Cholesterol Fluorometric Assay Kit

Item No. 10007640

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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

Item Number	Item	96 well Quantity/Size	480 well Quantity/Size
10008052	Cholesterol Assay Buffer (10X)	1 vial/3 ml	1 vial/15 ml
10008053	Cholesterol Assay Standard	1 vial/100 µl	1 vial/500 µl
10008054	Cholesterol Assay Detector	2 vials	5 vials
10008055	Cholesterol Assay Horseradish Peroxidase	1 vial	1 vial
10008056	Cholesterol Assay Oxidase	1 vial	1 vial
10008057	Cholesterol Assay Esterase	1 vial	1 vial
700001	DMSO Assay Reagent	1 vial/1 ml	1 vial/3 ml
400017	96 well Solid Plate (black)	1 plate	5 plates
400012	96 well Cover Sheet	1 cover	5 covers

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

Hours: M-F 8:00 AM to 5:30 PM EST

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 specified at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorometric plate reader capable of measuring fluorescence using an excitation wavelength between 530-540 nm and emission wavelengths between 585-595 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Cholesterol circulates in the blood as a free acid, as well as, esterified to long-chain fatty acids called cholesteryl esters. Cholesteryl esters are the preferred form for cholesterol transport and storage. Lecithin:cholesterol acyltransferase is a 67 kDa glycoprotein that is responsible for cholesterol esterification in plasma. The enzyme displays two activities: a phospholipase A_2 activity, which hydrolyzes the fatty acyl group from the *sn*-2 position of phosphatidylcholine; and a transacylase activity, which catalyzes the transfer of the fatty acyl group from the acyl-enzyme complex to the 3-β hydroxyl group of cholesterol to form cholesteryl ester.[1] Elevated levels of cholesterol and cholesteryl esters have been linked to atherosclerosis and heart disease.[2,3] This has resulted in a large amount of research focused in the understanding of cholesterol homeostasis. Quantitation of cholesterol in experimental samples is imperative to this research.

About This Assay

Cayman's Cholesterol Fluorometric Assay Kit provides a simple fluorometric method for the sensitive quantitation of cholesterol in serum and plasma. The assay is based on an enzyme-coupled reaction that detects both free cholesterol and cholesteryl esters as depicted in Figure 1 below. Cholesteryl esters are hydrolyzed by cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase to yield hydrogen peroxide and the corresponding ketone product. Hydrogen peroxide is then detected using 10-acetyl-3,7-dihydroxyphenoxazine (ADHP), a highly sensitive and stable probe for hydrogen peroxide.[4] In the presence of horseradish peroxidase, ADHP reacts with hydrogen peroxide with a 1:1 stoichiometry to produce highly fluorescent resorufin.[4]

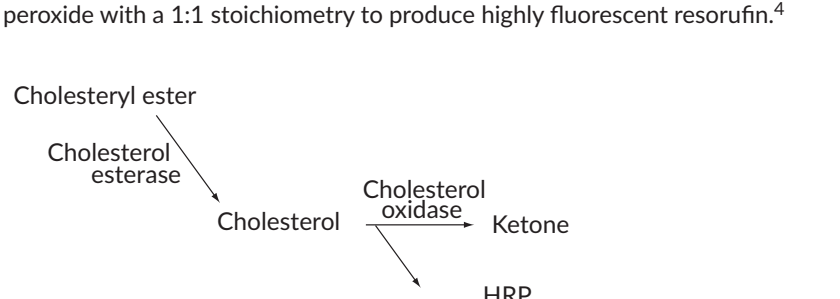


Figure 1. Scheme 1

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Cholesterol Assay Buffer (10X) - (Item No. 10008052)**

   Dilute 3 ml of assay buffer concentrate with 27 ml of HPLC-grade water. This final assay buffer (100 mM potassium phosphate, pH 7.4, containing 50 mM sodium chloride and 5 mM cholic acid) should be used for the preparation of standards and the dilution of samples. The diluted assay buffer should be stable for at least one week if stored at room temperature or 4°C.

2. **Cholesterol Assay Standard - (Item No. 10008053)**

   The vial contains 10 mM cholesterol (5-cholestan-3β-ol) in ethanol. The reagent is ready to use for preparation of the diluted cholesterol standards.

3. **Cholesterol Assay Detector - (Item No. 10008054)**

   The vial contains a lyophilized powder of ADHP. Prior to adding to the assay cocktail (See **Performing the Assay** on page 13 step 4), reconstitute the cholesterol detector with 100 µl of DMSO (Item No. 700001) and 100 µl of HPLC-grade water. The reconstituted cholesterol detector should be stable for 15 minutes. Each reconstituted vial is enough reagent to perform the entire 96-well plate.

4. **Cholesterol Assay Horseradish Peroxidase (HRP) - (Item No. 10008055)**

   The vial contains a lyophilized powder of HRP. Reconstitute the 1 each vial with 200 µl and the 5 each vial with 1 ml of HPLC-grade water. The reconstituted HRP should be stable for at least one week when stored at -20°C. Aliquot the 5 each vial into smaller aliquots before freezing so as to avoid repeated freeze/thaw cycles.

5. **Cholesterol Assay Oxidase - (Item No. 10008056)**

   The vial contains a lyophilized powder of cholesterol oxidase. Reconstitute the 1 each vial with 100 µl and the 5 each vial with 500 µl of HPLC-grade water. The reconstituted reagent should be stable for at least one week when stored at -20°C. Aliquot the 5 each vial into smaller aliquots before freezing so as to avoid repeated freeze/thaw cycles.

6. **Cholesterol Assay Esterase - (Item No. 10008057)**

   The vial contains a lyophilized powder of cholesterol esterase. Reconstitute the 1 each vial with 50 µl and the 5 each vial with 250 µl of HPLC-grade water. The reconstituted reagent should be stable for at least one week when stored at -20°C. Aliquot the 5 each vial into smaller aliquots before freezing so as to avoid repeated freeze/thaw cycles.

7. **DMSO Assay Reagent - (Item No. 700001)**

   The vial contains DMSO. The reagent is ready to use as supplied.

Sample Preparation

Plasma

Typically, cholesterol levels in human plasma are in the range of 2.5-7.5 mM.[5-7]

1. Collect blood using an anticoagulant such as heparin, EDTA, or citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample should be stable for at least one month.
3. Typically, a 1:200-400 dilution of plasma samples should produce results which fall within the standard curve.

Serum

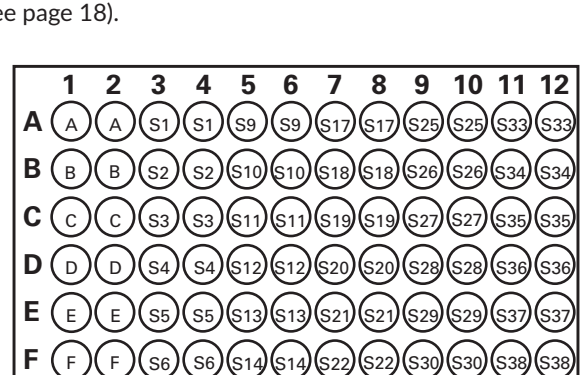
Typically, cholesterol levels in human serum are in the range of 2.5-7.5 mM.[8]

1. Collect blood without using an anticoagulant such as heparin or citrate. Allow blood to clot for 30 minutes at 25°C.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The sample should be stable for at least one month.
3. Typically, a 1:200-400 dilution of serum samples should produce results which fall within the standard curve.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of cholesterol standards and samples to be measured in duplicate is given below in Figure 2. We suggest you record the contents of each well on the template sheet provided (see page 18).



A-H = Standards
S1-S40 = Sample wells

Figure 2. Sample plate format

Pipetting Hints

- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well(s).

General Information

- The final volume of the assay is 100 µl in all of the wells.
- All reagents except samples must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- The cholesterol level in human serum and plasma ranges from 2.5-7.5 mM. Serum and plasma samples need to be diluted 1:200-400 with assay buffer before assaying.
- It is recommended that the samples and cholesterol standards be assayed at least in duplicate.

Standard Preparation

For the determination of cholesterol in plasma or serum, prepare the cholesterol standards according to Table 1. Dilute 20 µl of cholesterol assay standard (Item No. 10008053) with 980 µl of diluted assay buffer. Use this diluted standard (200 µM) to prepare the standard curve.

Take eight clean glass test tubes and mark them A-H. Add the amount of cholesterol standard and assay buffer to each tube as described in Table 1.

Tube	200 µM Cholesterol Standard (µl)	Assay Buffer (µl)	Final Concentration (µM cholesterol)
A	0	1,000	0
B	10	990	2
C	20	980	4
D	30	970	6
E	40	960	8
F	60	940	12
G	80	920	16
H	100	900	20

Table 1. Cholesterol standards to be assayed along with plasma and serum samples

Performing the Assay

1. **Cholesterol Standard Wells** - add 50 µl of cholesterol standard (tubes A-H) per well in the designated wells on the plate (see **Sample Plate Format** on page 10).
2. **Sample Wells** - add 50 µl of sample to two wells. To obtain reproducible results, sample cholesterol levels should fall within the standard curve.
3. Cover the plate with the plate cover provided.
4. Prepare the assay cocktail by mixing the following reagents in a test tube: assay buffer (4.745 ml), cholesterol detector (150 µl), HRP (50 µl), cholesterol oxidase (50 µl), and cholesterol esterase (5 µl).

   NOTE: This volume provides enough cocktail to run the entire 96-well plate. For best results, use the cocktail within 10 minutes of preparation. If only the concentration of free cholesterol is to be determined, do not add the cholesterol esterase to the assay cocktail.
5. Remove the plate cover and initiate the reactions by adding 50 µl of freshly prepared assay cocktail to all the wells being used.
6. Cover the plate with the plate cover and incubate for 30 minutes at 37°C protected from light.
7. Remove the plate cover and read the fluorescence using excitation wavelengths between 530-540 nm and emission wavelengths between 585-595 nm.

ANALYSIS

Calculations

1. Calculate the average fluorescence of each standard and sample.
2. Subtract the average fluorescence of standard A from itself and all other standards and samples. This is the adjusted fluorescence.
3. Plot the adjusted fluorescence of the standards (from step 2 above) as a function of the final concentration of cholesterol from Table 1. See Figure 3 for a typical standard curve.

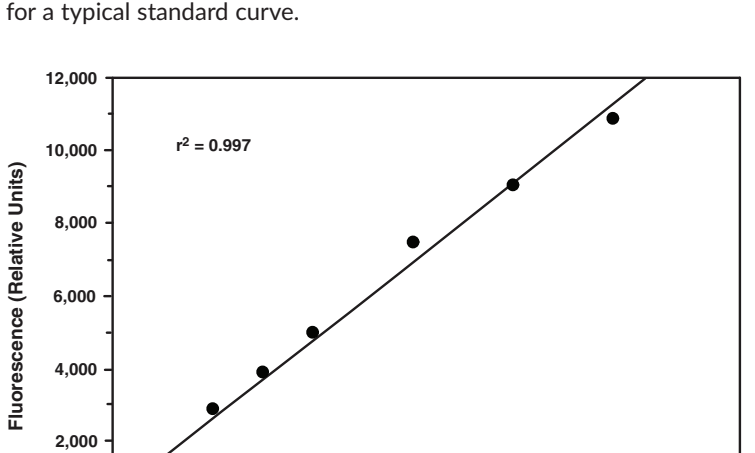


Figure 3. Cholesterol standard curve

4. Calculate the cholesterol concentration of the samples using the equation obtained from the linear regression of the standard curve substituting adjusted fluorescence values for each sample.

$$\text{Cholesterol (mM)} = \left[\frac{\text{Sample adjusted fluorescence - (y-intercept fluorescence)}}{\text{Slope (fluorescence/µM)}}\right] \text{x Sample dilution x 0.001 mM/µM}$$

NOTE: To convert the results from mM to mg/dl, divide the cholesterol concentration (mM) by 0.0259.

Performance Characteristics

Precision:

When a series of 65 plasma measurements at a 1:400 dilution were performed on seven different days under the same experimental conditions, the intra-assay coefficient of variation was 6.4% and the inter-assay coefficient of variation was 3.4%.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 2-20 µM cholesterol.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
Poor fluorescence of both standard and samples	Plate was not incubated at 37°C	Re-assay the sample at 37°C
Cholesterol was not detected in the sample	Sample was too dilute	Re-assay the sample using a lower sample dilution
Fluorescence of sample is higher than most concentrated cholesterol standard	The sample is too concentrated	Dilute your sample with assay buffer and re-assay
The cholesterol standard curve did not work	Either the cholesterol standards were not diluted properly or the cholesterol standard has deteriorated	Set-up the standards according to Table 1 and re-assay

References

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2. Matsuura, E. and Lopez, L.R. Are oxidized LDL/β2-glycoprotein I complexes pathogenic antigens in autoimmune-mediated atherosclerosis? *Clinical & Developmental Immunology* **11(2)**, 103-111 (2004).
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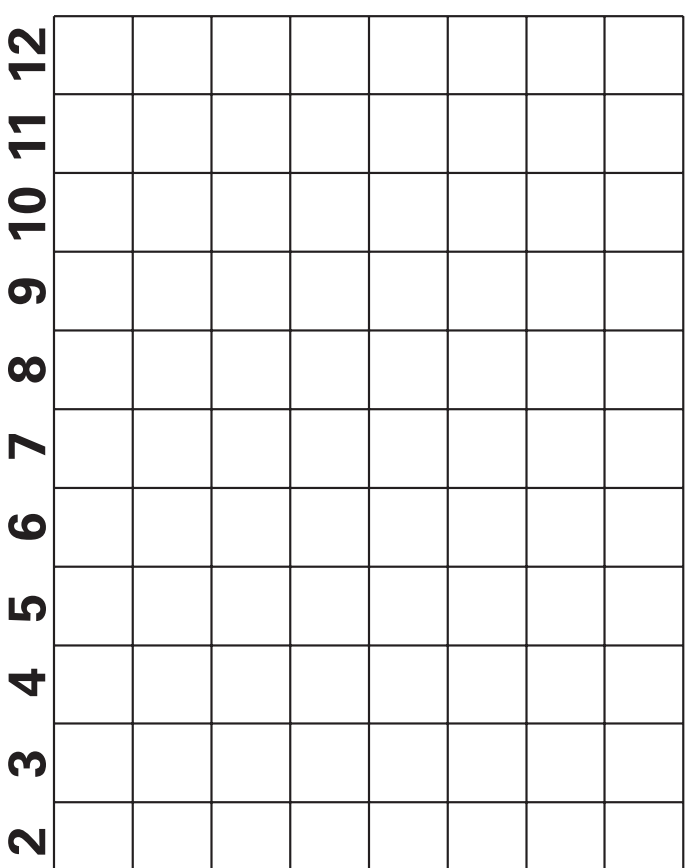

NOTES

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