1. Preparation of Calibration and Internal Standard Solutions

A. Preparation of Individual Acylcarnitine Stock Solutions: Individual acylcarnitine stock solutions from powders are prepared according to table below.

Acylcarnitine (AC)	Carrier	Cat. No.	Individual Stock Conc. (M)	Diluent
Acetylcarnitine, C2	Sigma-Aldrich	A6706	0.01	DI water
Propionylcarnitine, C3	R&D Systems	0611/50	0.01	DI water
Butyrylcarnitine, C4	Toronto Research Chemicals	B802700	0.01	DI water
Isovalerylcarnitine, C5-Iso	Larodan	17-0550-9	0.01	DI water
Hexanoylcarnitine, C6	R&D Systems	0526/50	0.01	DI water
Octanoylcarnitine, C8	R&D Systems	0605/50	0.01	50% MeOH
Decanoylcarnitine, C10	R&D Systems	0477/50	0.01	50% MeOH
Lauroylcarnitine, C12	R&D Systems	0548/50	0.01	50% MeOH
Myristoylcarnitine, C14	R&D Systems	0567/50	0.01	50% MeOH
Palmitoylcarnitine, C16	Sigma-Aldrich	P4509	0.01	50% MeOH
Stearoylcarnitine, C18	Larodan	17-1800-9	0.01	50% MeOH

B. Preparation of Acylcarnitine Working Solutions: Individual stock solutions are combined to prepare the highest calibrator working solution (C_{11} in the table below).

Acylcarnitine	Initial Individual Stock Conc. (M)	Final Combined C_{11} Stock Conc. (mM)
C2	0.01	0.5
C3	0.01	0.05
C4	0.01	0.05
C5-Iso	0.01	0.05
C6	0.01	0.05
C8	0.01	0.05
C10	0.01	0.05
C12	0.01	0.05
C14	0.01	0.05
C16	0.01	0.05
C18	0.01	0.05

Serial dilution of this working solution in 50/50 Acetonitrile/Water with 0.3% Formic Acid yields all eleven calibrator working solutions, C_1-C_{11} in the table below:

Calibrator Stock Soln.	Conc. of C2 (µM)	Conc. of All Other ACs (µM)
C_{11}	500	50
C_{10}	250	25
C_9	100	10
C_8	50	5
C_7	25	2.5
C_6	10	1
C_5	5	0.5
C_4	2.5	0.25
C_3	1	0.1
C_2	0.5	0.05
C_1	0.25	0.025

A 10 µL aliquot of the calibrator working solutions above produces individual calibration curve concentrations used for LC/MS/MS quantitation of acylcarnitines according to the table below:

Calibrator Stock Soln.	Conc. of C2 (µM)	Conc. of All Other ACs (µM)
C_{11}	50	5
C_{10}	25	2.5
C_9	10	1
C_8	5	0.5
C_7	2.5	0.25
C_6	1	0.1
C_5	0.5	0.05
C_4	0.25	0.025
C_3	0.1	0.01
C_2	0.05	0.005
C_1	0.025	0.0025

C. Preparation of Individual Acylcarnitine Internal Standard Stock Solutions: Individual acylcarnitine internal standard stock solutions are prepared from powders according to table below.

Acylcarnitine-Int. Std.	Carrier	Cat. No.	Stock Conc'n (M)	Diluent
Acetylcarnitine, C2-D3	Cambridge Isotopes	DLM-1871	0.01	DI water
Propionylcarnitine, C3-D3	Cambridge Isotopes	DLM-3973	0.01	DI water
Butyrylcarnitine, C4-D3	Cambridge Isotopes	DLM-3861	0.01	DI water
Isovalerylcarnitine, C5-Iso-D9	Cambridge Isotopes	DLM-3974	0.01	DI water
Hexanoylcarnitine, C6-D3	CDN Isotopes	D-6647	0.01	DI water
Octanoylcarnitine, C8-D3	Cambridge Isotopes	DLM-755	0.01	50% MeOH
Decanoylcarnitine, C10-D3	CDN Isotopes	D-6640	0.01	50% MeOH
Dodecanoylcarnitine, C12-D3	CDN Isotopes	D-6643	0.01	50% MeOH
Tetradecanoylcarnitine, C14-D3	CDN Isotopes	D-6661	0.01	50% MeOH
Palmitoylcarnitine, C16-D3	Cambridge Isotopes	D-1263	0.01	50% MeOH
Octadecanoylcarnitine, C18-D3	CDN Isotopes	D-6650	0.01	50% MeOH

D. Preparation of Acylcarnitine Internal Standard Working Solution I: Individual internal standard stock solutions are combined in 50/50 Acetonitrile/Water with 0.3% Formic Acid to prepare the internal standard stock working solution.

Acylcarnitine-Int. Std.	Initial Individual Stock Conc. (M)	Final Combined Stock Conc. (µM)
C2-D3	0.01	25
C3-D3	0.01	2.5
C4-D3	0.01	2.5
C5-Iso-D9	0.01	2.5
C6-D3	0.01	2.5
C8-D3	0.01	2.5
C10-D3	0.01	0.25
C12-D3	0.01	2.5
C14-D3	0.01	2.5
C16-D3	0.01	0.25
C18-D3	0.01	2.5

The internal standard working solution above is diluted 1:10 in 50/50 ACN/0.3% formic acid to generate the internal standard working solution II.

A 10 µL aliquot of internal standard working solution II is spiked into calibrator and study samples to produce the following concentrations for LC/MS/MS quantitation of acylcarnitines:

Sample	Conc. of "A" ISs (uM)	Conc. of "B" ISs (uM)	Conc. of "C" ISs (uM)
C_1-C_9	2.5	0.25	0.025
Tissue/Fluid	2.5	0.25	0.025

"A" Internal Standard: C2

"B" Internal Standards: C3, C4, C5-Iso, C6, C8, C12, C14, C18

"C" Internal Standards: C10, C16

2. Preparation of Calibration Curves and Biological Samples

A. Preparation of Calibration Curves

- To eleven Eppendorf tubes, add either 90 µL of either H_2O (for fluid analysis) or 50/50 ACN/0.3% formic acid (for tissue analysis).
- Spike in 10 µL of the appropriate calibrator working solution to each tube.
- Spike in 10 µL of internal standard working solution II to each tube and vortex thoroughly.
- For all remaining steps, beginning with the addition of MeOH, follow the steps used in "Preparation of Biological Samples" below.

B. Preparation of Biological Samples

- Aliquot 100 µL of either biological fluid or tissue homogenate (at a concentration of 100 mg tissue per mL of 50/50 ACN/0.3% formic acid) into an Eppendorf tube.
- Add 10 µL of internal standard working solution II to each tube.
- Add 800 µL of ice-cold MeOH to the tube and vortex thoroughly.
- Centrifuge the tube at 18,000 x g for five minutes at 4°C; precipitated protein will form a tight pellet at the bottom of the tube.
- After centrifugation, transfer 100 µL of methanolic supernatant to a 96-well plate.
- Dry the contents of the plate under nitrogen at 45°C until all wells are completely dry
- Meanwhile, prepare the derivatization reagents
    - Prepare 0.2 M o-benzylhydroxylamine (OBA) in 50% MeOH/50% 10 mM ammonium acetate, pH 5.5 (store at room temperature; this solution is stable for three days).
    - Prepare 2 M EDC in H_2O (this solution must be prepared daily).

- Once the wells are dry, reconstitute the samples in 100 µL of OBA and 10 µL of EDC. Seal the plate, vortex on a plate mixer for 30 seconds, and incubate for 10 minutes at room temperature.
- After incubation, transfer the plate to the autosampler of the LC/MS/MS.

3. LC/MS/MS Assay Conditions

- **Agilent 1290 Infinity HPLC**

Autosampler
  - Temperature: 4°C
  - Injection Volume: 2 µL
  - Needle Wash Solution: 80/20 Methanol/Water

Column
  - Waters 2 x 100 mm, 1.7 µm BEH C18 column
  - Temperature: 55°C
  - Maximum Pressure: 1000 bar

Binary Pump
  - Flow Rate: 0.4 mL/min
  - Solvent A: 0.1% Formic Acid in H_2O
  - Solvent B: 0.1% Formic Acid in ACN
  - Gradient Conditions:

Segment	Time (min.)	% B	Flow Rate (mL/min)
0 (Start)	0.00	1	0.4
1	3.00	20	0.4
2	5.00	30	0.4
3	12.50	95	0.4
4	12.60	95	0.8
5	14.10	95	0.8
6	14.20	1	0.8
7	16.00	1	0.8
Re-equil.	16.20	1	0.4

- **Agilent 6490 Triple Quadrupole Mass Spectrometer**
  - Gas Temperature: 325˚ C
  - Gas Flow: 11 L/min.
  - Nebulizer: 50 psi
  - Sheath Gas Temperature: 325˚ C
  - Sheath Gas Flow: 10 L/min.
  - Capillary Voltage: 3500 V
  - Nozzle Voltage: 500 V

- Electrospray ionization: Positive

- **Software**
  - MassHunter Acquisition: B.04.01
  - MassHunter Quantitative Analysis: B.05.00

- **MRM Transitions**

Compound Name	Precursor Ion	Product Ion	MS2 Res	Dwell	Fragmentor	Collision Energy	Cell Accelerator Voltage
C22	589.3	190.1	Unit	3	380	25	5
C22:1	587.3	190.1	Unit	3	380	25	5
C22:2	585.3	190.1	Unit	3	380	25	5
C22:3	583.3	190.1	Unit	3	380	25	5
C22:4	581.3	190.1	Unit	3	380	25	5
C22:5	579.3	190.1	Unit	3	380	25	5
C20-OH/C22:6	577.3	190.1	Unit	3	380	20	5
C20:1-OH	575.3	190.1	Unit	3	380	25	5
C20:2-OH	573.3	190.1	Unit	3	380	25	5
C20	561.3	190.1	Unit	3	380	25	5
C20:1	559.3	190.1	Unit	3	380	25	5
C20:2	557.3	190.1	Unit	3	380	25	5
C20:3	555.3	190.1	Unit	3	380	25	5
C20:4	553.3	190.1	Unit	3	380	25	5
C18-OH	549.3	190.1	Unit	3	380	25	5
C18:1-OH	547.3	190.1	Unit	3	380	25	5
C18:2-OH	545.2	190.1	Unit	3	380	20	5
C18-d3	536.3	190.1	Unit	3	380	25	5
C18	533.3	190.1	Unit	3	380	25	5
C18:1	531.3	190.1	Unit	3	380	25	5
C18:2	529.3	190.1	Unit	3	380	25	5
C16-OH	521.3	190.1	Unit	3	380	25	5
C16:1-OH	519.3	190.1	Unit	3	380	20	5
C16:2-OH	517.3	190.1	Unit	3	380	25	5
C16-d3	508.2	190.1	Unit	3	380	25	5
C16	505.2	190.1	Unit	3	380	25	5
C16:1	503.2	190.1	Unit	3	380	25	5
C16:2	501.2	190.1	Unit	3	380	25	5

C14-OH	493.2	190.1	Unit	3	380	25	5
C14:1-OH	491.2	190.1	Unit	3	380	25	5
C14:2-OH	489.2	190.1	Unit	3	380	25	5
C5-DC	486.2	190.1	Unit	3	380	30	5
C14-d3	480.3	190.1	Unit	3	380	25	5
C14	477.3	190.1	Unit	3	380	25	5
C14:1	475.3	190.1	Unit	3	380	25	5
C14:2	473.3	190.1	Unit	3	380	25	5
Ci4-DC	472.2	190.1	Unit	3	380	25	5
C4-DC	472.2	190.1	Unit	3	380	25	5
C12-OH	465.1	190.1	Unit	3	380	25	5
C3-DC	458.1	190.1	Unit	3	380	25	5
C12-d3	452.2	190.1	Unit	3	380	25	5
C12	449.2	190.1	Unit	3	380	25	5
C12:1	447.2	190.1	Unit	3	380	25	5
C10-OH	437.1	190.1	Unit	3	380	25	5
C10-d3	424.2	190.1	Unit	3	380	20	5
C10	421.2	190.1	Unit	3	380	20	5
C8-OH	409.1	190.1	Unit	3	380	20	5
C8:1-OH	407.1	190.1	Unit	3	380	20	5
C8-d3	396.2	190.1	Unit	3	380	20	5
C8	393.2	190.1	Unit	3	380	20	5
C6-OH	381.1	190.1	Unit	3	380	20	5
C6-d3	368.2	190.1	Unit	3	380	20	5
C5-OH	367.2	190.1	Unit	3	380	20	5
C6	365.2	190.1	Unit	3	380	20	5
C5-d9	360.2	190.1	Unit	3	380	20	5
C4-OH	353.2	190.1	Unit	3	380	20	5
C5 2-methylbutyryl	351.2	190.1	Unit	3	380	20	5
C5 isovaleryl	351.2	190.1	Unit	3	380	20	5
C5 valeryl	351.2	190.1	Unit	3	380	20	5
C5:1	349.2	190.1	Unit	3	380	20	5
C4-d3	340.2	190.1	Unit	3	380	20	5
C4 butyryl	337.2	190.1	Unit	3	380	20	5
C4 isobutyryl	337.2	190.1	Unit	3	380	20	5
C4:1	335.2	190.1	Unit	3	380	20	5
C3-d3	326.2	190.1	Unit	3	380	20	5
C3	323.2	190.1	Unit	3	380	20	5

C2-d3	312.2	190.1	Unit	3	380	20	5
C2	309.2	190.1	Unit	3	380	20	5

4. Data Analysis Considerations

Because we only include eleven acylcarnitine standards and eleven internal standards in our calibrator curve, the majority of acylcarnitine species are quantitated using a surrogate curve. The table below depicts which acylcarnitines are quantitated using each curve:

Acylcarnitine Standard/Int. Std.	AC(s) Quantitated Using this Curve
C2/C2-D3	C2
C3/C3-D3	All “C3” species
C4/C4-D3	All “C4” species
C5-Iso/C5-Iso-D9	All “C5” species
C6/C6-D3	All “C6” species
C8/C8-D3	All “C8” species
C10/C10-D3	All “C10” species
C12/C12-D3	All “C12” species
C14/C14-D3	All “C14” species
C16/C16-D3	All “C16” species
C18/C18-D3	All “C18”, “C20” and “C22” species

Created By:	Jeffrey A. Culver	Date: August 7, 2014
Reviewed By:	Christopher Petucci	Date: August 7, 2014
Approved By:	Christopher Petucci	Date: August 7, 2014

Revision Number	Name	Reason for Revision	Effective Date
01			
02			