Eicosanoid Mass Spectra Protocol
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Synopsis:
This protocol describes the standard method for quantitating the eicosanoids found in media via LCMSMS. The media is removed from the cells and methanol is added along with deuterated eicosanoids that serve as internal standards. The eicosanoids are then isolated via column chromatography and then concentrated for LCMSMS analysis. **Note the media cannot contain any indicator dyes. These swamp the mass spec.** The samples are then run on the mass spec along with a set of primary eicosanoid standards (also containing the same deuterated internal standards as the samples). The primary standards are not deuterated and their concentrations are accurately known.
All of the MS parameters are available in the MS data files. All other conditions are listed here.

Storage: All samples and standards are stored under argon at -20° C.

Solutions:
1. Buffer A
37% Acetonitrile
0.02% Formic Acid
63% Water

2. Buffer B
50% Acetonitrile
50% Isopropanol
Buffer A and B are sparged with argon for 1 min and then filtered through a Millipore 5μm LSWP 47 mm filter (cat #LSWP04700).

3. Internal Standards
The internal standard contains 0.1 ng/μl of each of the following deuterated eicosanoids in 50/50 ethanol in water:

Compound	Deuteration	Cayman Cat#
$PGF_{2\alpha}$	D4	316010
PGE_2	D4	314010
PGD_2	D4	312010
5 Hete	D8	334230
AA	D8	390010

The stocks are usually 1 ng/μl.

4. Primary Standards
We are currently using four primary standards. PGF2α, PGE_2, PGD_2, and AA. These standards are obtained from Cayman with accurately determined quantities in 1 mg amounts. Stock solutions were made of each of these by dissolving the compound in the same solvent that Cayman uses to ship the given compound normally. All stock solutions were 500 ng/ μl (exact).

Compound	Cayman Cat #	Stock Conc.
PGF_2	16010	500 ng/ μl
PGE_2	14010	500 ng/ μl
PGD_2	12010	500 ng/ μl
AA	90010	500 ng/ μl

From these stocks, a 50 ng/μl stock is made and then serial dilutions of this stock (50 ul plus 450 ul of ethanol) are made to provide the following Working Dilutions for each standard. 5 μl of each Working Dilution is then add to 100 μl of the Internal Standard to give the Final Primary Standard Solutions.

Working Dilutions	Final Primary Standards
50 ng/μl	2.38 ng/ μl
5 ng/ μl	0.238 ng/ μl
.5 ng/ μl	0.0238 ng/ μl
0.05 ng/ μl	0.00238 ng/ μl

All solvents used are EMD Omnisolv grade reagents (including water)

5. Current Compounds: We are currently monitoring the following compounds via MRM. This will vary from run to run. The actual numbers are in the wiff files.

Compound	Ret. Time Min	Precursor m/z	Product m/z	Deut Precursor m/z	Deut Product m/z
11β PGF2α	4.53	353	193	357	197
PGF2α	4.93	353	193	357	197
PGE2	5.49	351	271	355	275
PGD2	5.98	351	271	355	275
LTB4	9.42	335	195		
LTE4	11.83	438	235		
20 HETE		319	145		
15 HETE		319	113		
11 HETE	10.74	319	167		
12 HETE		319	179		
8 HETE		319	155		
5 HETE		319	115	327	116
AA	14.5	303	259	311	267

HPLC Conditions:

1. Column Information

Company: Vydac
Model: 201TP52
S/N: NE981208-3-1
Packing: Reverse Phase C18
Particle Size 5 um
Diameter: 2.1 mm
Length: 250 mm

The column is maintained at 35° C with column heater.

Media Collection and Separation:

Cell Media Preparation Principle: The eicosanoids are separated from the other media components by purification on Strata-X columns (Phenomenex cat # 8B-S100-UBJ strata-X 33 μm Polymeric Sorbent). The 3 ml columns are used for 4 ml of media. The columns are run via a vacuum using a Supelco Visiprep 24 vacuum chamber.

Media Collection: The media is decanted off of the cells and spun in a 2 ml Eppendorf tube at 2000rpm for 5 min to remove detached cells. Then 400 μL of 100% MeOH (10% final concentration) and 200 μL 10% glacial acetic acid (0.5% final concentration) are added per 4 ml of media. 100 μl of the internal standards are added to each sample.

Columns:
Set the manometer on the vacuum chamber to 5 mmHg.
Do not let the column run dry in steps 1, 2, and 3.

1. Precondition columns: Elute 2 ml MeOH and then elute 2 ml H_2O.
2. Apply sample: Load sample.
3. Wash: Add 2 ml of 10% MeOH to the Sample vial. Vortex and apply to the column under vacuum.
4. Elution: Apply 1 ml 100% methanol to column. Elute and run dry with vacuum for 30 seconds.
5. Concentration: The solvent is removed by speed vac and the eicosanoids are redissolved in 100 μl of buffer A.
6. The samples are spun in a microfuge to remove a water insoluble ppt and the supernatant is decanted and stored.

7. Storage: These samples can be stored at -20 C for several days at least.