Oxysterol Derivatization MaxSpec® Kit

Item No. 601540

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Customer Service 800.364.9897
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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below. *Sold under license from Swansea University*

Item Number	Item	Volume/Size	Storage
601541	Girard's Reagent P	50 mg	-20°C
22005	Girard's Reagent P-d_5	50 mg	-20°C
601543	Oxysterol Derivatization MaxSpec® Cholesterol Oxidase	20 U/1 vial	-20°C
601542	Oxysterol Derivatization MaxSpec® System Suitability Mixture*	1 ml at 1 µg/ml	-20°C

*Oxysterol Derivatization MaxSpec® System Suitability Mixture

Item Number	Item	Concentration
11097	25-hydroxy Cholesterol	1 µg/ml in ethanol
16339	7-keto Cholesterol	1 µg/ml in ethanol
11032	7α,25-dihydroxy Cholesterol	1 µg/ml in ethanol

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. Methanol, LCMS grade or equivalent
2. Acetonitrile, HPLC grade or equivalent
3. Deionized water
4. HPLC autosampler vials (such as Phenomenex: AR0-9921-13-C)
5. 1.7 mL polypropylene Eppendorf Tubes (such as VWR: 87003-294)
6. Waters Sep-Pak Vac C18 SPE 3 cc cartridge, 200 mg (WAT054945)
7. Waters Oasis HLB SPE cartridges, 30 mg (WAT094225)
8. Acetic Acid (>99%) (such as Sigma-Aldrich: A6283)
9. Isopropanol, HPLC grade or equivalent
10. Ethanol, Absolute 200 proof
11. Formic Acid (>95%) (such as Sigma-Aldrich: F0507)

Equipment

1. Oven or incubator controlled at 37°C
2. Microcentrifuge (optional)
3. SpeedVac concentrator (optional)
5. Pipettors (10 µl, 200 µl, and 1,000 µl)
6. LC-MS system (a Thermo Scientific Ultimate 3000 UHPLC coupled with a Q Exactive Hybrid Quadrupole Orbitrap mass spectrometer was used to develop the procedures outlined in this kit)

INTRODUCTION

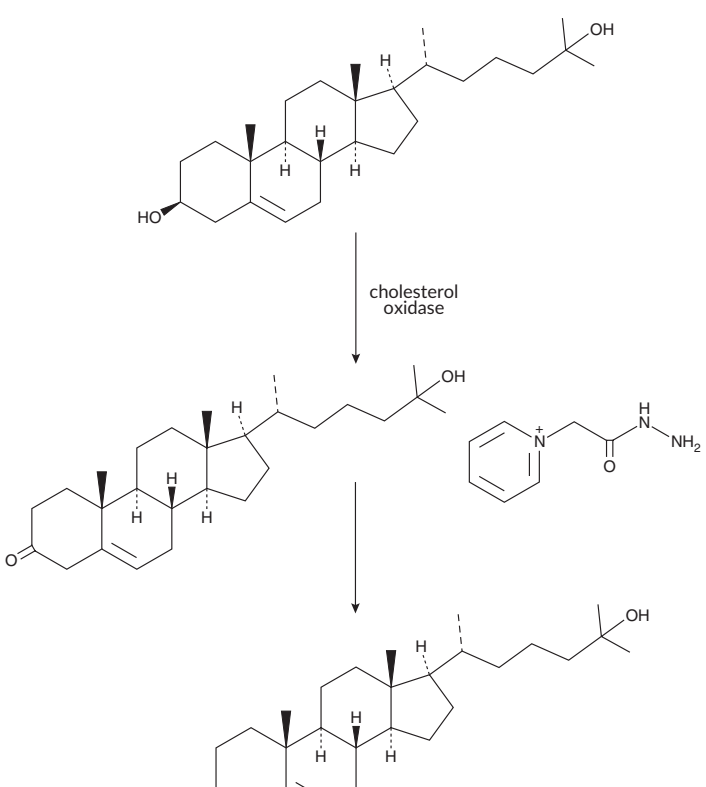
Background

Oxysterols are produced through enzymatic or non-enzymatic oxidation of cholesterol.[1] They function as metabolic intermediates or bioactive lipids and are involved in the regulation of cholesterol and lipid metabolism and have roles in cell signaling and innate and adaptive immunity, among others. For example, 24(S)-hydroxy cholesterol is important for sterol homeostasis in the CNS, several oxysterols act as liver X receptor (LXR) ligands, and 25-hydroxy cholesterol regulates cholesterol biosynthesis. 25-hydroxy Cholesterol also inhibits replication of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) *in vitro* and increased rapidly prior to death in the serum of a patient with COVID-19, the primarily respiratory virus caused by SARS-CoV-2.[2]

About This Assay

Oxysterols are challenging to study directly by LC-MS due to the abundance of structurally similar oxysterols and their metabolites and lack of method sensitivity.[3] Derivatization of oxysterols is often necessary to improve sensitivity and allow for their identification and quantification. The technology employed in Cayman's Oxysterol Derivatization MaxSpec® Kit, developed under license, employs derivatization with a charged functional group to improve LC-MS analysis of oxysterols.[3-6]

Based on the quantitative charge-tag procedure developed by Griffiths *et al.* for sterol and oxysterol analysis, Cayman has designed this kit for the sensitive detection and relative quantification of oxysterols in biological samples.[7] Using Cayman's Oxysterol Derivatization MaxSpec® Kit, 3-hydroxy sterols are converted to 3-oxo sterols with cholesterol oxidase (ChO), followed by derivatization using an enzyme-assisted oxidation step to produce GP hydrazones. Because of a quaternary nitrogen in the GP hydrazones, the derivatized oxysterols are easily ionized during LC-MS analysis and sensitive detection of oxysterols can be achieved. The charged functional group improves electrospray ionization of the sterols and thus improves sensitivity in the detection of these species. Two types of isotope-labeled GP reagents, light and heavy, are included in the kit. The use of light and heavy GP reagents allows for the implementation of multiple versatile workflows as described in the Workflow Examples section of this booklet (see page 14). Scheme 1, on page 8, shows the stepwise oxidation and derivatization steps for 25-hydroxy cholesterol.



Scheme 1. Stepwise oxidation and derivatization steps for 25-hydroxy cholesterol

PRE-ASSAY PREPARATION

Derivatization Reagent Preparation

Reconstitution of Cholesterol Oxidase: Dissolve 20 U ChO in 350 ul of deionized water. Add 3ul of the ChO solution will be added into each sample. Aliquot the remaining ChO solution as needed to avoid deactivation of the enzymes caused by multiple freeze-thaw cycles. The aliquoted ChO solutions should be stable at -80°C for several months.

ASSAY PROTOCOL

Derivatization

The following derivatization procedure is recommended for oxysterols in pure solvent such as ethanol. If the oxysterol sample is dissolved in pure organic solvent the sample should be diluted 10-fold in water (or 50 mM KH_2PO_4) before adding 3 µl of the ChO solution. For dried samples, reconstitute the sample in a small volume of ethanol first. Then dilute sample 10-fold with water (or 50 mM KH_2PO_4) before adding 3 µl of ChO solution.The organic content should be less than 10% in the final solutions. Gently vortex the dissolved sample, then incubate at 37°C for one hour.

Prior to derivatization, prepare a 2 mg/ml solution of the GP-d_0 reagent by dissolving 2 mg in 1 ml of methanol contining 1% acetic acid.[8] After ChO oxidation, add three volumes of the 2 mg/ml GP-d_0 solution into the sample mixture and vortex. Incubate the mixture in dark at 37°C overnight to derivatize the oxysterol sample. The entire 50 mg vial of the Girard's P reagent is designed for use with 50 samples. For a 50-sample set, transfer the entire contents of the reagent vial to a 25 ml volumetric flask and dilute to volume with diluent. Otherwise, the reagent may be weighed and used as needed for smaller sample sets (1 mg is required per every 3 samples).

If using the heavy labeled GP-d_5 reagent as described in the Workflow examples follow the same procedure as above for the unlabeled reagent.

For oxysterol samples in matrices, oxysterol extraction and purification are required. The detailed protocol for sample preparation in plasma samples can be referred to in the papers published by Griffiths W.J. *et al.*[3-5,9]

System Suitability Experiment

This system suitability test is intended to provide a positive control for the kit and method. We recommended you run this with each sample set or following established quality control practices. Prepare 100 ng/ml oxysterol standard mix by adding 10 µl of the Oxysterol System Suitability Mix into 90 µl water. Add 3 µl of the ChO solution into the diluted oxysterol mixture and incubate at 37°C for one hour.

After ChO oxidation, add 300 µl of the 2 mg/ml GP reagent solution and place in the dark at room temperature overnight for derivatization. The derivatized oxysterols can be analyzed directly by LC-MS.

1. LC-MS/MS Analysis

The derivatized sample can be analyzed by any LC-MS/MS system. High resolution mass spectrometer offers exact mass measurement of both precursor and product ions. A Thermo Scientific UltiMate 3000 UHPLC coupled with Q-Exactive hybrid Quadrupole-Orbitrap mass spectrometer was used for LC-MS/MS analysis of oxysterols and derivatized oxysterols. Full mass followed by data dependent MS/MS acquisition was used to profile and identify the GP derivatized oxysterols in the samples.

2. UHPLC setup

An Acquity UHPLC 1.7 µM BEH C8 column (2.1 x 100 mm) operated at ambient temperature is used to remove excess GP reagent and separate derivatized oxysterols. Set up a gradient elution method with Mobile Phase A (water with 0.1% formic acid) and Mobile Phase B (acetonitrile with 0.1% formic acid) as outlined in Table 1, below. The HPLC flow rate is 300 µl/minute. Sample injection volume is 5 µl.

Time (minutes)	%A	%B
0-0.5	98	2
1	85	15
10	20	80
10.2-13	5	95
13.1-16	98	2

Table 1. UHPLC Gradient for oxysterol and derivatized oxysterol mixture

3. Mass spectrometer setup

The eluted oxysterols and derivatized-oxysterols are ionized by electrospray ionization in positive ion mode with nitrogen as desolvation gas and collision gas. The following mass spectrometric parameters are set for ESI source: spray voltage, 3.8 kV; capillary temperature, 320°C; S-lens RF level is 50. Mass resolution for full mass analysis is 70,000, and mass range is m/z 380-650. AGC target 1e5. Data dependent acquisition of MS/MS spectra of the ions is enabled automatically based on ion intensity threshold 4e4. An inclusion list is used during MS/MS for oxysterols and GP derivatized oxysterols. Dynamic exclusion is set to one second. To reduce the contamination of mass spectrometer ion source, the HPLC eluate is diverted to waste in the period of 0-3 and 13-16 minutes during each HPLC-MS/MS run.

Name	Derivatized Formula	Accurate Mass	Retention (minutes)
25-hydroxy Cholesterol-GP-d_0	$C_{34}H_{52}N_3O_2^+$	534.4054	8.1
7-keto Cholesterol-DP-d_0*	$C_{34}H_{52}N_3O_3^+$	532.3898	9
7α,25-dihydroxy Cholesterol-DP-d_0	$C_{34}H_{52}N_3O_3^+$	550.4003	7.2
25-hydroxy Cholesterol-DP-d_5	$C_{34}H_{47}D_5N_3O_2^+$	539.4368	8.1
7-keto Cholesterol-DP-d_5*	$C_{34}H_{47}D_5N_3O_3^+$	537.4211	9
7α,25-dihydroxy Cholesterol-DP-d_5	$C_{34}H_{47}D_5N_3O_3^+$	555.4317	7.2

**Monoderivatized at 7-position*

Table 2. Derivatized oxysterol mass spectrometry and retention time information

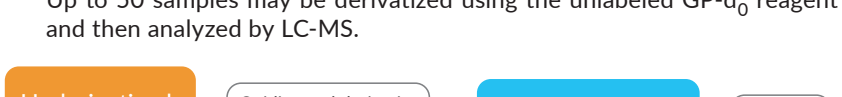
ANALYSIS

Workflow Examples

The kit contains both an unlabeled and labeled derivatization reagent which are intended to provide versatility in use of the kit. The following diagrams show four workflows that may be followed using this kit.

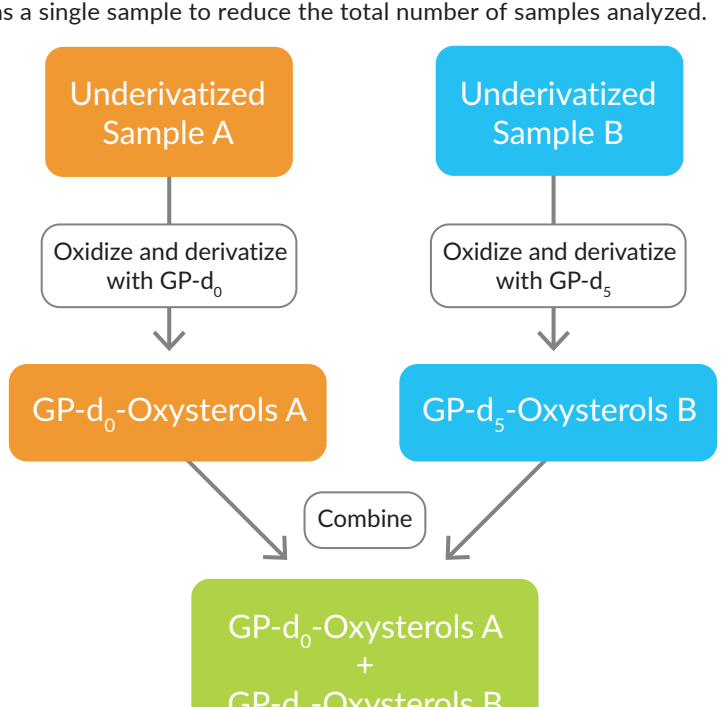
1. Standard Oxysterol Derivatization Workflow

Up to 50 samples may be derivatized using the unlabeled GP-d_0 reagent and then analyzed by LC-MS.



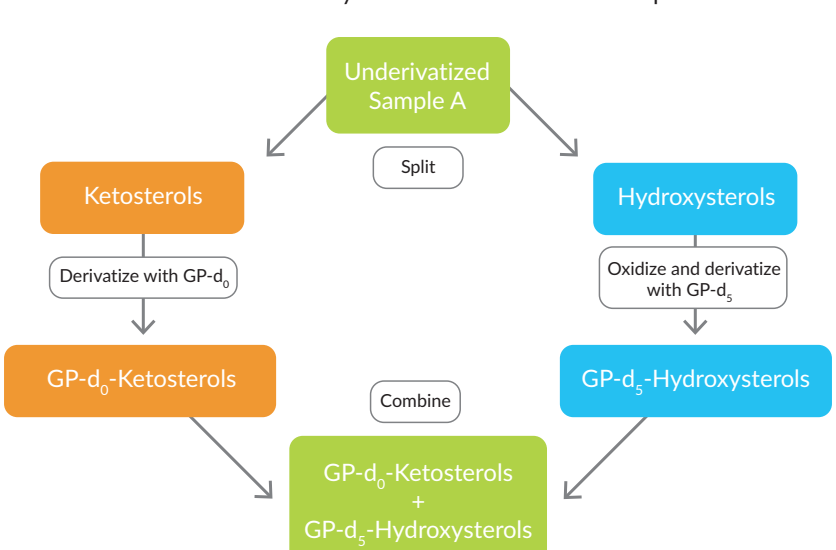
2. Multiple Cohort Derivatization Workflow

In a two-population study, up to 50 samples from population 1 may be derivatized with the GP-d_0 reagent and up to 50 samples from population 2 may be derivatized with the labeled GP-d_5 reagent (GP-d_5). Pairs of population 1/population 2 samples may then be recombined and analyzed as a single sample to reduce the total number of samples analyzed.



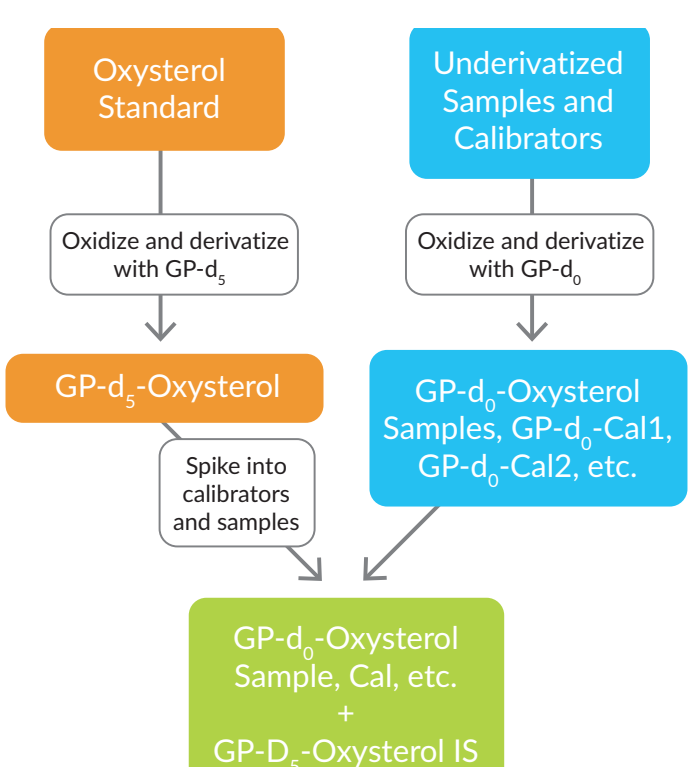
3. Hydroxysterols and Ketosterols Workflow

Up to 50 samples may be split into two equal portions. The first portion can be treated directly with the GP-d_0 reagent to derivatize the ketosterols. The second portion may be oxidized and then treated with the GP-d_5 reagent to derivatize the combined oxysterols and ketosterols. The two portions can then be analyzed separately or recombined to differentially measure ketosterols and oxysterols within the same sample.



4. Quantitative Analysis of Oxysterols

An oxysterol standard can be derivatized using the labeled Girard's P reagent (GP-d_5) and used as the internal standard for quantitation of up to 50 oxysterol samples.



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NOTES

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