Technical Manual

Total Cholesterol (TC) and Cholesterol Esters (CE) Fluorometric Assay Kit

- Catalogue Code: MAES0007
- Size: 96T
- Research Use Only

1. Key features and Sample Types

Detection method:

Fluorimetric method

Specification:

96T

Range:

0.12-30 μmol/L

Sensitivity:

0.12 μmol/L

Storage:

-20°C for 6 months

Expiry:

See Kit Label

Experiment Notes:

This kit is for **research use only.**

Instructions should be strictly followed. Changes of operation may result in unreliable results.

The validity of kit is 6 months.

Do not use components from different batches of kit.

2. Background

Cholesterol is a kind of sterol and lipid in cell membrane. Most of cholesterol in blood exists in the form of cholesterol ester. Lecithin-cholesterol acyltransferase in human plasma is an enzyme that catalyzes the formation of cholesterol ester. Cholesterol synthesized or deposited in peripheral cells returns to the liver through the reverse cholesterol transport system for reusing or regaining bile acids.

3. Intended Use

This kit can be used for determination of total cholesterol (TC) and cholesterol esters (CE) content in serum, plasma, animal tissue and cell samples.

4. Detection Principle

Total cholesterol (TC) includes free cholesterol (FC) and cholesteryl esters (CE). Cholesterol ester can be hydrolyzed by cholesterol esterase to produce cholesterol and free fatty acid. Cholesterol is oxidized by cholesterol oxidase to produce △4-cholestenone and hydrogen peroxide. In the presence of the enzyme and probe, hydrogen peroxide can be catalyzed to produce the fluorescence substrate. The fluorescence intensity at the excitation wavelength of 535 nm and emission wavelength of 590 nm is proportional to the cholesterol concentration.

5. Kit components & storage

Item	Specification	Storage
Buffer Solution	60 mL × 1 vial	-20°C, 6 months
Substrate	0.12 mL × 1 vial	-20°C, 6 months, avoid direct sunlight
Enzyme Reagent 1	0.3 mL ×1 vial	-20°C, 6 months, avoid direct sunlight
Enzyme Reagent 2	0.3 mL ×1 vial	-20°C, 6 months
Cholesterol Standard Solution (5.17 mmol/L)	0.2 mL×1 vial	-20°C, 6 months
Extracting Solution	60 mL × 1 vial	-20°C, 6 months, avoid direct sunlight
Black Microplate	96 wells	No requirement
Plate Sealer	2 pieces	

Materials required but not supplied

- Micropipettor
- Incubator
- Centrifuge
- Fluorescence microplate reader (Ex/Em=535 nm/587 nm)
- Tips (10 µL, 200 µL, 1000 µL)
- EP tubes (1.5 mL, 2 mL)
- Double distilled water

6. Assay Notes:

1. If the sample content is beyond the maximum limit, please dilute the sample with buffer solution before detection, and multiply the result by the dilution ratio.

2. Prevent the formulation of bubbles when the reagents are added into the microplate.

3. Substrate, enzyme reagent 1 and enzyme reagent 2 should avoid repeated freezing and thawing, and it is recommended to aliquot the reagent into smaller quantities for optimal storage.

4. If the sample are tissue or cells, the control well is essential. And it can set one control well if the dilution factor of all tissue or cell samples is the same. Every sample needs a control well if the dilution factor of the tissue or cell samples is different.

7. Reagent Preparation

1. The preparation of **cholesterol standard (50 µmol/L):** Mix the cholesterol standard solution (5.17 mmol/L) and buffer solution at a ratio of 5:512. Prepare the fresh solution before use. Cholesterol standard solution (5.17 mmol/L) can be incubated at 65°C for 30 min if it doesn't dissolve completely.)

2. The preparation of **chromogenic agent 1:** Mix the buffer solution, substrate, enzyme reagent 1 and enzyme reagent 2 at a ratio of 45:1:2:2 and store with avoid direct sunlight. Prepare the fresh solution before use.

3. The preparation **of chromogenic agent 2:** Mix the buffer solution, substrate and enzyme reagent 1 at a ratio of 47:1:2 and store with avoid direct sunlight. Prepare the fresh solution before use.

8. Sample Preparation

1. Serum sample:
Collect fresh blood and stand at 25°C for 30 min to clot the blood. Then centrifuge at 2000 g for 15 min at 4°C. Take the serum (which is the upper light yellow clarified liquid layer) and preserve on ice before detection. If not detected on the same day, the serum can be stored at -80°C for a month.

2. Plasma sample:
Take fresh blood into the tube which has anticoagulant (Heparin is recommended), centrifuge at 700-1000 g for 10 min at 4°C. Take the plasma (which is the upper light yellow clarified liquid layer, don't take white blood cells and platelets in the middle layer) and preserve on ice before detection. If not detected on the same day, the plasma can be stored at -80°C for a month.

3. Cell sample:
Collect the cells and wash the cells with PBS (0.01 M, pH 7.4) for 1~2 times. Centrifuge at 1000 g for 10 min and then discard the supernatant and keep the cell sediment. Add homogenization medium at a ratio of cell number (2×10^6): extracting solution (μL) =1: 300. Sonicate the sample on an ice water bath. Centrifuge at 10000 g for 10 min, then take the supernatant and preserve on ice before detection. If not detected on the same day, the cells sample (without homogenization) can be stored at -80°C for a month.

4. Tissue sample:
Take 0.02-1g fresh tissue to wash with PBS (0.01 M, pH 7.4) at 2-8°C. Use filter paper to absorb excess water and weigh. Homogenize at the ratio of the volume of extracting solution (2-8°C) (mL): the weight of the tissue (g) =9:1, then centrifuge the tissue homogenate for 10 min at 10000 g at 4°C. Take the supernatant and preserve on ice before detection. If not detected on the same day, the tissue sample (without homogenization) can be stored at -80°C for a month.

Sample Notes:

The concentration should be determined before preforming the assay. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity.

Dilution of Samples:

Large variances in results may be seen when performing pre-experiments. Dilute the sample according to the result of the pre-experiment and the detection range (0.12-30 μmol/L).

The recommended dilution factor for different samples is as follows (for reference only).

Sample Type:	Dilution Factor
Human serum	100-300
Rat serum	100-300
Mouse plasma	100-300
Rabbit serum	100-300
10% Rat liver tissue homogenate	50-150
10% Mouse kidney tissue homogenate	50-200
10% Rat brain tissue homogenate	200-400
10% Rat spleen tissue homogenate	50-200
Jukat cells	20-50

Note: The diluent is buffer solution.

9. Assay Protocol

Ambient Temperature: 25-30°C

Optimum detection wavelength: Ex/Em=535 nm/590 nm

Plate Set Up:

Determination of total cholesterol and free cholesterol

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73
B	B	B	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74
C	C	C	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75
D	D	D	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76
E	E	E	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77
F	F	F	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78
G	G	G	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79
H	H	H	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80

Note: A-H, standard wells; S1-S80, sample wells.

Determination of Cholesterol ester

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S9	S17	S25	A'	A'	S1'	S9'	S17'	S25'
B	B	B	S2	S10	S18	S26	B'	B'	S2'	S10'	S18'	S26'
C	C	C	S3	S11	S19	S27	C'	C'	S3'	S11'	S19'	S27'
D	D	D	S4	S12	S20	S28	D'	D'	S4'	S12'	S20'	S28'
E	E	E	S5	S13	S21	S29	E'	E'	S5'	S13'	S21'	S29'
F	F	F	S6	S14	S22	S30	F'	F'	S6'	S14'	S22'	S30'
G	G	G	S7	S15	S23	S31	G'	G'	S7'	S15'	S23'	S31'
H	H	H	S8	S16	S24	S32	H'	H'	S8'	S16'	S24'	S32'

Note: A-H, standard wells of total cholesterol; S1-S32, sample wells of total cholesterol; A'-H', standard wells of free cholesterol; S1'-S32', sample wells of free cholesterol.

10. Operation Steps

The preparation of standard curve

Dilute cholesterol standard with buffer solution (50 μmol/L) to a serial concentration. The recommended dilution gradient is as follows: 0, 2, 5, 10, 15, 20, 25, 30 μmol/L.

Preparation of extracting application solution (for determination of tissue or cell samples in control wells)

Dilute the extracting solution with buffer solution according to the dilution factor of sample. For example, the tissue or cell samples was diluted for 100 times, so dilute the extracting solution with buffer solution at a ratio of 1:99.

Determination of total cholesterol

1. **Standard well:** Add 50 μL of standard with different concentrations into the well.
   Sample well: Add 50 μL of sample into the wells.
   Control well: Add 50 μL of extracting application solution into the well. Add 50 μL of chromogenic agent 1 to each well.

2. Mix fully with microplate reader for 10 s and incubate at 37°C for 10 min with avoid direct sunlight.

3. Measure the fluorescence intensity at the excitation wavelength of 535 nm and the emission wavelength of 590 nm.

Determination of free #cholesterol

1. **Standard well:** Add 50 μL of standard with different concentrations into the well.
   Sample well: Add 50 μL of sample into the wells.
   Control well: Add 50 μL of extracting application solution into the well.

2. Add 50 μL of chromogenic agent 2 to each well.

3. Mix fully with microplate reader for 10 s and incubate at 37°C for 10 min with avoid direct sunlight.

4. Measure the fluorescence intensity at the excitation wavelength of 535 nm and the emission wavelength of 590 nm.

Operation Table

Determination of total cholesterol

	Standard well	Sample well	Sample control well
Standard with different concentrations (μL)	50		
Sample (μL)		50	
Extracting application solution (μL)			50
Chromogenic agent 1 (μL)	50	50	50
Mix fully with microplate reader for 10 s and incubate at 37℃ for 10 min. Measure the fluorescence intensity at the excitation wavelength of 535 nm and the emission wavelength of 590 nm.			

Determination of free cholesterol

	Standard well	Sample well	Sample control well
Standard with different concentrations (μL)	50		
Sample (μL)		50	
Extracting application solution (μL)			50
Chromogenic agent 2 (μL)	50	50	50
Mix fully with microplate reader for 10 s and incubate at 37℃ for 10 min. Measure the fluorescence intensity at the excitation wavelength of 535 nm and the emission wavelength of 590 nm.			

11. Calculations

Calculation of total cholesterol:

1. Serum (plasma) sample:

$$\text{TC content (μmol/L)} = (\Delta F - b) \div a \times f$$

2. Tissue sample:

$$\text{TC content (μmol/g fresh weight)} = (\Delta F_1 - b) \div a \times f \div \frac{m}{V}$$

3. Cell sample:

$$\text{TC content (μmol/}10^6\text{ cells)} = (\Delta F_1 - b) \div a \times f \div \frac{N}{V}$$

Calculation of free cholesterol:

1. Serum (plasma) sample:

$$\text{FC content (μmol/L)} = (\Delta F_2 - b_1) \div a_1 \times f$$

2. Tissue sample:

$$\text{FC content (μmol/g fresh weight)} = (\Delta F_3 - b_1) \div a_1 \times f \div \frac{m}{V}$$

3. Cell sample:

$$\text{FC content (μmol/10^6 cells)} = (\Delta F_3 - b_1) \div a_1 \times f \div \frac{N}{V}$$

Calculation of cholesteryl esters:

$$\text{CE content} = \text{TC content} - \text{FC content}$$

y: The absolute fluorescence value of standard, $F_{Standard} - F_{Blank}$ (F_{Blank} is the F value when the standard concentration is 0)
x: The concentration of standard
a: The slope of standard curve
b: The intercept of standard curve
ΔF: Absolute fluorescence intensity of serum (plasma) sample ($F_{Sample} - F_{Blank}$)
ΔF_1: Absolute fluorescence intensity of tissue or cell sample ($F_{Sample} - F_{Control}$)
f: Dilution factor of sample before tested
m: The weight of tissue sample, g.
V: The volume of extracting solution added during the preparation of tissue or cell samples, L.
N: The number of cells. For example, the number of cells is $5*10^6$, N is 5.

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12. Performance Characteristics

Detection Range	0.12-30 μmol/L
Sensitivity	0.12 μmol/L
Average recovery rate (%)	96
Average inter-assay CV (%)	7.3
Average intra-assay CV (%)	1.7

Analysis

Determination of total cholesterol

Dilute human serum with buffer solution for 300 times, take 50 μL of diluted human serum, carry the assay according to the operation table.

The results are as follows:

Standard curve: y = 284.28 x + 139.79, the average fluorescence value of the sample is 4522, the average fluorescence value of the blank is 148, and the calculation result is:

TC content (μmol/L) = （4522 - 148 - 139.79）÷ 284.28 × 300
= 4468 μmol/L

Determination of free cholesterol

Dilute human serum with buffer solution for 300 times, take 50 μL of diluted human serum, carry the assay according to the operation table.

The results are as follows:

standard curve of free cholesterol: y = 274.85 x + 280.99, the average fluorescence value of the sample is 830, the average fluorescence value of the blank is 125, and the calculation result is:

FC content (μmol/L) = (830 - 125 - 280.99) ÷ 274.85 × 300
= 463 μmol/L

Standard curve of total cholesterol: y = 273.49 x + 221.46, the average fluorescence value of the sample is 3239, the average fluorescence value of the blank is 149, and the calculation result is:

TC content (μmol/L) = (3239 - 149 – 221.46) ÷ 273.49 × 300
= 3147 μmol/L

CE content = TC content - FC content = 3147- 463
= 2684 μmol/L

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Safety Notes

Some of the reagents in the kit contain dangerous substances. Avoid touching skin and clothing.

Wash immediately with plenty of water if touching it carelessly.

All the samples and waste material should be treated according to the relevant rules of laboratory's biosafety.

Before the experiment, read the instructions carefully, and wear gloves and work clothes.

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