AO-GLP-SOP-03

Valid in AcureOmics AB

Title: **PREPARATION OF CELL MEDIA FOR METABOLIC PROFILING BY GC-MS**

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1. AIM

This SOP applies to the preparation of cell media samples for metabolic profiling by gas chromatography mass spectrometry (GC-MS).

2. OBJECTIVE

This document describes the procedure for the extraction of metabolites from cell media prior to GC-MS analysis. These samples have been shipped and stored following the procedures detailed in the SOP for Sample Shipment.

3. RESPONSIBILITY

3.1 Each employee is responsible for the work performed in this SOP
3.2 This SOP should be reviewed and revised within 24 months from the validity date.

4. MATERIALS

4.1 **Solvents:** Methanol (HPLC grade) (Fisher Scientific, Loughborough, UK),
4.2. **Reagents:** N-Methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), 1 % trimethylchlorosilane (TMCS) and pyridine (Thermo Fisher Scientific, Rockford, IL, USA). Methoxyamine (Sigma-Aldrich, St Louis, MO, USA).
4.3 MilliQ H_2O
4.4 1.5 ml Eppendorf tubes (Sarstedt, Germany) (Item no. 72690)
4.5 Gloves
4.6 300 µl microvials (Chromacol, Herts, UK) (Part no. 03-FISV)
4.7 GC vial caps (Chromacol, Langerwehe, Germany) (Part no. 9-SC(B)-8RT1)

5. EQUIPMENT

5.1 MM400 Vibration Mill (Retsch GmbH & Co. KG, Haan, Germany)
5.2 VWR VX-2500 Multi-Tube Vortexer (VWR International, Stockholm, Sweden)
5.3 Vortex Genie 2 (Scientific Industries, Inc., NY, USA)
5.4 Speed Vac (Savant Instrument, Framingdale, NY, USA)
5.5 Refrigerated microcentrifuge (Hettich, Tuttlingen, Germany)
5.6 -80 ^{o}C Freezer

6. STANDARDS

Eleven internal standards (IS) are used for GC-MS analysis and are obtained from Cambridge Isotope Laboratories (CIL) (Andover, MA, USA), Campro Scientific (Veenendaal, The Netherlands) and Icon (Summit, NJ, USA) (stated below). The standards are stored in the following solutions:

[$^{13}C_5$] - Proline	H_2O	(CIL)
[$^{2}H_4$] - Succinic acid	H_2O	(CIL)
[$^{13}C_5$,^{15}N] - Glutamic acid	H_2O	(CIL)
[1,2,3-$^{13}C_3$] - Myristic acid	Methanol	(CIL)
[$^{2}H_7$] - Cholesterol	Methanol	(CIL)
[$^{13}C_4$] - α-Ketoglutarate	H_2O	(CIL)
[$^{13}C_{12}$]- Sucrose	H_2O	(Campro)
[$^{13}C_4$] - Hexadecanoic acid	Methanol	(Campro)
[$^{2}H_4$] - Putrescine	H_2O	(Campro)
[$^{13}C_6$] - Glucose	H_2O	(Campro)
[$^{2}H_6$] - Salicylic acid	Methanol	(Icon)

Stock solutions of each standard are stored in the fridge at a concentration of 500 ng/µl. Methyl stearate is used as a standard for the sensitivity test and is stored in the fridge (in heptane) at a concentration of 5 ng/µl and 15 ng/µl. Methyl stearate is obtained from Sigma-Aldrich (St Louis, MO, USA).

7. PREPARATION OF SOLUTIONS

The following extraction mix is prepared:
MeOH/H_2O (9:1 v/v) + each internal standard (2.5 ng/µl).
A solution of methoxyamine (15 µg/µl) in pyridine is stored in the fridge.

8. TUBES AND LABELLING

Use Eppendorf tubes with a 'Safety Cap', for example Sarstedt type 72.690. Tubes with an 'easy-cap' leak during the extraction procedure.
Label the Eppendorf tubes from 1-number of samples. Label on the cap and the side of the tube. All information including sample number, sample name, age, disease state, genetic information is stored in an excel spread sheet.

9. PROCEDURE FOR METABOLITE EXTRACTION

1. Thaw cell media samples at RT (thawing times may vary depending on sample volume, but try to keep as consistent as possible and transfer to ice as soon as thawed)
2. Vortex samples briefly for 5 seconds
3. Add 900 µl of extraction mix to 100 µl cell media on ice
   a. A blank sample should be prepared, containing 900 µl Extraction mix and 100 µl MilliQ H_2O
4. Agitate samples vigorously in a vibration mill for 2 min, at a frequency of 30 Hz
5. Cool samples in an ice bath (4 ^oC) for 2 h (in the fridge on ice)
6. Centrifuge for 10 min at 4 ^oC, 14000 rpm
7. Transfer 900 µl of supernatant into fresh Eppendorf tubes (1.5 ml)

Note: It is possible to stop the protocol here and store the samples at -80 ^oC.

8. Transfer 200 µl of the supernatant into microvials (with no cap). The remaining supernatant should be stored at -80^oC.
9. Evaporate samples until dryness in a Speed Vac (no heat, should take less than 2 hours)
10. Transfer vials to a storage box (13x13x5 cm), cover with foil (no cap required) and store at -80 ^oC ready for GC-MS analysis.

Note, Quality control (QC) samples should also be prepared using a pooled aliquot of all cell media samples (if too many, pool a representative selection i.e. from all groups). QC samples should be prepared in exactly the same way following the protocol above and alongside all other samples.

10. DERIVATISATION FOR GCMS ANALYSIS

1) If taken from freezer, evaporate samples (from step 9.10) until dryness in the Speed Vac concentrator (no heat, 15 min)
2) Add 30 µl methoxyamine (15 µg/µl) in pyridine and cap the vial
3) Agitate samples for 10 min using a multi-tube vortexer
4) Let the reaction continue for 16 h at room temperature
5) Add 30 µl MSTFA, 1 % TMCS
6) Shake for 1 min using a multi-tube vortexer

7) Leave samples for 1 h at room temperature
8) Add 30 µl heptane including methyl stearate (15 ng/µl)
9) Shake the samples for 1 min using a multi-tube vortexer
10) Samples are now ready for GC-TOF analysis (must be analysed on the same day and cannot be stored)

A SOP for the use of the GC-MS instrument for metabolic profiling will take over here

11. DOCUMENTATION

11.1 SOP for Sample Shipment

11.2 SOP describing the Guidelines for the use of the GC-MS instrument

12. SAFETY

12.1 Wear gloves at all times
12.2 All work with solvents (especially methanol) must be performed in the fume hood
12.3 Dispose solvents into designated waste labeled container if necessary