Total Cholesterol and Cholesteryl Ester Fluorometric Assay Kit

Catalog No: E-BC-F032

Method: Fluorimetric method

Specification: 96T (Can detect 80 samples for total cholesterol without duplication, or detect 32 samples for cholesteryl ester without duplication)

Measuring instrument: Fluorescence Microplate Reader

Sensitivity: 0.12 μmol/L

Detection range: 0.12-30 μmol/L

- Please kindly provide us the lot number (on the outside of the box) of the kit for more efficient service.

General information

▲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.

▲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.

▲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 Δ4-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.

▲Kit components & storage

Item	Component	Specification	Storage
Reagent 1	Buffer Solution	60 mL × 1 vial	-20℃ , 6 months
Reagent 2	Substrate	0.12 mL × 1 vial	-20℃ , 6 months, shading light
Reagent 3	Enzyme Reagent 1	0.3 mL ×1 vial	-20℃ , 6 months, shading light
Reagent 4	Enzyme Reagent 2	0.3 mL ×1 vial	-20℃ , 6 months
Reagent 5	5.17 mmol/L Cholesterol Standard Solution	0.2 mL × 1 vial	-20°C , 6 months
Reagent 6	Extracting Solution	60 mL × 1 vial	-20℃ , 6 months, shading light
	Black Microplate	96 wells	
	Plate Sealer	2 pieces	

▲Materials prepared by users

Instruments

Fluorescence microplate reader (Ex/Em=535 nm/590 nm), Micropipettor, Vortex mixer, Centrifuge

Consumptive material

Tips (10 μL, 200 μL, 1000 μL), EP tubes (1.5 mL, 2 mL)

Reagents

Double distilled water

▲Safety data

Some of the reagents in the kit contain dangerous substances. It should be avoided to touch the 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.

▲Precautions

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

▲The key points of the assay

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

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

3. Reagent 2, reagent 3 and reagent 4 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.

Pre-assay preparation

▲Reagent preparation

1. The preparation of 50 μmol/L cholesterol standard:
   Mix the reagent 5 and reagent 1 at a ratio of 5:512. Prepare the fresh solution before use. (Reagent 5 can be incubated at 65℃ for 30 min if it doesn't dissolved completely.)
2. The preparation of chromogenic agent 1:
   Mix the reagent 1, reagent 2, reagent 3 and reagent 4 at a ratio of 45:1:2:2 and store with shading light. Prepare the fresh solution before use.
3. The preparation of chromogenic agent 2:
   Mix the reagent 1, reagent 2 and reagent 3 at a ratio of 47:1:2 and store with shading light. Prepare the fresh solution before use.

▲Sample preparation

The samples should be prepared as conventional methods. Also please refer to appendix II.

▲Dilution of sample

It is recommended to take 2~3 samples with expected large difference to do pre-experiment before formal experiment and 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 reagent 1.

Assay protocol	
Ambient temperature	25-30℃
Optimum detection wavelength	Ex/Em=535 nm/590 nm

Instructions for the use of transferpettor:

(1) Equilibrate the pipette tip in that reagent before pipetting each reagent.

(2) Don't add the liquid outside the tips into the reaction system when pipetting each reagent.

Assay protocol

▲Plate set up

1.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.
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.

2.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 .
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.

▲Operating steps

1. The preparation of standard curve
   Dilute 50 μmol/L cholesterol standard with reagent 1 to a serial concentration. The recommended dilution gradient is as follows: 0, 2, 5, 10, 15, 20, 25, 30 μmol/L.
2. Preparation of reagent 6 application solution (for determination of tissue or cell samples in control wells): dilute the reagent 6 with reagent 1 according to the dilution factor of sample. For example, the tissue or cell samples was diluted for 100 times, so dilute the reagent 6 with reagent 1 at a ratio of 1:99.
3. 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 well.
   Control well: add 50 μL of reagent 6 application solution into the well.
2) Add 50 μL of chromogenic agent 1 to each well.
3) Mix fully with microplate reader for 10 s and incubate at 37℃ for 10 min with shading light.
4) Measure the fluorescence intensity at the excitation wavelength of 535 nm and the emission wavelength of 590 nm.

4. 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 well.
   Control well: add 50 μL of reagent 6 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℃ for 10 min with shading light.
4) Measure the fluorescence intensity at the excitation wavelength of 535 nm and the emission wavelength of 590 nm.

▲Operation table

1. Determination of total cholesterol

	Sample well	Sample well	Sample control well
Standard with different concentrations (μL)	50		
Sample (μL)		50	
Reagent 6 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 with shading light. Measure the fluorescence intensity at the excitation wavelength of 535 nm and the emission wavelength of 590 nm.			

2. Determination of free cholesterol

	Standard well	Sample well	Sample control well
Standard with different concentrations (μL)	50		
Sample (μL)		50	
Reagent 6 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 with shading light. Measure the fluorescence intensity at the excitation wavelength of 535 nm and the emission wavelength of 590 nm.			

▲Calculation

Plot the standard curve by using fluorescence value (F) of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve with graph software (or EXCEL). The concentration of the sample can be calculated according to the formula based on the F value of sample.

1. Calculation of total cholesterol

The standard curve is: y= ax + b.

1) Serum (plasma) sample:

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

2) Tissue sample:

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

3) cell sample:

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

Note:

y: $F_{Standard} - F_{Blank}$. (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 samples ($F_{Sample} - F_{Control}$)

f: Dilution factor of sample before tested.

m: the weight of tissue sample, g.

V: the volume of reagent 6 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.

2. Calculation of free cholesterol

The standard curve is: $y= a_1x + b_1$.

1) For serum (plasma):

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

2) For tissue:

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

3) For cell sample:

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

Note:

y: $F_{Standard} - F_{Blank}$. (F_{Blank} is the F value when the standard concentration is 0)

x: The concentration of standard.

a_1: The slope of standard curve.

b_1: The intercept of standard curve.

ΔF_2: Absolute fluorescence intensity of serum (plasma) ($F_{Sample} - F_{Blank}$)

ΔF_3: Absolute fluorescence intensity of tissue or cells ($F_{Sample} - F_{Control}$)

f: Dilution factor of sample before tested.

m: the weight of tissue sample, g.

V: the volume of reagent 6 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.

3. Calculation of free cholesterol

CE content = TC content-FC content

▲Notes

1. This kit is for research use only.
2. Instructions should be followed strictly, changes of operation may result in unreliable results.
3. The validity of kit is 6 months.
4. Do not use components from different batches of kit.

Appendix I Performance characteristics

Appendix I Performance characteristics			
Detection range	0.12-30 μmol/L	Average intra-assay CV (%)	1.7
Sensitivity	0.12 μmol/L	Average inter-assay CV (%)	7.3
Average recovery rate (%)	96		

▲Example analysis

1. Determination of total cholesterol

Dilute human serum with reagent 1 for 300 times, take 50 μL of diluted human serum and 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

2. Determination of cholesterol ester

Dilute human serum with reagent 1 for 300 times, take 50 μL of diluted human serum and 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

Appendix II Sample preparation

The following sample pretreatment methods are for reference only.

▲Serum:

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

▲Plasma:

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

▲Tissue sample:

Take 0.02-1g fresh tissue to wash with PBS (0.01 M, pH 7.4) at 2-8℃ . Absorb the water with filter paper and weigh. Homogenize at the ratio of the volume of homogenized medium (2-8℃) (mL): the weight of the tissue (g) =9:1, then centrifuge the tissue homogenate for 10 min at 1500 g at 4℃ . Take the supernatant to preserve it on ice for detection. If not detected on the same day, the tissue sample (without homogenization) can be stored at -80℃ for a month.

▲Cells:

Suspension cells:

Centrifuge at 1000 g for 10 min to collect the cells. Add homogenization medium (reagent 6) at a ratio of cell number (10^6): homogenization medium (μL) =1: 200-400. Sonicate or mechanical homogenate. Centrifuge at 10000 g at 4 ℃ for 10 min, then take the supernatant and preserve it on ice for detection. If not detected on the same day, the cells sample (without homogenization) can be stored at -80℃ for a month.

Adherent cells:

Discard the medium, wash the cells with PBS (0.01 M, pH 7.4) for 1~2 times. Scratch the cells with cell scraper, add 2-5 mL of PBS(0.01 M, pH 7.4) to collect the cell suspension. The post-treatment method refers to the suspension cell treatment method.

Note:

1. Homogenized medium: Reagent 6.

2. Homogenized method:

(1) Hand-operated: Weigh the tissue and mince to small pieces (1 mm^3), then put the tissues pieces to glass homogenized tube. Add homogenized medium into homogenized tube, place the tube into the ice bath with left hand, and insert the glass tamping rod vertically into the homogenized tube with the right hand to grind up and down for 6-8 min.

Or put the tissue into the mortar, and add liquid nitrogen to grind fully. Then add the homogenized medium to homogenize.

(2) Mechanical homogenate: Weigh the tissue to EP tube, add the homogenized medium to homogenize the tissue with homogenizer instrument (60 Hz, 90s) in the ice bath. (For samples of skin, muscle and plant tissue, the time of homogenization can be properly prolonged.)

▲ Notes for sample

1.Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.

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

3.If a lysis buffer is used to prepare tissue homogenates, there is a possibility of causing a deviation due to the introduced chemical substance.