HILIC UPLC-QTOF Analysis of Small Molecules in Human Urine

National Phenome Centre

NPC.SOP.MS006 Version 2.1

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1. Purpose

The purpose of this standard operating procedure (SOP) is to provide step-by-step instructions for the sample preparation and hydrophilic interaction chromatographic (HILIC) analysis of small molecules urine 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 human urine, and to ensure accurate reproducible results through efficient and consistent sample handling. This SOP must be used alongside proforma NPC.PRO.MS006.

2. Scope

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

This SOP details analysis of the HILIC methodology for human urine samples. After formatting there will be a 96-well plate labeled for HILIC analysis, containing 25 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 method reference standards to each QC sample. This SOP then details the procedure and methodology required to analyse the samples by HILIC UPLC-MS.

3. Analysis Notes

In addition to sample analysis the analytical run consists of: conditioning runs, blanks, quality control (QC) samples of the study reference (SR) and long term reference (LTR) samples, and two dilution series. 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 are 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 runs helps to maintain consistency between the run thus reducing the level of pre-processing sample normalisation required.

Blanks help identify any system peaks present within the sample set.

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 should this be required due to instrumental drift for example.

The dilution series serves a different function in that it acts as a data filtering tool that gives insight into which features respond to dilution, and thus, 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 consist of more than a 1000 customer samples. 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 provide additional information in terms of fragmentation data/spectra, thus assisting in feature identification and.

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 urine HILIC sample plate
- HILIC study reference
- Urine long term reference
- Eppendorf 2.5 mL green combitip
- Eppendorf 1 mL yellow combitip
- Eppendorf 1.5 or 2 mL tubes
- Analytical plate barcode labels
- Eppendorf microplate 96/U-PP
- Heat seal foil
- Box of 300 µL eppendorf orange tips
- PTFE/Silicone Septa, 300 µL volume
- Total recovery glass vials
- 300 µL polypropylene plastic screw top vials
- Borosilicate glass bottles grade 3.3 (0.25,0.5,1.0,2.0,5.0L)
- LCMS Ammonium formate
- LCMS grade 0.1% formic acid in water
- LCMS grade 0.1% formic acid in acetonitrile
- LCMS grade acetonitrile (stored at -20°C)
- LCMS grade water
- LCMS grade isopropanol
- Leucine enkephalin (prepared as per NPC.SOP.MS001)
- HILIC standards (see Tables 1 and 2)

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
- Fridge and -80ºC freezer
- 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, 1.7µm, 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 solution (IStd-Soln)

PART A of Proforma NPC.PRO.MS006

5.1.1. The stock solutions of the two internal standards for HILIC analysis are prepared in LCMS grade water in the concentrations outlined in Table 1 (IStd-Stock).

5.1.2. Prepare a mixed solution of each internal standard in LCMS grade water to achieve the final concentration outlined in Table 1 (IStd-Soln).

5.1.3. The quantity of the internal standard solution can be scaled up or down depending on the volumes required for the project (approx. 30 mL for a batch of 1000 samples).

5.1.4. Dispense the final solution into 3 mL aliquots.

5.1.5. Store the prepared IStd-Soln solution at -80 °C for a maximum of 12 months.

Table 1. Concentration of IStd-Stock and IStd-Soln

Material	Supplier	Product number	Concentration of IStd-Stock		Concentration of IStd-Soln	
			mM	mg/mL	mM	mg/mL
L-Phenylalanine-$^{13}C_9$,^{15}N	Sigma	608017	114	20	1.6	0.280
Hippuric Acid-$^{13}C_6$	QMX	IS9117	8.1	1.5	2.0	0.370

5.2. HILIC method reference solution (MR-Soln)

PART B of Proforma NPC.PRO.MS006

5.2.1. Stock solutions of the method reference standards for HILIC analysis are prepared in LCMS grade water in the concentrations outlined in Table 2 (MR-Stock).

5.2.2. Prepared a mixed solution of each MR-Stock standards to achieve the final concentration (as detailed in Table 3) and label as MR-Soln.

5.2.3. Cap vessel and place at -80^{o}C for length of study, unless being used immediately for LTR and SR generation.

5.2.4. The quantity of the MR solution can be scaled up or down depending in the volumes required for the project (approx. 5 mL for a batch of 1000 samples).

Table 2. Concentration of MR-Stock and MR-Soln

Material	Supplier	Product number	Concentration of MR-Stock		Concentration MR-Soln	
			mM	mg/mL	mM	mg/mL
Adenosine-2-d1	CDN isotopes	D-1827	18.6	5.0	0.16	0.043
Adenine-2-d1	CDN isotopes	D-6291	5.9	0.8	0.08	0.011
Taurine ^{15}N	Sigma	605956	190.3	24.0	1.6	0.202
Creatine-(methyl-d3) monohydrate	Sigma	616249	32.9	5.0	0.16	0.025
L-Arginine-$^{13}C_6$ hydrochloride	Sigma	643440	230.8	50.0	1.6	0.345
L-Tryptophan-d5(indole-d5)	Sigma	15862	23.9	5.0	1.6	0.335
Uracil-2-^{13}C,$^{15}N_2$	Sigma	608459	17.4	2.0	3.2	0.360

PART C of Proforma NPC.PRO.MS006 – *On the Day of MS-SR preparation*

5.3. Analytical long term reference (analytical LTR)

5.3.1. Defrost urine 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 urine LTR with LCMS grade water and the MR-Soln together in a 1:1:1 ratio v/v *(e.g. 1mL LTR, 1 mL LCMS grade water, and 1 mL MR-Soln*). 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. Aliquot the analytical LTR solution into Eppendorfs one for each sample plate to be analysed. The volume required for each plate is 700 µL.

5.3.5. Store at -80°C until required.

5.4. Analytical study reference (analytical SR)

5.4.1. Combine the HILIC SR stock aliquot (see NPC.SOP.CC005, section 6.3) with LCMS grade water and the MR-Soln together in a 1:1:1 ratio v/v *(e.g. 1 mL SR, 1 mL LCMS grade water, and 1 mL MR-Soln).* 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. Aliquot the analytical SR solution into Eppendorfs: one for each sample plate to be analysed. The volume required for each plate is 700 µL.

5.4.4. Prepare at least two 150 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 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 urine stock MS- SR with MR-Soln in a ratio of 2:1 v/v. Per 1000 sample batch, recommended volumes are 1 mL SR and 0.5 mL MR.

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

5.5.3. For conditioning and DIDA runs directly prepare 4x 150 µL aliquots of SR+MR solution in separate 2 mL Eppendorf tubes for instrument conditioning (3x) 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 at -80°C until required.

5.6. Blanks

5.6.1. Prepare three blanks by adding 50 µL MR to 100 µL of LCMS grade water in 2.0 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, 50 µL IStd-Soln, 50 µL of MR-Soln and 600 µL of ice cold acetonitrile for each blank.
- Vortex mix and briefly spin.
- 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

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

5.7.1.2. A dilution series of the SR+MR will be performed and run at the beginning and end of every batch of 1000 samples. Prepare the following master dilution sequence (Table 3)

of the SR+MR (containing MR-Soln), IStds and water in 2 mL Eppendorf tubes using appropriately sized automatic pipettes.

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

5.7.1.4. For each master dilution point, the Eppendorf tubes will be vortex mixed and briefly spun.

5.7.1.5. Once the master dilution series has been completed, each master dilution point will be divided into six separate sets of aliquots (two sets for the beginning and end of each batch [per polarity] and 4 sets as a spare) as per the volumes in Table 3. The dilutions will be aliquoted into 1.5 mL Eppendorf tubes and then be stored at -80°C until required.

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

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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 defrost at 2-8°C. Once thawed, add the corresponding volume of ice cold acetonitrile detailed in Table 3 to the dilution series and mix for 2 minutes at 1200 rpm at 4ºC.

5.7.2.2. On the first day of analysis, remove 2x instrument conditioning samples (150 µL each) and 2x blank samples (150 µL each) and allow to defrost at 2-8°C. Each aliquot is single use only; once thawed left overs must be discarded and not re-frozen.

5.7.2.3. On the last day of analysis, remove 1x blank sample (150 µL) and the remaining instrument condition and DIDA samples (150 µL each) from storage and allow to defrost at 2-8°C. Each aliquot is single use only; once thawed left overs must be discarded and not re-frozen.

5.7.2.4. Once thawed, using an automatic pipette, add 50 µL of IStd-Soln and 600 µL of ice cold acetonitrile (stored at -20°C) to the instrument conditioning, blank and/or DIDA sample, respectively, and mix for 2 minutes at 1200 rpm at 2-8°C.

5.7.2.5. Centrifuge all samples and blanks at 3486 g for 10 minutes at 4ºC.

5.7.2.6. Remove supernatant from all samples and the blanks into an appropriate UPLC vial taking care not to disturb the pellet.

Table 3. Dilution series preparation for one sample batch (6 dilution sets)

Dil No.	Percentage of SR (%)	Vol. analytical SR (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	225	0	75	300	50	*150*
2	80	105	26.3	43.8	175	29.2	*87.6*
3	60	57.4	38.3	31.9	127.5	21.7	*65.1*
4	40	38.3	57.4	31.9	127.5	21.7	*65.1*
5	20	26.3	105	43.8	175	29.2	*87.6*
6	10	22.5	202.5	75	300	50	*150*
7	1	2.3	222.8	75	300	50	*150*
8	0	0	247.5	82.5	300	55	*165*

More restart SR samples can be made when needed (i.e. when ready after instrument failure), using a freshly thawed analytical SR prepared in 5.4.4.

For conditioning runs use analytical SR (or LTR if SR volume is limited) prepared as follows:

- In an Eppendorf combine: 150 µL analytical SR + 50 µL IStd-Soln.
- Add 600 µL of ice cold acetonitrile (stored at -20°C).
- Mix for 2 minutes at 1200 rpm at 2-8°C (if possible), otherwise vortex to mix.
- Centrifuge samples at 3486 g for 10 minutes at 4°C.
- Remove supernatant into an appropriate UPLC vial.

5.8 Sample Preparation

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5.8.1. Remove the appropriate HILIC plate and appropriate analytical LTR and analytical SR aliquots for the required project from storage at -80 °C and thaw 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, centrifuge the sample plate at 3486 g for 1 minute at 4°C to pool sample into the bottom of the well.

5.8.4. Remove cap mat and add 50 µL of LCMS grade water to all samples excluding the wells designated for the SR and LTR using an Eppendorf multipette (CombiTip).

5.8.5. Dispense 75 µL of analytical LTR into column 11 wells and 75 µL of analytical SR to column 12 wells. *(Please refer to Figure 1 for a row guide for the sample plate).*

5.8.6. Add 25 µL of IStd-Soln as prepared as detailed in section 4.1 to all wells on the plate.

5.8.7. Add 300 µL of ice cold acetonitrile (stored at -20°C) to each well on the plate.

5.8.8. Heat seal sample plate with heat seal foil (2.0 seconds at 160 ^{o}C, followed by rotation of the plate by 180° and a further 2.0 seconds of sealing).

5.8.9. Mix for 2 minutes on a plate mixer at 1200 rpm at 2-8 °C.

5.8.10. Centrifuge sample plate at 3486 g for 10 minutes at 4 ^{o}C.

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

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

5.8.13. Transfer 145 µL of each well to both analytical plates (HPOS and backup) using a multichannel pipette.

5.8.14. 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: 1 mL CombiTip with Prog = Dis, Vol = 50 µL, No. of steps = 20, aspirate speed = max, dispense speed = max.
- Analytical SR/LTR addition: 1 mL CombiTip with Prog = Dis, Vol = 75 µL, No. of steps = 8, aspirate speed = max, dispense speed = max.
- IStd addition: 1 mL CombiTip with Prog = Dis, Vol = 25 µL, No. of steps = 12, aspirate speed = max, dispense speed = max.
- Acetonitrile addition: 10 mL CombiTip with Prog = Dis, Vol = 300 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 = 145 µL, No. of steps = 2, aspirate speed = 2/10, dispense speed = 4/10.

6. Procedure - Mobile Phases and Wash Solutions

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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 aluminum 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 the autosampler weak wash bottle.

6.4.2. To this, add 300 mL of acetonitrile using a measuring cylinder.

6.4.3. Cap the bottle and mix until homogenous.

6.4.4. 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.MS006

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 on the subsequent pages.

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	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_UxWxx_LTR Project_HPOS/HNEG_ToFxx_UxWxx Project_HPOS/HNEG_ToFxx_UxWxx_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	Conditioning samples	MS only	Project_HPOS/HNEG_TOFxx_IC34-41
	10	SR	DIDA*	Project_HPOS/HNEG_TOFxx_DIDA01-10

** 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 conditioning SR 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 conditioning SR, 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 samples as emergency instrument conditioning at the beginning of the remaining run.

8. Related Documents

Document Number	Title
NPC.PRO.MS006	HILIC UPLC-QTOF Analysis of Small Molecules in Human Urine 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/MD	Revision and minor changes	all

Previous Version

Version number	Author	Changes and justification	Section(s) updated
V2.0	VHS/MD	Changed for new diluted MS protocols	all
V1.1	VHS/AA/ BC	Revision and minor changes	all
V1.0	MD	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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