**Standard Protocol Harmonization Ceramides**

**Summary:** This protocol describes the extraction and LC-MRM procedure for absolute quantification of Cer d18:1/16:0, Cer d18:1/18:0, Cer d18:1/24:0 and Cer d18:1/24:1 in human plasma according to Kauhanen et al, Anal Bioanal Chem (2016) 408:3475–3483.

A calibration curve is built by injecting several dilutions of the non-labelled standards mixed with a fixed concentration of labelled standards. Each calibration point is prepared and analysed in triplicates (n=3).

To establish the endogenous concentration of the four selected ceramides in the study samples (NIST SRM1950, NIST high TAG, NIST T1D, and NIST young AA) each sample is extracted and analyzed in six replicates (n =6).

Prepare the pooled plasma quality control sample (pooled plasma QC) by mixing 100 L each of NIST SRM1950, NIST high TAG, NIST T1D, and NIST young AA (to obtain in total 400 L of pooled plasma).

We suggest proceeding first to measure the ceramides concentration in the four study samples and then continue with the intra-assay validation to avoid wasting material.

**Extraction**

Table 1.

|  |
| --- |
| Chemicals, solvents and materials |
| Ethyl acetate (EtAc) |
| 2-propanol (IPA) |
| 5% Bovine Serum Albumin (BSA) |
| 96-well plates/tubes |
| Sealing foil |
| 2 mL deep well plates/tubes/LC vials |

Table 2.

|  |  |  |  |
| --- | --- | --- | --- |
| Sample type |  |  |  |
| Pooled Quality Control (QC) samples | 10 L pooled plasma QC | 20 L Labelled IS | 570 L EtAc:IPA 2:8 (v/v) |
| Matrix Blank (MB) | 10 L Matrix (pooled plasma QC) only | - | 590 L EtAc:IPA 2:8 |
| Study Samples (S) | 10 L Human plasma (NIST SRM1950, high TAG, T1D, young AA) | 20 L Labelled IS | 570 L EtAc:IPA 2:8 |
| Total Blank (TB) | 10 L 5% BSA | - | 590 L EtAc:IPA 2:8 |
| Calibration line standards (STD) | 10 L 5% BSA + 20 L non-labelled STDx mixture (Table 5) | 20 L Labelled IS | 550 L EtAc:IPA 2:8 |

Table 3. **Labelled internal standard** mixture solution (provided). Keep at -20° C. Equilibrate at RT and sonicate 10 min before use.

|  |  |  |
| --- | --- | --- |
| Labelled internal standard mixture in EtAc:IPA 2:8 (v/v) | Concentration, pmol/L [M] | Vendor |
| D7-Cer d18:1/16:0 | 0.125 | Avanti Polar Lipids |
| D7-Cer d18:1/18:0 | 0.050 | Avanti Polar Lipids |
| D7-Cer d18:1/24:0 | 1.500 | Avanti Polar Lipids |
| D7-Cer d18:1/24:1 | 0.500 | Avanti Polar Lipids |

Table 4. **STD1** mixture solution (provided)

|  |  |  |  |
| --- | --- | --- | --- |
| Non-labelled standards in EtAc:IPA 2:8 (v/v) | | Concentration [pmol/L] | Vendor |
| Non-labelled standards mixture | Cer d18:1/16:0 | 2 | Avanti Polar Lipids |
| Cer d18:1/18:0 | 2 | Avanti Polar Lipids |
| Cer d18:1/24:0 | 20 | Avanti Polar Lipids |
| Cer d18:1/24:1 | 20 | Avanti Polar Lipids |

Table 5. Serial dilutions of STD1 non-labelled standards **for calibration line**.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Add 20 L of | | **STD1** | STD2 | STD3 | STD4 | STD5 | STD6 |  |
| Non-labelled STD mixture | Cer d18:1/16:0 | **2.00** | 1.00 | 0.10 | 0.02 | 0.01 | 0.008 | pmol/L |
| Cer d18:1/18:0 | **2.00** | 1.00 | 0.10 | 0.02 | 0.01 | 0.008 |
| Cer d18:1/24:0 | **20.00** | 10.00 | 1 | 0.20 | 0.1 | 0.08 |
| Cer d18:1/24:1 | **20.00** | 10.00 | 1 | 0.20 | 0.1 | 0.08 |

Extraction procedure:

1. Prepare different dilutions of STD1 by diluting the provided mixture (table 4) with EtAc:IPA 2:8 as suggested in Table 5.
2. Thaw plasma samples on ice (+4C) and bring them to room temperature prior to extraction. Mix by pipetting up and down several times before use.
3. Prepare samples in plates/tubes manually or with a robotic liquid handler, according to specifications in Column 2 of Table 2.
4. Add 20 L of labelled internal standard mixture to samples (Column 3 Table 2).
5. For the calibration line, add 20 L of different dilutions of the non-labelled mixture (STD1-6).
6. Add 590 L of ethyl acetate:isopropanol (2:8, vol/vol) to Total Blank and Matrix Blank; 550 L to calibration line (STD); 570 L to study samples and QCs (Column 4 Table 2)
7. Mix samples 10 min by automated pipetting or vortexing
8. Centrifuge samples for 10 min at 3000 x *g* at room temperature
9. Transfer 50 L of the clear supernatant to MS vials or MS plates, close/seal, store samples at -20C prior to LC-MRM analysis.

**LC-MRM**

Table 6.

|  |
| --- |
| Chemicals and solvents |
| Ammonium formate |
| Formic acid |
| Ultra-pure water |
| Acetonitrile |

Table 7.

|  |  |
| --- | --- |
| LC-MS details | Specifications |
| Mass spectrometry | QQQ |
| LC | UPLC |
| Injection volume (indicative) | 5 L |
| Weak wash | Water |
| Strong wash | Acetonitrile |
| Pre-column | Waters Acquity BEH C18, 1.7 µm VanGuard Pre-Column |
| Column | Waters Acquity BEH C18, 2.1 × 50 mm id. 1.7 µm |
| Column temperature | 60 C |
| Mobile phase A | 10 mM ammonium acetate in water with 0.1% formic acid |
| Mobile phase B | 10 mM ammonium acetate in acetonitrile:2-propanol (4:3, vol/vol) with 0.1% formic acid |

Table 8.

|  |  |  |  |
| --- | --- | --- | --- |
| LC gradient | | | |
| Time | A% | B% | Flow (L/min) |
| 0.00 | 15 | 85 | 500 |
| 0.50 | 15 | 85 | 500 |
| 1.50 | 0 | 100 | 500 |
| 4.00 | 0 | 100 | 500 |
| 4.10 | 15 | 85 | 500 |
| 5.00 | 15 | 85 | 500 |

Table 9 (example based on the published reference).

|  |  |
| --- | --- |
| MS conditions (this only applies to Sciex QTRAP 6500) | |
| Polarity mode | Positive |
| Ion spray | 5000 V |
| Curtain gas | 25 psi |
| Source temperature | 300 C |
| Gas 1 | 50 psi |
| Gas 2 | 35 psi |
| DP | 30 V |
| EP | 10 V |
| Collision cell exit potential | 20 V |
| Collision energy CE | 40 eV |

Table 10.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Mass transitions used for absolute quantification. With different systems RT and CE might be different. | | | | | |
| Analyte | Mass transitions (MRM) | IS | Mass transitions (MRM) | RT (min) | CE (not applicable to different instruments) |
| Cer d18:1/16:0 | 538.5 – 264.3  C34H68NO3 – C18H34N | D7- Cer d18:1/16:0 | 545.6 - 271.3 | 1.9-2.0 | 40 |
| Cer d18:1/18:0 | 566.6 – 264.3  C36H72NO3 – C18H34N | D7- Cer d18:1/18:0 | 573.6 – 271.3 | 2.1-2.2 | 40 |
| Cer d18:1/24:0 | 650.6 – 264.3  C42H84NO3 – C18H34N | D7- Cer d18:1/24:0 | 657.7 – 271.3 | 2.4-2.5 | 40 |
| Cer d18:1/24:1 | 648.6 – 264.3  C42H82NO3 – C18H34N | D7- Cer d18:1/24:1 | 655.7 – 271.3 | 2.3-2.4 | 40 |

Suggested sample sequence. Samples and QC for intra-assay validation can be measured in one single batch

|  |
| --- |
| Total Blank 1 |
| Matrix blank |
| Calibration line 1 from STD6 to 1 (n=3) |
| […] |
| Pooled QC |
| Pooled QC |
| SRM 1950-1 |
| SRM 1950-2 |
| SRM 1950-3 |
| SRM 1950-4 |
| SRM 1950-5 |
| SRM 1950-6 |
| Pooled QC |
| Pooled QC |
| T1D-1 |
| T1D-2 |
| T1D-3 |
| T1D-4 |
| T1D-5 |
| T1D-6 |
| Total Blank 2 |
| Pooled QC |
| Pooled QC |
| Young AA-1 |
| Young AA-2 |
| Young AA-3 |
| Young AA-4 |
| Young AA-5 |
| Young AA-6 |
| Pooled QC |
| Pooled QC |
| highTAG-1 |
| highTAG-2 |
| highTAG-3 |
| highTAG-4 |
| highTAG-5 |
| highTAG-6 |
| Pooled QC |
| Pooled QC |
| Calibration line 2 from STD1 to 6 (n=3) |
| LLQC 1  LLQC 2  LLQC 3  LLQC 4  LLQC 5  LLQC 6  LQC 1  LQC 2  LQC 3  LQC 4  LQC 5  LQC 6  MQC 1  MQC 2  MQC 3  MQC 4  MQC 5  MQC 6  HQC 1  HQC 2  HQC 3  HQC 4  HQC 5  HQC 6  HLQC 1  HLQC 2  HLQC 3  HLQC 4  HLQC 5  HLQC 6 |
| Matrix blank |
| Total Blank 3 |

**Clarifications:** In the corresponding Excel table we have 3 cells for each STD concentration (1-6), so we mean that we inject only once from each vial (each vial is a replicate that you prepared). The calibration curves are 2: one at the beginning and one at the end.

Then for the study samples we ask to prepare 6 replicates. Then in the Excel we have 3 cells for each sample, which means this time that each vial is injected 3 times.

Table 11.

|  |  |
| --- | --- |
| Data processing | |
| Processing software | (Add here name and version of the software used) |

**Quality controls for intra-assay variation**

Prepare the pooled plasma QC sample containing equal volumes of the four NIST reference plasma you received.

The pooled QC representing middle quality control (MQC), low QC (LQC), and lowest level of QC (LLQC) is prepared by diluting MQC (e.g. two-(1:1) and three-fold (1:2)) with water. High QC (HQC) and highest level of QC (HLQC) are prepared by adding to MQC additional 10 L and 20 L respectively of STD3 endogenous standards from table 5. Internal standards are added to all QC samples.

**Only intra-assay variation will be measured**

The precision and accuracy of the assay is determined for each of the four ceramides measured in LLQC, LQC, MQC, HQC, and HLQC.

Intra-assay variation, precision, and accuracy is calculated for each of the ceramide in replicates of six (n = 6) at each QC concentration.

The intra-assay precision (percentage coefficient variance, %CV) and accuracy (percentage accuracy, %Accuracy) are calculated from the nominal concentrations according to the formulas reported in the corresponding Excel tables attached to this document.

Recovery estimation is not necessary for the published method but only for different ones. Stability estimation is not necessary for the published method but only for different ones.

Calibration line is prepared in 5% BSA in water, no specific purity/type of BSA is required.

**Results**

Please **report the peak areas** values for Calibration lines, Samples and Intra assay QC in tables 12-15 of the corresponding Excel file.