Core J Extraction and Analysis of Cellular Sterol Lipids

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Synopsis: This protocol describes the standard method for the extraction and analysis of sterols following a LIPID MAPS time course protocol. Cells should be grown and treated according to a LIPID MAPS protocol. Sterols are extracted via a modified Bligh-Dyer method and separated using a reverse phase binary liquid chromatography (LC) gradient. Sterols are quantitated using a MRM method with positive electrospray ionization mass spectrometry (ESI-MS) and normalized to DNA.

I. Extraction of Sterol Lipids

The extraction protocol outlined below is for cells grown in 60 or 100mm dishes suspended in 2 mL of DPBS. After extraction and quantification of lipids, sterols are normalized to mass of DNA.

Reagents Required:

Chloroform	High purity water
DPBS	MeOH
EDTA	

A. Lipid Extraction from Medium

B. Cell Harvest and Lipid Extraction

1. Remove medium to a 15 mL conical tube. Centrifuge at 2400 rpm for 10 min (eppendorf 5810 R with swinging bucket rotor). Transfer supernatant to a new tube and add 10 µL of each surrogate mix. Store at -80°C until extraction and analysis.
2. After washing cells twice with 3 mL DPBS, add 2 mL DPBS/1mM EDTA and scrape cells loose from dish surface.
3. Transfer the cells to a 15 mL polypropylene conical tube. Pipette 20 times to suspend cells.
4. Transfer 400 µL to a 1.5 mL eppendorf tube for DNA assay. To these, add 20 µL 50% EtOH in H_2O. Store at -80˚C until assay.
5. To the remaining 1.6 mL cells, add 6 mL $CHCl_3$/MeOH (1:2 *v*:*v*).
6. Add 10 µL of each surrogate mix. Make note of the concentrations of these standards. Vortex well.
7. Centrifuge at 2400 rpm for 5 minutes (eppendorf 5810 R with swinging bucket rotor).
8. Decant supernatant into a fresh 15 mL polypropylene conical tube. Discard pellet.
9. To the supernatant, add 2 mL each of $CHCl_3$ and DPBS. Vortex well.
10. Centrifuge at 2400 rpm for 5 minutes.
11. Remove organic (lower) phase to a fresh 4 mL glass vial with Teflon-lined cap using a 9 inch Pasteur pipette.
12. Dry the organic phase under nitrogen with gentle heating (37˚C).
13. Resolve sterols in 400 µL of 5% water in methanol.

Storage: DNA samples are stored at -80°C until analysis. Sterol samples are stored at 4°C

II. Positive ESI Liquid Chromatography Mass Spectrometry (ESI- LC/MS)

The LC/MS protocol outlined below is for the analysis of sterols in purified cell extracts (part I). Sterols were resolved by reverse-phase HPLC using a binary solvent system and gradient elution was performed on a C18 RP-HPLC column. The HPLC was coupled to a triple quadrupole MS with an ESI source. The MS was operated in multiple reaction monitoring (MRM) mode with transitions optimized for each sterol of interest. Sterols were quantified using the internal standards, surrogate, and relative response factor (RRF) of each sterol of interest.

A. Solutions:

1. **Mobile Phase A**
   Methanol with 5mM ammonium acetate

2. **Mobile Phase B**
   15% High Purity water in methanol with 5mM ammonium acetate

Mobile phases A and B were sparged with Helium for 5 minutes.

3. Surrogates

Two deuterated surrogates, 10 μL each, are added to cells before extraction:

Table 1: Surrogate composition

SURROGATE MIX 1	SOURCE	CONCENTRATION [PPM]
25-Hydroxycholesterol (D_3) in MeOH	Avanti Polar Lipids	2.348
24,25-Epoxycholesterol (D_6) in MeOH	Avanti Polar Lipids	1.656
7α-Hydroxycholesterol (D_7) in MeOH	Avanti Polar Lipids	1.983
7-Oxocholesterol (D_7) in MeOH	Avanti Polar Lipids	2.042
4β-Hydroxycholesterol (D_7) in MeOH	Avanti Polar Lipids	0.390
SURROGATE MIX 2		
Cholesterol (D_7) in MeOH	Avanti Polar Lipids	78.200
Desmosterol (D_6) in MeOH	Avanti Polar Lipids	78.278

4. Internal Standard

27-Hydroxycholesterol (D_5) 5.258 ppm from Avanti Polar Lipids

B. Compounds of interest

We are monitoring the following compounds via Selected Reaction Monitoring

Table 2: Compounds monitored via Selected Reaction Monitoring

COMPOUND	MRM PAIR
22r-Hydroxycholesterol	420/385
24-Hydroxycholesterol	420/385
25-Hydroxycholesterol	420/367
26-Hydroxycholesterol	420/385
24,25-Epoxycholesterol	418/383
7α-Hydroxycholesterol	385/367
7-Ketocholesterol	401/383
5/6β Epoxycholesterol	420/385
5/6α Epoxycholesterol	420/385
4β-Hydroxycholesterol	420/385
Zymosterol	385/367
Desmosterol	402/367
7-Dehydrocholesterol	385/367
3keto cholestene	385/367
Lathosterol	404/369
Cholesterol	404/369
Lanosterol	444/409
Cholestanol	404/387
24-Dihydrolanosterol	429/411
3,16dioxo cholestenoic acid	429/411
TriOH cholesterol	401/383
4-chol-27acid-3one	415/397
4-chol-22OH-3one	401/383

4-chol-24OH-3one	401/383
4-chol-25OH-3one	401/383
4-chol-2OH-3one	401/383
20-Hydroxycholesterol	385/367
4-chol-26(25r)OH-3one	401/383
4-chol-26(25s)OH-3one	401/383
3keto,26cholestene	401/383
8(14) cholesten 3β,15α diol	385/367
3β,15α cholestanol	422/369
8(14) cholesten 3βOH 15one	401/383
cholestan 3oh 15one	403/385
7α hydroxycholestenone	401/383
8(14) cholesten 3β,15β diol	385/367
3β,15β cholestanol	422/369
7ketocholestanone	401/383
dihydroxyketocholesterol	401/383
19-Hydroxycholesterol	420/385
4,6 Chlestadiene -3-one	383/365
Lathosterone	385/367
5-chol-3-one	385/367
cycloartenol	444/409
Bsitosterol	432/397
Bsitosterone	413/413
3,16dioxo cholestenoic acid	429/411
TriOH cholesterol	401/383
4-chol-27acid-3one	415/397
4-chol-22OH-3one	401/383
4-chol-24OH-3one	401/383
4-chol-25OH-3one	401/383
4-chol-2OH-3one	401/383
20-Hydroxycholesterol	385/367
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Lathosterone	385/367
5-chol-3-one	385/367
cycloartenol	444/409
Bsitosterol	432/397
Bsitosterone	413/413
DEUTERATED COMPOUND	**MRM Pair**
7β-Oxocholesterol (D_7)	408/390
7β-Hydroxycholesterol (D_7)	391/373
4β-Hydroxycholesterol (D_7)	426/391
7α-Hydroxycholesterol (D_7)	391/373
25-Hydroxycholesterol (D_3)	423/370
27- Hydroxycholesterol (D_5)	425/390

24,25 Epoxycholesterol (D_6)	424/389
Cholesterol (D_7)	411/376
Desmosterol (D_6)	408/373

C. Instrumentation

1. Column Information

Company: Phenomenex
Packing: Reverse Phase C18
Particle Size: 3µ
Diameter: 2mm
Length: 150mm

This column is maintained at 25°C.

2. HPLC conditions

Total Flow: 0.25 mL/min

Table 3: HPLC Gradient

TIME (MIN)	% MOBILE PHASE B
0	100
2	100
8	0
18	0
23	100

3. API 4000 Q Trap Conditions

CUR: 15.00
CAD: Medium
IS: 5500.00
GS1: 60.00
GS2: 20.00
DP: Variable Depending on MRM pair (45.00-120.00)
EP: 10.00
CE: Variable Depending on MRM pair (10.00-65.00)
CXP: 10.00