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Standard Operating Procedure

Automated Sample Prep-LCHRMS

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Overview

Purpose of SOP: This procedure details steps for preparing biofluids such as plasma, serum, urine, and follicular fluids for LC analysis using Opentrons Flex automated liquid handlers. **Required BioRAFT**

Safety Training:

- BBP for Research
- Biosafety
- Research Lab Safety

Cautions

- Sample preparation involves hazardous chemicals and biohazardous materials. Technician must be familiar with the SDS forms for all chemicals and must follow proper procedures for handling biohazardous materials.
- PPE (eye protection, lab coat, and gloves) should be worn throughout processing the samples to prevent contamination of samples and exposure to the technician. Gloves should be changed when they become contaminated or when touching items such as cell phones not directly involved in sample prep. If computer or other items do become contaminated, clean with 70% ethanol.
- Clean worksurfaces daily with 70% ethanol.
- Waste materials should be collected and disposed of in biohazardous waste bins.

Definitions/ explanations

- **Water blanks:** These are prepared and processed exactly like samples, except using an identical volume of LCMS grade water in place of biofluid.

Abbreviations

- **DWP:** Deep well plate.
- **LC:** Liquid chromatography
- **QA/QC:** Quality Assurance/ Quality Control. Plasma or urine pools 1 (young) and 2 (older) are typically used, but different biological sample types may require the use of different types of QAQCs that more closely match the study sample type.

Equipment

Protocol Name	Manufacturer	Item
Opentron	Opentrons	Flex liquid handling robot
Pipette	Opentrons	1000ul 96-channge pipette
Centrifuge	Eppendorf	Centrifuge 5810R
ThermoMixerC or Vortex	Eppendorf	ThermoMixerC
Station One	JDM Lab Solutions	StationOne rack thawing station

Important Notes:

- Samples prepared for LC analysis must be kept cold. Use chilled aluminum blocks when aliquoting from vials, keep thawed samples in the refrigerator when not actively aliquoting, and cap and return samples to their original box in the freezer as soon as possible after aliquoting.
- All solvents used for LC sample preparation must be kept at 4C.
- When volatile solvents or samples extracts are involved, do not perform labware position checks using exposed solvents or sample plates. Rather, use empty or extra labware. Labware used for position checks must not have been formerly heat-sealed, as this affects the labware height.
- **Opentrons robot/app upgrades require retesting of all protocols**

Preliminary Software Requirements: Each Opentrons user must perform these one-time tasks in order to operate the Opentrons from their user setting on a computer.

1. Download the [Opentron application](#)
2. Ensure that the version is 8.3.1. Follow the steps in [this link](#) to select the correct software version. Make sure to update both the application and the robot!
3. Install Notepad++ for protocol editing. Can also use other GUIs for .py files.
4. Download all custom labware definitions (included on GitHub)
 - a. Download all .json files
 - b. Open Opentrons app and select “labware” from the lefthand panel.
 - c. Select “Import” from the top right corner, then locate your file.
5. Create a study folder with protocols and excel file to place your sample lists/run orders. You may choose to include labware definitions in this folder as well.

Sample Aliquoting

Overview: This is the first step of sample preparation. The LC plate (Abgene 800ul 96-well plate) receives 40uL of sample followed by 120ul of extraction solvent.

Materials:

Protocol Name	Manufacturer	Item	Item number
Wellplate	Thermo Scientific	800ul Abgene PP plates	AB0859
Heat Seal	Thermo Scientific	Easy Pierce 20um Foil	AB-1720
Reservoir	Opentrons	195ml Nest Reservoir	999-00078
200ul Pipette Tips	Opentrons	200ul Filter Pipette Tips	991-00111
50ul Pipette Tips	Opentrons	50ul Filter Pipette Tips	991-00110
15mL tube	Corning	Falcon 15 mL PP Conical Tube	352097
50mL tube	Corning	Falcon 50 mL PP Conical Tube	352098
Acetonitrile	Fisher	UHPLC-MS Acetonitrile*	A956-1
Water	Fisher	UHPLC-MS Water*	W8-1

*UHPLC grade solvents are used instead of Optima LCMS because of lower PFAS contamination.

Pre-Prep:

1. Prepare solvents for the week.
 - a. Solvents will be stored in glass bottles. These must be cleaned and made fresh weekly.
Cleaning procedure: each bottle will be rinsed 3x. Each rinse will be performed 1x in the following order:
 - i. 1:1 acetonitrile/basic water
 - ii. UHPLC methanol
 - iii. The primary solvent that will be used in the bottle (UHPLC water or acetonitrile)
 - b. For each plate, the following volumes will be needed:
 - i. Extraction Solvent

Number of plates	LC-IS (ml)	PFAS-IS (ml)	Acetonitrile (ml)
1	0.50	0.50	30
2	0.75	0.75	45
3	1.00	1.00	60
4	1.25	1.25	75
5	1.5	1.50	90
6	1.75	1.75	105

- ii. Water: 30ml/ plate
 - iii. 1:1 Acetonitrile/Water: 30 ml/ plate
2. Using micronic scanner, scan in plate to create plate loading order document. Save.
3. Save Opentrons protocols to the study folder.
4. Thaw samples (1-3hrs, 4°C).

While samples are thawing:

- a) You may choose to document via a sample prep worksheet. If so, fill that out.
- b) Turn everything on:

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- i) Opentrons and computer
 - ii) Set temperature modules, centrifuge, and thermomixers to 4°C
 - iii) Preheat heat-sealer to 165°C.
- c) Upload the first Opentrons protocol (“...ES_and_Sample_Aliquot...py”) to the app and start setup.
- d) Set out labware and perform labware position check.
- 2) **Vortex thawed samples:** Thermomixer at 4C and 1100 RPM for 2 minutes. *Ensure cover is secured over caps. Otherwise, the caps will be unscrewed!*
- 3) **Centrifuge thawed samples:** 4°C/ 2 minutes/4000rpm. *Ensure centrifuge is balanced!*
- 4) **Run protocol in Opentrons (~2min).** Items to set out:
 - a) Source plate: un-cap samples and save caps in a clean, empty pipette tip rack.
 - b) Extraction solvent: add 30ul of extraction solvent to reservoir. *Measure this roughly using a clean Falcon tube.*
- 5) **Heat seal receiving plate.**
- 6) **Vortex:** 4°C/2 min/700rpm. During the vortex:
 - a) Return left over extraction solvent to the bottle.
 - b) Cap remaining samples and return to -80C
- 7) **Equilibrate:** 4°C/ 30 min (place in refrigerator + set timer). During this time, also:
 - a) Update worksheet.
 - b) Start set up for supernatant transfer protocol (“...LC_Supernatant_transfer_and_dilution...py”)
 - c) Prepare deck for final protocol:
 - i) Set up labware
 - ii) Perform labware position check
 - iii) Ensure all labware is covered between protocols!
 - d) Prepare plate labels
- 8) **Centrifuge:** 4°C/2 minutes/4000rpm. During this time, proceed to the LC Supernatant Transfer and begin following the steps in the procedure.

Supernatant transfer

Overview: This is the second step of sample preparation. The LC plate made in the last step has been vortexed, equilibrated, and centrifuged. In the second and final step, the supernatant from that plate is delivered in equal amounts to two (2) 96-well plates, each diluted with 60 uL of their respective solvents (water for C18, 1:1 Acetonitrile/Water for HILIC. The Opentrons protocol does the dilution step first (while the plate finishes in the centrifuge), then the supernatant transfer step.

Material

Protocol Name	Manufacturer	Description	Part number
Wellplate	Thermo Scientific	800ul Abgene PP plates	AB0859
Plate mat	Thermo Scientific	Thermo Scientific SureSTART WebSeal Clear Alcohol Resistant EVA 96-Well Plate Sealing Mats, 7 mm Diameter	60180-M179
Reservoir	Opentrons	195ml Nest Reservoir	999-00078
200ul Pipette Tips	Opentrons	200ul Filter Pipette Tips	991-00111
200ul Pipette Tips	Opentrons	200ul Filter Pipette Tips	991-00111
Acetonitrile	Fisher	UHPLC-MS Acetonitrile	A956-1
Water	Fisher	UHPLC-MS Water	W8-1

Procedure: This assumes preparations were already completed during equilibration of the extracts.

- 1) Label the C18 and HILIC wellplates
- 2) Add 30ml of solvent to the reservoir:
 - a) HILIC: 1:1 Acetonitrile/Water - reservoir in B2
 - b) C18: Water – reservoir in C2
- 3) Click “Start Run.”
- 4) After transfers are complete, cap plates with plate mats. Use roller to press into place, then use UltraSeal Cap-Lite to secure in place.
- 5) Vortex: 2 minutes/ 700 rpm/ 4°C.
- 6) Place sample plate in autosampler as directed by instrument technician.
- 7) Deliver completed worksheet to instrument technician and discuss any sample prep issues which may affect analysis.