（FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSIS !）

Catalog No：E-BC-F032

Specification：48T(32 samples)/96T(80 samples)

Measuring instrument：Fluorescence Microplate Reader

(Ex/Em=535 nm/590 nm)

Detection range：0.12-30 μmol/L

Elabscience®Total Cholesterol And Cholesterol Esters Fluorometric Assay Kit

This manual must be read attentively and completely before using this product.
If you have any problem, please contact our Technical Service Center for help：

Toll-free: 1-888-852-8623
Tell: 1-832-243-6086
Fax: 1-832-243-6017
Email: techsupport@elabscience.com
Website: www.elabscience.com

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

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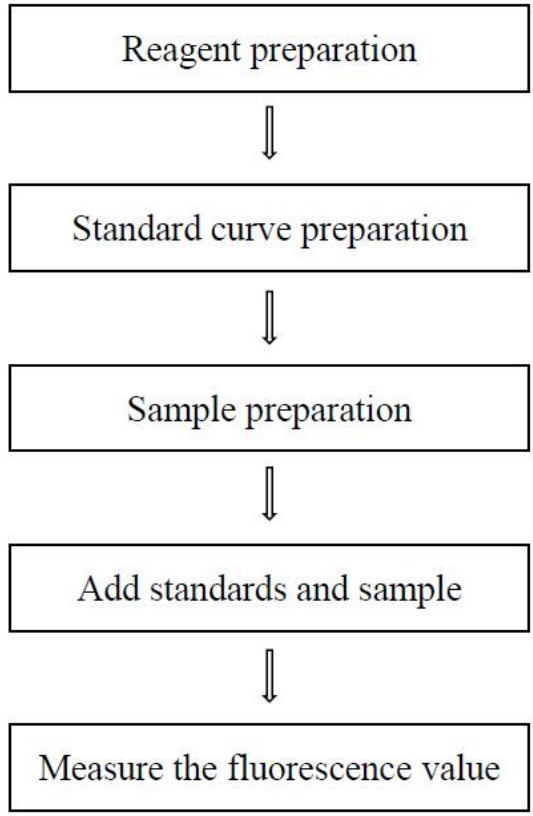
Assay summary



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.

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.

Kit components & storage

Item	Component	Size 1(48 T)	Size 2(96 T)	Storage
Reagent 1	Buffer Solution	30 mL × 1 vial	60 mL × 1 vial	-20℃, 12 months
Reagent 2	Substrate	0.06 mL × 1 vial	0.12 mL × 1 vial	-20℃, 12 months, shading light
Reagent 3	Enzyme Reagent 1	0.3 mL × 1 vial	0.3 mL × 1 vial	-20℃, 12 months, shading light
Reagent 4	Enzyme Reagent 2	0.3 mL × 1 vial	0.3 mL × 1 vial	-20℃, 12 months
Reagent 5	5.17 mmol/L Cholesterol Standard Solution	0.2 mL × 1 vial	0.2 mL × 1 vial	-20℃, 12 months
Reagent 6	Extracting Solution	30 mL × 1 vial	60 mL × 1 vial	-20℃, 12 months, shading light
	Black Microplate	96 wells		No requirement
	Plate Sealer	2 pieces		

Note: The reagents must be stored strictly according to the preservation conditions in the above table. The reagents in different kits cannot be mixed with each other. For a small volume of reagents, please centrifuge before use, so as not to obtain sufficient amount of reagents.

Materials prepared by users

Instruments:

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

Reagent preparation

① Equilibrate all the reagents to room temperature before use.

② The preparation of 50 μmol/L cholesterol standard:
Dilute 12.5 μL of 5.17 mmol/L cholesterol standard solution with 1280 μL of buffer solution, mix well. Prepare the fresh solution before use. (5.17 mmol/L cholesterol standard solution can be incubated at 65℃ for 30 min if it doesn't dissolved completely.)

③ The preparation of chromogenic agent 1:
For each well, prepare 50 μL of chromogenic agent 1 (mix well 45 μL of buffer solution, 1 μL of substrate, 2 μL of enzyme reagent 1 and 2 μL of enzyme reagent 2). The chromogenic agent 1 should be prepared on spot.

④ The preparation of chromogenic agent 2:
For each well, prepare 50 μL of chromogenic agent 2 (mix well 47 μL of buffer solution, 1 μL of substrate and 2 μL of enzyme reagent 1). The chromogenic agent 2 should be prepared on spot.

⑤ The preparation of standard curve：
Always prepare a fresh set of standards. Discard working standard dilutions after use.

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

Item	①	②	③	④	⑤	⑥	⑦	⑧
Concentration (μmol/L)	**0**	**2**	**5**	**10**	**15**	**20**	**25**	**30**
50 μmol/L standard (μL)	0	20	50	100	150	200	250	300
Buffer solution (μL)	500	480	450	400	350	300	250	200

Sample preparation

① Sample preparation

Serum and plasma: detect directly. If not detected on the same day, the serum or plasma can be stored at -80℃ for a month.

Tissue sample:

① Harvest the amount of tissue needed for each assay (initial recommendation 20 mg).

② Wash tissue in cold PBS (0.01 M, pH 7.4).

③ Homogenize 20 mg tissue in 180 μL extracting solution with a dounce homogenizer at 4℃.

④ Centrifuge at 10000×g for 10 min to remove insoluble material. Collect supernatant and keep it on ice for detection.

⑤ Meanwhile, determine the protein concentration of supernatant (E-BC-K318-M).

Cell (adherent or suspension) samples:

① Harvest the number of cells needed for each assay (initial recommendation 1×10^6 cells).

② Wash cells with PBS (0.01 M, pH 7.4).

③ Lyse 1× 10^6 cells with 200-400 μL extracting solution. Mix well for 5 s and place on the ice box, lyse for 10 min.

④ Centrifuge at 10000×g for 10 min to remove insoluble material at 4℃. Collect supernatant and keep it on ice for detection.

⑤ Meanwhile, determine the protein concentration of supernatant (E-BC-K318-M).

② Dilution of sample

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	1

Note: The diluent is buffer solution. For the dilution of other sample types, please do pretest to confirm the dilution factor.

The key points of the assay

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 is 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.

Operating steps

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.

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.

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.

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.

Calculation

1. Calculation of total cholesterol

The standard curve:

1. Average the duplicate reading for each standard.

2. Subtract the mean fluorescence value of the blank (Standard #①) from all standard readings. This is the absoluted fluorescence value.

3. Plot the standard curve by using absoluted fluorescence value of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve ($\mathbf{y = a_1x + b_1}$) with graph software (or EXCEL).

1) Serum (plasma) sample:

$$\frac{\text{TC content}}{(\mu\text{mol/L})} = (\Delta F - b_1) \div a_1 \times f$$

2) Tissue and Cell sample:

$$\frac{\text{TC content}}{(\mu\text{mol/g gprot})} = (\Delta F_1 - b_1) \div a_1 \times f \div C_{pr}$$

[Note]

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

ΔF_1: Absolute fluorescence intensity of tissue or cell samples ($F_{Sample} - F_{Blank}$)

f: Dilution factor of sample before tested.

C_{pr}: Concentration of protein in sample, mgprot/mL

2. Calculation of free cholesterol:

The standard curve:

1. Average the duplicate reading for each standard.

2. Subtract the mean fluorescence value of the blank (Standard #①) from all standard readings. This is the absoluted fluorescence value.

3. Plot the standard curve by using absoluted fluorescence value of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve ($\mathbf{y = a_2x + b_2}$) with graph software (or EXCEL).

1) Serum (plasma) sample:

$$\frac{\text{FC content}}{(\mu\text{mol/L})} = (\Delta F_2 - b_2) \div a_2 \times f$$

2) Tissue and Cell sample:

$$\frac{\text{FC content}}{(\mu\text{mol/gprot})} = (\Delta F_3 - b_2) \div a_2 \times f \div C_{pr}$$

[Note]:

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

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

f: Dilution factor of sample before tested.

C_{pr}: Concentration of protein in sample, mgprot/mL

3. Calculation of cholesterol esters:

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

Appendix I Performance Characteristics

1. Parameter:

Intra-assay Precision

Three human serum samples were assayed in replicates of 20 to determine precision within an assay. (CV = Coefficient of Variation)

Parameters	Sample 1	Sample 2	Sample 3
Mean (μmol/L)	1.50	14.00	25.00
%CV	1.8	1.6	1.7

Inter-assay Precision

Three human serum samples were assayed 20 times in duplicate by three operators to determine precision between assays.

Parameters	Sample 1	Sample 2	Sample 3
Mean (μmol/L)	1.50	14.00	25.00
%CV	7.9	6.8	7.2

Recovery

Take three samples of high concentration, middle concentration and low concentration to test the samples of each concentration for 6 times parallelly to get the average recovery rate of 96%.

	Standard 1	Standard 2	Standard 3
Expected Conc.(μmol/L)	2.5	12	23
Observed Conc. (μmol/L)	2.5	11.4	21.9
Recovery rate (%)	98	95	95

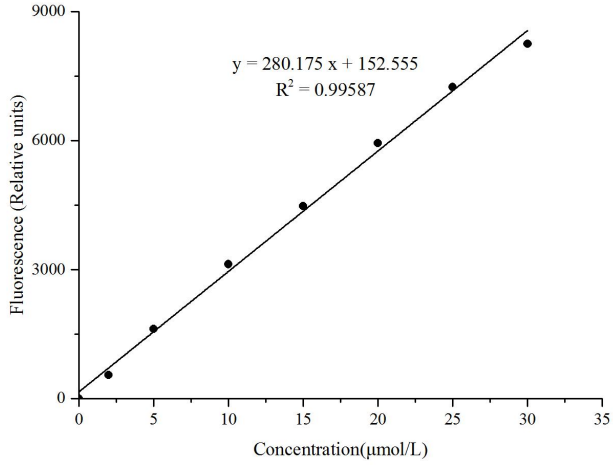
Sensitivity

The analytical sensitivity of the assay is 0.12 μmol/L. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 20 times, and calculating the corresponding concentration.

2. Standard curve:

As the fluorescence value of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique or temperature effects), so the standard curve and data are provided as below for reference only:

Concentration (μmol/L)	**0**	**2**	**5**	**10**	**15**	**20**	**25**	**30**
Fluorescence value	210	741	1834	3334	4630	6141	7432	8394
	216	786	1828	3346	4745	6165	7482	8524
Average fluorescence value	213	764	1831	3340	4688	6153	7457	8459
Absoluted fluorescence value	0	550	1618	3127	4475	5940	7244	8246



Appendix II Example Analysis

Example analysis:

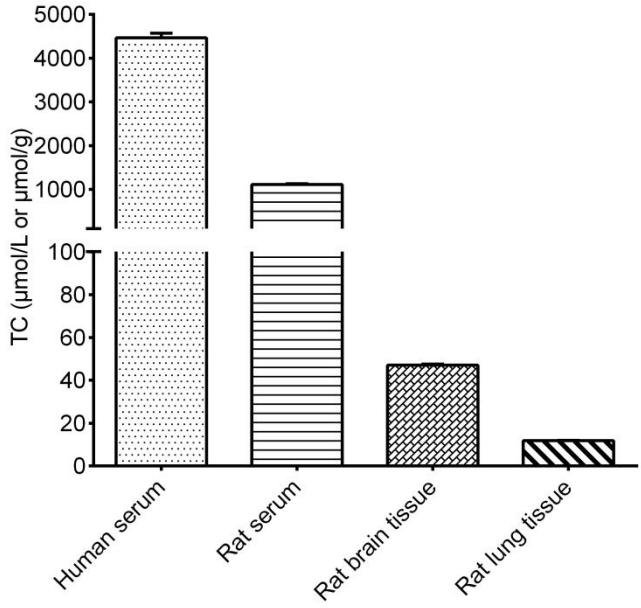
1. Determination of total cholesterol

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

$$\text{TC content } (\mu mol/L) = (4522 - 148 - 139.79) \div 284.28 \times 300 = 4468\ \mu mol/L$$

Detect human serum (dilute for 300 times), rat serum (dilute for 300 times), 10% rat brain tissue homogenate (dilute for 200 times) and 10% rat lung tissue homogenate (dilute for 50 times) according to the protocol, the result is as follows:



2. Determination of cholesterol ester

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

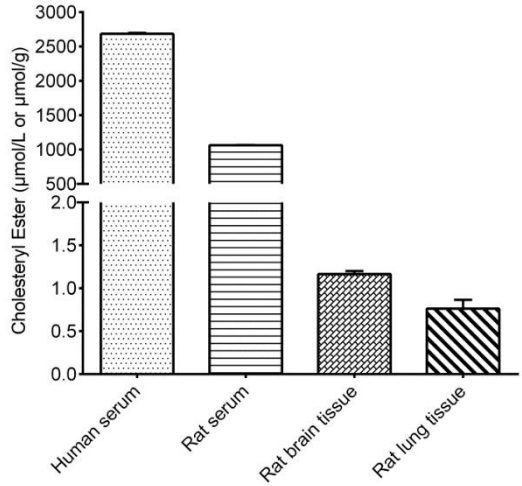
$$\text{FC content } (\mu\text{mol/L}) = (830 - 125 - 280.99) \div 274.85 \times 300 = 463\ \mu\text{mol/L}$$

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

$$\text{TC content } (\mu\text{mol/L}) = (3239 - 149 - 221.46) \div 273.49 \times 300 = 3147\ \mu\text{mol/L}$$

$$\text{CE content} = \text{TC content} - \text{FC content} = 3147 - 463 = 2684\ \mu\text{mol/L}$$

Detect human serum (dilute for 300 times), rat serum (dilute for 300 times), 10% rat brain tissue homogenate (dilute for 200 times) and 10% rat lung tissue homogenate (dilute for 50 times) according to the protocol, the result is as follows:



Statement

1. This assay kit is for Research Use Only. We will not response for any arising problems or legal responsibilities causing by using the kit for clinical diagnosis or other purpose.
2. Please read the instructions carefully and adjust the instruments before the experiments. Please follow the instructions strictly during the experiments.
3. Protection methods must be taken by wearing lab coat and latex gloves.
4. If the concentration of substance is not within the detection range exactly, an extra dilution or concentration should be taken for the sample.
5. It is recommended to take a pre-test if your sample is not listed in the instruction book.
6. The experimental results are closely related to the situation of reagents, operations, environment and so on. Elabscience will guarantee the quality of the kits only, and NOT be responsible for the sample consumption caused by using the assay kits. It is better to calculate the possible usage of sample and reserve sufficient samples before use.