Synopsis:
This protocol describes the standard method for quantitating the eicosanoids found in media via LCMS. The media is removed from the cells and acetic acid and methanol are added along with deuterated eicosanoids that serve as internal standards. The eicosanoids are then isolated via column chromatography and then concentrated for LCMS 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 2 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:
   - **PGF2α D4** 316010
   - **PGE2 D4** 314010
   - **PGD2 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, PGE2, PGD2, 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).
   - **PGF2α** 16010 500 ng/µl
   - **PGE2** 14010 500 ng/µl
   - **PGD2** 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
     - 0.5 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

<table>
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<th>Compound</th>
<th>Ret. Time</th>
<th>Parent m/z</th>
<th>Daughter m/z</th>
<th>Deut. Parent m/z</th>
<th>Deut. Daughter m/z</th>
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<td>6-KETO</td>
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</table>

**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 400 μL of 10% MeOH (10% final concentration) and 20 μL glacial acetic acid (0.5% final concentration) per 4 ml of media is added. 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) and (2).

1. **Precondition columns:** Elute 2 ml MeOH, stop, and then elute 2 ml H2O.
2. **Apply sample:** Load sample.
3. **Wash:** Add 2 ml of 5% MeOH to the Sample vial. Vortex and apply to the column under vacuum. Allow to run dry for 30 seconds.
4. **Elution:** Apply 2 ml Isopropyl alcohol to column and equilibrate for 1 min. 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 50% ethanol in water.

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