.

5.7.1.7. Three dilution series aliquots are needed per batch. If a project contains more than one batch then the number of dilution series must be increased (e.g. 6 aliquots for 2 batches, 9 aliquots for 3 batches etc).

N.B.: Start here with the UPLC Q-TOF system performance checks as given in 7.1.1-7.1.3 of section "7. Procedure – Acquisition" of this SOP.

5.7.2. On day of analysis

PART E of Proforma NPC.PRO.MS004

5.7.2.1. On the day of use (either first or last day of analysis), remove a set with each dilution point from storage and thaw at 2-8 °C. Once thawed, add the corresponding volume of ice cold acetonitrile (stored at -20 °C) detailed in Table 3. Mix for 2 minutes (1400 rpm at 2-8 °C). Incubate for 2 hours at 2-8 °C.

5.7.2.2. On the first day of analysis, remove 1x instrument conditioning samples (165 µL), 1x blank samples (165 µL), and the Start SR sample (165 µL); on the last day of analysis, 1x blank sample (165 µL), the End SR sample (165 µL) and the DIDA samples (165 µL) from storage and thaw at 2-8 °C. Each aliquot is single use only; once thawed it must be discarded and not re-frozen.

5.7.2.3. Once thawed briefly vortex mix and spin down.

5.7.2.4. Using an automatic pipette, add 15 µL of IStd solution and 540 µL of ice cold acetonitrile to each sample respectively. Mix for 2 minutes (1400 rpm at 4 °C). Incubate for 2 hours at 2-8 °C.

5.7.2.5. Centrifuge all blanks and all samples for 10 minutes (3486 g at 4 °C). Dispense each supernatant into UPLC vials taking care not to disturb the pellet.

Table 3. SR dilution series preparation for one sample batch

Dil No.	Percentage of SR (%)	Vol. of SR+MR (µL)	Vol. of water (µL)	Vol. of IStd (µL)	Total Vol. (µL)	Vol. in aliquot (µL)	*Vol. of ACN added per aliquot (µL)*
1	100	275	0	25	300	90	*270*
2	80	132	33	15	180	55	*165*
3	60	99	66	15	180	55	*165*
4	40	66	99	15	180	55	*165*
5	20	33	132	15	180	55	*165*
6	10	33	297	30	360	100	*300*
7	1	3.3	326.7	30	360	100	*300*
8	0	0	330	30	360	100	*300*

More restart SR samples can be made when needed (i.e. when ready after instrument failure), using a freshly thawed analytical SR prepared in 5.5.1. Proceed as follows:

- In an Eppendorf combine: 165 µL analytical SR + 15 µL IStd.
- Add 540 µL of ice cold acetonitrile.
- Mix for 2 minutes (1400 rpm at 4°C). Incubate for 2 hours at 2-8 °C.
- Centrifuge samples at 3486 g for 10 minutes at 4 °C.
- Remove supernatant into an appropriate UPLC vial.

5.8 Sample preparation

PART F of Proforma NPC.PRO.MS004

N.B.: Sample plates for HILIC analysis have been prepared previously according to NPC.SOP.CC005

5.8.1. Remove the appropriate HILIC plate, plasma/serum analytical LTR aliquot, analytical study reference sample and IStd aliquot from storage at -80 °C. Thaw the sample plate, analytical SR and LTR, and IStd aliquots at 2-8 °C for a minimum of 2 hours.

5.8.2. Ensure centrifuge and heat sealer are at the required temperatures of 4 °C and 160 °C respectively.

5.8.3. Once thawed, briefly vortex mix all aliquots and spin down.

5.8.4. Once thawed, centrifuge the sample plate for 1 minute (3486 g at 4 °C) to pool sample into the bottom of the well.

5.8.5. Remove carefully seal cap mat and add 60 µL of LCMS grade water to all samples (columns 1-10) excluding the wells designated for the SR and LTR (columns11-12) using an Eppendorf multipette (CombiTip).

5.8.6. Dispense 110 µL of analytical LTR (containing MR) into column 11 and 110 µL of analytical SR (containing MR) to column 12 (refer to Figure 1 for a row guide for the sample plate).

5.8.7. Add 10 µL of IStd solution to all wells.

5.8.8. Add 360 µL of ice cold acetonitrile to all wells.

5.8.9. Heat seal sample plate with heat seal foil (2 seconds, followed by rotation of the plate by 180° and a further 2 seconds of sealing). Care must be taken to exert only minimal pressure to avoid melting the top of the wells, which causes improper sealing and evaporation.

5.8.10. Mix for 2 minutes (1400 rpm at 2-8 °C) and incubate for a further 2 hours at 2-8 °C.

5.8.11. Centrifuge sample plate for 10 minutes (3486 g at 4 °C).

5.8.12. Label two analytical plates with a unique plate barcode label, one for analysis and one for backup purposes.

5.8.13. Carefully remove foil from the sample plate (without disturbing the pelleted material).

5.8.14. Transfer 125 µL of each well to both analytical plates (HPOS and backup) using an automatic multichannel pipette and jig.

5.8.15. Heat seal both analytical plates (as per 5.8.8) with heat seal foil and transfer the analytical HPOS plate to the autosampler ready for analysis. Store the backup plate at -80 °C until required.

	1	2	3	4	5	6	7	8	9	10	11	12
Rows A-H	S	S	S	S	S	S	S	S	S	S	LTR	SR

*S = sample, LTR = long term reference, SR = study reference

Figure 1: Row guide for sample plate.

Eppendorf Multipette Xstream pipette settings:

- Water addition: 2.5 mL CombiTip with Prog = Dis, Vol = 60 µL, No. of steps = 40, aspirate speed = max, dispense speed = max.
- Analytical SR/LTR addition: 1 mL CombiTip with Prog = Dis, Vol = 110 µL, No. of steps = 8, aspirate speed = max, dispense speed = max.
- IStd addition: 0.5 mL CombiTip with Prog = Dis, Vol = 10 µL, No. of steps = 48, aspirate speed = max, dispense speed = max.
- Acetonitrile addition: 10 mL CombiTip with Prog = Dis, Vol = 360 µL, No. of steps = 27, aspirate speed = max, dispense speed = 4/10 (discard the first 3 dispensing steps as the ACN when very cold has a tendency to drip out of the multipette tip. Discarding the first 3 steps prevents this).
- Aliquoting: 12 channel 15-300 µL pipette with Prog = Dis, Vol = 125 µL, No. of steps = 2, aspirate speed = 2/10, dispense speed = 4/10.

6. Procedure - Mobile Phases and Wash Solutions

PART G of Proforma NPC.PRO.MS004

6.1. Mobile phase A *(0.1% formic acid in water + 20 mM ammonium formate)*

6.1.1. To make 2 L of mobile phase A, weigh 2.52 g ± 0.0252 g of ammonium formate into a pre-weighed glass weigh boat.

6.1.2. Transfer the ammonium formate into a 2 L volumetric flask. Re-weigh the weighing boat to calculate how much of the ammonium formate was transferred.

6.1.3. Transfer 2000 mL of 0.1% formic acid in water into the 2 L volumetric flask making it up to volume, cap, mix by inverting and correcting the flask until the ammonium formate has dissolved, which is almost instantaneous. Transfer to an appropriately sized Duran bottle and sonicate for 5 minutes.

6.1.4. Decant 50 mL of the mobile phase to a separate vessel and measure the pH using a calibrated pH meter. pH should be in the range of 3.50 +/- 0.1.

6.2. Mobile phase B *(0.1 % formic acid in acetonitrile)*

Use 0.1 % formic acid in acetonitrile as supplied.

6.3. Preparation of seal wash *(isopropanol:water 1:9)*

6.3.1. Using a measuring cylinder, transfer 100 mL of isopropanol to a 1 L Duran bottle.

6.3.2. To this, add 900 mL of LCMS grade water.

6.3.3. Cover the bottle with aluminium foil and sonicate for 5 minutes.

6.3.4. Assign an expiry date of one month from the date of preparation

6.4. Preparation of Weak needle wash *(water:acetonitrile 1:3)*

6.4.1. Using a measuring cylinder, transfer 100 mL of LCMS grade water into a 0.5 L Duran bottle.

6.4.2. Using a measuring cylinder, add 300 mL of acetonitrile to the above.

6.4.3. Cap the bottle and mix until homogenous

6.4.4. Transfer contents to the autosampler weak wash bottle.

6.4.5. Assign an expiry date of one month from the date of preparation before connecting to autosampler weak wash line.

6.5. Preparation of strong needle wash (*100% isopropanol*)

Transfer isopropanol into the strong wash bottle and assign an expiry date of three months from the date of preparation before connecting to the autosampler strong wash line.

Please remember to purge lines when new mobile phases or solvents are added.
It is also good practice to purge all lines prior to use if the system has not been running for >1 day.

7. Procedure - Acquisition

PART H of Proforma NPC.PRO.MS004

7.1. Instrument checks and sample loading

7.1.1. Perform UPLC Q-TOF system performance check in the assay specific polarity only as per the NPC.SOP.MS002 and confirm that all checks meet acceptance criteria.

7.1.2. Check that all solvent lines match the assay specific buffers and solutions as outlined in Table 4.

7.1.3. Confirm that all instrument parameters and settings correspond with the values outlined in the Tables 4, 5 and 6.

7.1.4. Load the correct sequence (*SampleList.spl*) into MassLynx and confirm that sample injection order corresponds with the template below in Table 7.

7.1.5. Place the samples (either vials or analytical plate) in the correct autosampler tray position

Table 4. LC instruments parameters

Variable	Description
Mobile Phase A	Water + 0.1% formic acid + 20 mM ammonium formate
Mobile Phase B	Acetonitrile + 0.1% formic acid
Seal Wash	Water:isopropanol 9:1, set to run every 0.5 minutes
Weak Wash	Acetonitrile:water 3:1
Strong Wash	Isopropanol
Lockspray	Leucine enkephalin 200 pg/µL (prepared in 1:1 water:acetonitrile+0.05% formic ac.)
Column	Waters Acquity UPLC BEH HILIC, 1.7 µm, 2.1 x 150 mm
PEEK tubing	ID = 0.004 inch, Length = 33 cm
Column Temp.	40 °C
Sample Loop	2 µL
Injection Volume	15 µL
Run Time	12.65 minutes (N.B. with the addition of autogain each injection will be 15 minutes)
Autosampler cycle variables/ arguments	Injector programme: LCinj5 Pre-cleans or filling strokes = 0, Filling & injection speed = 5 µL/sec, Pre-injection delay = 500 ms, Post-injection delay = 500 ms, Inject 10 µL to injector, followed by 5 µL to waste. Needle wash sequence = Weak, strong, weak, Wait time = 720 Secs, Valve wash sequence = Weak, strong, weak.
Sample Cooler	4 °C with low nitrogen flow to prevent condensation

Table continues

Table 4. LC instruments parameters - continuation

Gradient					
#	**Time (Mins)**	**Flow (ml/min)**	**% A**	**% B**	**Curve**
1	Initial	0.600	5.0	95.0	Initial
2	0.1	0.600	5.0	95.0	6
3	4.6	0.600	20.0	80.0	6
4	5.5	0.600	50.0	50.0	6
5	7.0	0.600	50.0	50.0	6
6	7.1	0.605	5.0	95.0	6
7	7.2	0.610	5.0	95.0	6
8	7.3	0.620	5.0	95.0	6
9	7.4	0.650	5.0	95.0	6
10	7.5	0.700	5.0	95.0	6
11	7.6	0.800	5.0	95.0	6
12	7.7	0.900	5.0	95.0	6
13	7.8	1.000	5.0	95.0	6
14	12.50	1.000	5.0	95.0	6
15	12.65	0.600	5.0	95.0	6

Table 5. MS instruments parameters

Variable	Description
MS Function	ToF MS
Analyser Mode	Sensitivity
Dynamic Range	Extended
Mass Range	50 – 1200 Da
Scan Time	0.07 Secs
Data Format	Centroid
Lockspray function Settings	Acquire lockspray & apply correction, Scan time = 0.07 Secs, Interval = 60 Secs, Scans to average = 4, Mass window = ±0.5.
Method Events	At initial conditions lockspray is infused, at 7.50 minutes lockspray is refilled, at 8.00 minutes lockspray starts infusing again

Table 6. MS source settings

Variable	Description *(positive mode only)*
Sample Probe Position	7
Capillary Voltage (kV)	1.5
Sampling Cone	20
Source Offset	80
Source Temperature (°C)	120
Desolvation Temperature (°C)	600
Cone Gas Flow (L/hr)	150
Desolvation Flow (L/hr)	1000
Collision Energy (v)	Off but with default set to 4
Gas	On
Detector Auto Gain, Tune Page:	Automatic Gain control ticked
Detector Auto Gain, System View:	Optimise Detector Gain Usage: Background ions = ticked, Background Ions Settings: Timeout = 120 sec, Use Max Voltage Adjust (V): ticked and set to 1.0
Stepwave 2 Offset	10
Quad Profile	Auto
Lockspray Flow (µL/min)	≤15
Lockspray Capillary Voltage (kV)	3.0

7.2. Sample acquisition

7.2.1. Export the batch sample list from LIMS. In the event of samples missing (noted during formatting), modify the sample list accordingly: The missing sample injection is replaced by the last injected SR or LTR, with "_X" added at the end of the sample name.

7.2.2. The analytical batch is structured as described in Table 7.

Table 7. Acquisition order

Phase	Number of injections	Description	Acquisition Method	File Naming Convention
Start	5-10	Pre-check blanks	MS only	Project_HPOS/HNEG_TOFxx _test01-05
	10	Blanks	MS only	Project_HPOS/HNEG_TOFxx_Blank01-10
	30	Instrument conditioning samples	MS only	Project_HPOS/HNEG_TOFxx_IC01-30
	2	Blanks (for dilution series)	MS only	Project_HPOS/HNEG_TOFxx_Blank11-12
	46	Dilution series (low to high % SR)	MS only	Project_HPOS/HNEG_TOFxx_BxSRD01-46
	3	SR conditioning samples	MS only	Project_HPOS/HNEG_TOFxx_IC31-33
	5	Batch start SR	MS only	Project_HPOS/HNEG_TOFxx_BxS01-05_SR
Sample Analysis	X	Analytical plates	MS only	Project_HPOS/HNEG_ToFxx_S*xWxx_LTR Project_HPOS/HNEG_ToFxx_S*xWxx Project_HPOS/HNEG_ToFxx_S*xWxx_SR
End	5	Batch end SR	MS only	Project_HPOS/HNEG_TOFxx_BXE01-05_SR
	46	Dilution series (high to low % SR)	MS only	Project_HPOS/HNEG_TOFxx_BXSRD92-47
	2	Blanks (for dilution series)	MS only	Project_HPOS/HNEG_TOFxx_Blank13-14
	8	DIDA conditioning samples	MS only	Project_HPOS/HNEG_TOFxx_IC34-41
	10	SR	DIDA**	Project_HPOS/HNEG_TOFxx_DIDA01-10

** S for Serum, P for Plasma, ** DIDA = data independent/dependent acquisition, for compound identification assistance. This can be DDA, MS^e, SONAR or a combination of all three modes depending upon availability.*

Start: Pre-check blanks to ensure the system is functioning as expected in terms of accuracy and sensitivity, followed by blanks, conditioning SR, 2 blanks (for dilution series), dilution series run low to high % SR (see Table 8 for the number of injections of each dilution), followed by 3 SR conditioning and the 5 batch start SR.

Table 8. Number of injections for each SR dilution point

Dilution point (% SR)	Number of injections
1	10
10	10
20	5
40	3
60	3
80	5
100	10

Sample Analysis: For sample analysis each row of the 96 well plate should be analysed in the following order: one LTR, 5 samples, one SR, 5 samples. Plates are sequentially located in trays 2, 4 and 6.

End: Batch end SR, dilution series run high to low % SR (see Table 8 for the number of injections of each dilution), 2 blanks (for dilution series), 8 DIDA conditioning, 10 DIDA.

7.2.3. Load the full batch sequence (*SampleList.spl*) into MassLynx

N.B. In case of a run interruption, reset instrument, prepare fresh restart SR, check with a SR sample whether the instrument performance is still fine, and restart run with additional 8 SR sample as emergency instrument conditioning at the beginning of the remaining run.

8. Related Documents

Document Number	Title
NPC.PRO.MS004	HILIC UPLC-QTOF Analysis of Small Molecules in Human Plasma and Serum - Proforma
NPC.SOP.CC005	Formatting and Replication of Samples
NPC.SOP.MS002	UPLC & Q-ToF System Performance Check

9. Version History

Current Version

Version number	Author	Changes and justification	Section(s) updated
V2.1	VHS/BC	Revision and minor changes	all

Previous Version

Version number	Author	Changes and justification	Section(s) updated
V2.0	VHS/BC	Changed for new diluted MS protocols	all
V1.1	VHS/BC	Revision and minor changes	all
V1.0	VHS	Correction of column titles	5.6, Table 3
V1.0	DB	New SOP	N/A

10. Responsibilities

Centre management is responsible for ensuring that laboratory technical personnel are appropriately qualified to perform the procedures outlined in this SOP. The appointed laboratory personnel are in turn responsible for conducting the procedure as outlined in accordance with health and safety standards.

Health and safety statement: before commencing any activities described in this document personnel must be adequately trained e.g. staff having completed local institution Chemical Safety Training and staff having read and understood the relevant risk assessments. Chemical, biological and general waste should be disposed of according to local policies.

11. Approval

Prepared by Dr Verena Horneffer-van der Sluis	Date
Reviewed by Dr Maria Gomez-Romero	Date
Authorised by Dr Matthew Lewis	Date

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