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| date: 11/08/2012 | Sample preparation of blood plasma or serum samples for GCTOF analysis | Code no.: blood-GCTOF- 11082012 |

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| Issued: 11/08/2012 Valid from: 11/08/2012 | Validity area: UC Davis Genome Center, Metabolomics Core and Research Laboratories |
| Responsible: Mine Palazoglu | Secondary: Mimi Swe |
| This SOP supersedes: SOP blood 03-04-2008 and SOP blood 05282008 | approved: Oliver Fiehn |

Sample preparation of blood plasma/serum samples for GCTOF analysis

1. Purpose:

This SOP describes sample extraction and sample preparation for primary metabolism profiling by gas chromatography / time of flight mass spectrometry (GCTOF)

2. References:

Fiehn O, Kind T (2006) Metabolite profiling in blood plasma. In: Metabolomics: Methods and Protocols. Weckwerth W (ed.), Humana Press, Totowa NJ

3. Starting material:

Plasma/serum: 30 µl sample volume or aliquot

4. Equipment:

- Centrifuge Eppendorf 5415 D
- calibrated pipettes 1-200µl and 100-1000µl
- Eppendorf tubes 1.5 ml, uncoloured, cat. # 022363204
- MiniVortexer VWR
- ThermoElectron Neslab RTE 740 cooling bath
- Orbital Mixing Chilling/Heating Plate (Torrey Pines Scientific Instruments)
- Speed vacuum concentration system (Labconco Centrивap cold trap)
- Precision balance with accuracy ± 0.1mg
- 2mL crimp vials with Target Micro-Serts
- Agilent Electronic crimper and decapper

5. Chemicals:

- Acetonitrile LCMS quality, JT Baker #9829-02
- Isopropanol HPLC solvent, JT Baker #9095-02
- pH paper 5-10, EMD Chem. Inc. Gibbstown NJ 08027
- Nitrogen line with pipette tip
- 18 mΩ pure water (MilliQ)
- Methoxyamine hydrochloride [MeOX] (Aldrich: Cat. No. 226904)
- Pyridine (Acros Organics: Cat. No. 270970)
- N-methyl-N-(trimethylsilyl)-trifluoroacetamide [MSTFA] (Aldrich: Cat. No. 394866)
- FAME markers (refer to FAME marker SOP for preparation)

6. Preparation of extraction mix before experiment:

1. Check pH of acetonitrile and isopropanol (pH7) using wetted pH paper
2. Acetonitrile, isopropanol and water are mixed in volumes in proportion 3 : 3 : 2

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- Rinse the extraction solution mix for 5 min with nitrogen with small bubbles. Make sure that the nitrogen line was flushed out of air before using it for degassing the extraction solvent solution

7. Sample Preparation:

- Switch on bath to pre-cool at -20°C ($\pm 2^{\circ}\text{C}$ validity temperature range)
- Gently rotate or aspirate the blood samples for about 10s to obtain a homogenised sample.
- Aliquot 30 μL of plasma sample to a 1.0 mL extraction solution. The extraction solution has to be pre-chilled using the ThermoElectron Neslab RTE 740 cooling bath set to -20°C .
- Vortex the sample for about 10s and shake for 5 min at 4°C using the Orbital Mixing Chilling/Heating Plate. If you are using more than one sample, keep the rest of the sample on ice (chilled at $<0^{\circ}\text{C}$ with sodium chloride).
- Centrifuge samples for 2min at 14000 rcf using the centrifuge Eppendorf 5415 D.
- Aliquot two 450 μL portions of the supernatant. One for analysis and one for a backup sample. Store the backup aliquot in -20°C freezer.
- Evaporate one 450 μL aliquots of the sample in the Labconco Centrivap cold trap concentrator to complete dryness.
- The dried aliquot is then re-suspended with 450 μL 50% acetonitrile (degassed as given above).
- Centrifuged for 2 min at 14000 rcf using the centrifuge Eppendorf 5415.
- Remove supernatant to a new Eppendorf tube.
- Evaporate the supernatant to dryness in the Labconco Centrivap cold trap concentrator.
- Submit to derivatization.

8. Derivatization

- Prepare 40mg/mL MeOX solution in pyridine. Weigh out methoxyamine hydrochloride in 1.5mL Eppendorf tube on balance and add appropriate amount of pyridine.
- Vortex MeOX solution and sonicate at 60°C for 15 minutes to dissolve.
- Add 10 μL of 40mg/mL Methoxyamine hydrochloride [MeOX] solution to each dried sample and standard
- Shake at maximum speed at 30°C for 1.5 hours.
- To 1mL of MSTFA and add 10 μL of FAME marker. Vortex for 10sec.
- Add 91 μL of MSTFA + FAME mixture to each sample and standard. Cap immediately.
- Shake at maximum speed at 37°C
- Transfer contents to glass vials with micro-serts inserted and cap immediately.
- Submit to GCTOF MS analysis

9. Quality assurance

- For each sequence of sample extractions, perform one blank negative control extraction by applying the total procedure (i.e. all materials and plastic ware) without biological sample.
- Use one commercial plasma/serum pool sample per 10 authentic subject samples as control. If no suitable commercial blood sample is available, prepare a large pool sample during the thawing/mixing step by aliquoting 100 μL per 1 mL plasma sample, and aliquot such pool sample for 1 pool extract per 10 authentic subject samples.
- Prepare at least one NIST plasma extract in the same manner

10. Disposal of waste

- Collect all chemicals in appropriate bottles and follow the disposal rules.
- Collect residual plasma / serum samples in specifically designed red 'biohazard' waste bags.