Cholesterol Assay Kit

Catalog Number KA1303

96 assays

Version: 08

Intended for research use only

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Introduction

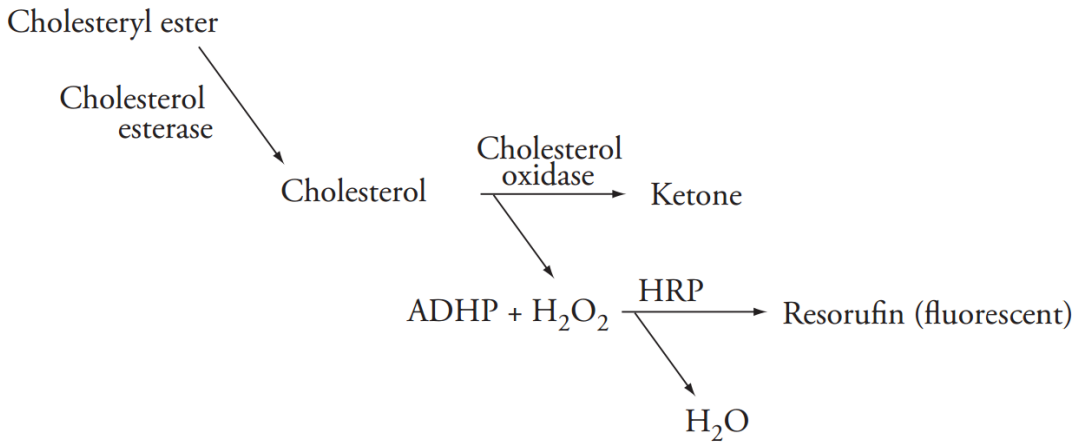
Background

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 (LCAT) is a 67-kDa glycoprotein that is responsible for cholesterol esterification in plasma. The enzyme displays two activities: a phospholipase A2 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.

Principle of the Assay

The Cholesterol 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 Scheme 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 ADHP (10-acetyl-3,7-dihydroxyphenoxazine), 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



General Information

Materials Supplied

List of component

Component	Amount
Cholesterol Assay Buffer (10X)	3 mL
Cholesterol Assay Standard	100 µL
Cholesterol Assay Detector	2 vials
Cholesterol Assay Horseradish Peroxidase	1 vial
Cholesterol Assay Oxidase	1 vial
Cholesterol Assay Esterase	1 vial
DMSO Assay Reagent	1 mL
96-Well Solid Plate (black)	1 plate
96-Well Cover Sheet	1 cover

Storage Instruction

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Required but Not Supplied

- ✓ A fluorometric plate reader capable of measuring fluorescence using an excitation wavelength between 530-580 nm and emission wavelengths between 585-595 nm.
- ✓ Adjustable pipettes and a repeating pipettor.
- ✓ A source of pure water; glass distilled water or HPLC-grade water is acceptable.

Precautions for Use

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

- ✓ 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.

Assay Protocol

Reagent Preparation

- ✓ Cholesterol Assay Buffer (10X)

  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 is stable for at least one week if stored at room temperature or 4°C.

- ✓ Cholesterol Assay Standard

  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.

- ✓ Cholesterol Assay Detector

  The vial contains a lyophilized powder of ADHP (10-acetyl-3,7-dihydroxyphenoxazine). Prior to adding to the Assay Cocktail (See under step 4 of Performing the Assay), reconstitute the Cholesterol Detector with 100 µL of DMSO and 100 µL of HPLC-grade water. The reconstituted Cholesterol Detector is stable for 15 minutes. Each reconstituted vial is enough reagent to perform the entire 96-well plate.

- ✓ Cholesterol Assay Horseradish Peroxidase

  The vial contains a lyophilized powder of horseradish peroxidase (HRP). Reconstitute the 1 each vial with 200 µL and the 5 each vial with 1 mL of HPLC-grade water. The reconstituted HRP is stable for at least a week when stored at -20°C. Aliquot the 5 each vial into smaller aliquots before freezing so as to avoid repeated freeze/thaw cycles.

- ✓ Cholesterol Assay Oxidase

  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 is stable for at least a week when stored at -20°C. Aliquot the 5 each vial into smaller aliquots before freezing so as to avoid repeated freeze/thaw cycles.

- ✓ Cholesterol Assay Esterase

  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 is stable for at least a week when stored at -20°C. Aliquot the 5 each vial into smaller aliquots before freezing so as to avoid repeated freeze/thaw cycles.

✓ DMSO Assay Reagent

The vial contains dimethylsulfoxide (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 will be stable for one month.
3. Typically, a 1:200-400 dilution of plasma samples will 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 will be stable for at least one month.
3. Typically, a 1:200-400 dilution of serum samples will produce results which fall within the standard curve.

Assay Procedure

✓ 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 (below). Dilute 20 µL of Cholesterol Assay Standard 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, below.

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

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

✓ 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 Plate Layout).
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 530-580 nm and emission wavelengths between 585-595 nm.

Data Analysis

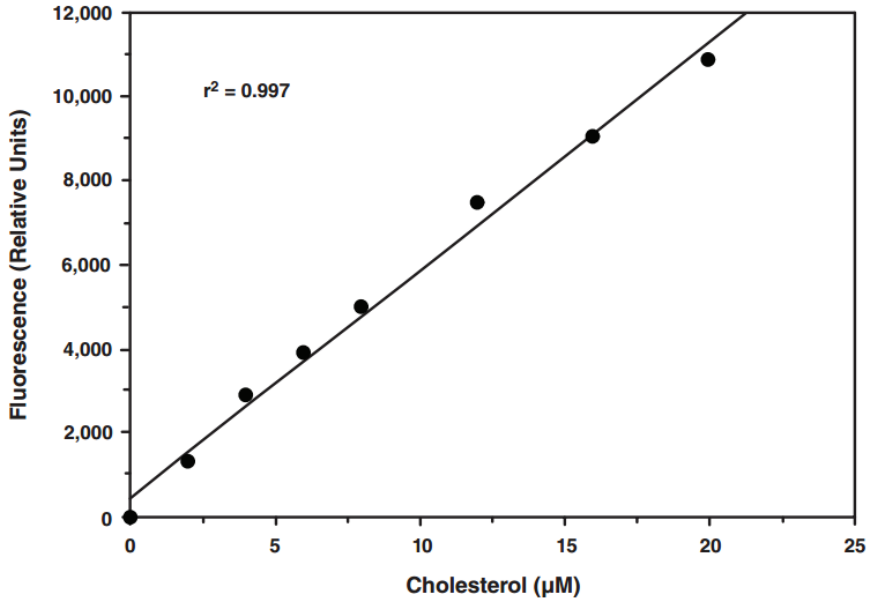
Calculation of Results

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 2 for a typical 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.

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

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

Figure 2. Cholesterol standard curve



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 0-20 µM cholesterol.

Resource

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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Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard A	Standard A	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
B	Standard B	Standard B	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
C	Standard C	Standard C	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
D	Standard D	Standard D	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
E	Standard E	Standard E	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
F	Standard F	Standard F	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
G	Standard G	Standard G	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
H	Standard H	Standard H	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample