Analysis of Neutral Lipids
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This protocol describes the analysis of neutral lipids from cells and is to follow a Lipid MAPS cell treatment (time course) protocol, which includes addition of internal standard to time course samples and controls. The steps are extraction and purification of neutral lipids, and analysis by mass spectrometry for each sample.

I. Extraction and Purification of Neutral Lipids

This extraction of total lipids is for cells suspended in 0.5 mL DPBS and 1.25 mL methanol. Subsequent purification of the neutral lipid fraction is by solid phase extraction (SPE) using cartridges obtained from Supelco (Discovery DSC-NH2, 1 mL tube, 100 mg, Cat #52636-U). Steps 1-4 of this procedure are carried out in an argon atmosphere by removing air from a glove bag (I^2R, cat # X-17-17H) by vacuum and filling with argon.

Reagents required:

Methylene chloride (CH_2Cl_2)
Methanol (MeOH)
Deionized water
Isopropyl alcohol (IPA)
Hexanes
Aminopropyl SPE cartridges

1. Prepare a monophase by adding 0.625 mL CH_2Cl_2 to each time course sample.
2. Vortex for 30 seconds using a Vortex Genie 2 (VWR Scientific).
3. Add 0.5 mL water and 0.625 mL CH_2Cl_2.
4. Vortex for 30 seconds with the stopper in place.
5. Centrifuge for 1 min at 1000x*g*. Two phases should be clearly visible.
6. Activate SPE cartridges by washing with 4 mL of hexanes, being sure not to allow the packing to become dry.
7. Carefully remove the bottom organic layer (step 5) and deliver into an activated aminopropyl cartridge.
8. Apply vacuum and draw the CH_2Cl_2 through the cartridge into a clean tube.
9. Elute neutral lipid fraction with 2 mL of CH_2Cl_2:IPA (2:1) into the same tube.
10. Dry sample under N_2
11. Store at -20 °C in IPA/MeOH/50 m<u>M</u> NH_4OAc, 4:3:2 (the electrospray solution without the addition of the CH_2Cl_2).
12. Use a LIMS "Mass Spectrometry" application to assign a barcode to each sample.

II. Neutral Loss Mass Spectrometry

This method is to survey the diacyl- and triacylglycerol species present in purified extracts of cells (part I). The samples are infused into an API 4000 QTRAP with a nanoMate ion source using the parameters listed below.

Solutions:

- Electrospray solution
  - 40% Isopropanol (IPA)
  - 30% Methanol (MeOH)
  - 20% 50 mM Ammonium Acetate (NH_4OAc)
  - 10% Methylene chloride (CH_2Cl_2)

- Internal Standard

  These deuterium labeled DAG and TAG species are obtained from Avanti Polar Lipids individually and mixed as described in protocol "Core E - Neutral Lipid Internal Standard" to form the stock solution added to the samples.

	m/z		
d5-Standard	$[M+H]^+$	$[M+NH_4]^+$	Avanti ID/LM ID
14:0/14:0 DAG	518.8	535.8	110535
15:0/15:0 DAG	546.9	563.9	110536
16:0/16:0 DAG	574.9	591.9	110537
17:0/17:0 DAG	602.9	619.9	110538
19:0/19:0 DAG	658.9	675.9	110539
20:0/20:0 DAG	686.9	703.9	110540
14:0/16:1/14:0 TAG	754.7	771.7	LMGL03010009
15:0/18:1/15:0 TAG	810.8	827.8	LMGL03010010
16:0/18:0/16:0 TAG	840.8	857.8	LMGL03010011
17:0/17:1/17:0 TAG	852.8	869.8	LMGL03010012
19:0/12:0/19:0 TAG	840.8	857.8	LMGL03010013
20:0/20:1/20:0 TAG	978.9	995.9	LMGL03010014
20:2/18:3/20:2 TAG	938.5	955.5	LMGL03010015
20:4/18:2/20:4 TAG	932.8	949.8	LMGL03010016
20:5/22:6/20:5 TAG	976.5	993.5	LMGL03010008

1. To samples stored in IPA/MeOH/50 mM NH_4OAc, 4:3:2 (Step II. 1 above), add 10% CH_2Cl_2 immediately prior to analysis on the nanoMate/API 4000 QTRAP.

2. nanoMate Conditions**:**

Gas	0.30 psi
IS	+1.38 kV
Asp. depth	2 mm
Sample volume	15 μL
Vented headspace	

3. API 4000 Conditions:

CUR	10
CAD	Medium
IS	0
GS1	0
GS2	0
IHT	0
DP	120
EP	15
CE	37
CXP	6.5
Mass range	500-1100 amu
Scan time	3 seconds
Total time	2 min/neutral loss (40 cycles)

4. Neutral Loss Scans:
The losses listed correspond to each of the fatty acid moieties in the internal standards.

Neutral loss (*m/z*)	Fatty acyl group ($+NH_4$)
217.2	12:0
245.2	14:0
259.2	15:0
271.3	16:1
273.3	16:0
285.3	17:1
287.3	17:0
297.3	18:2
299.3	18:1
301.3	18:0
315.3	19:0
321.3	20:4
327.3	20:1
329.3	20:0
325.3	20:2
295.3	18:3
319.3	20:5
345.3	22:6