TECHNICAL MANUAL

Total Cholesterol and Cholesteryl Ester Fluorometric Assay Kit

- **SKU CODE:** MAES0007
- **SIZE:** 48 Tests
- **DETECTION PRINCIPLE:** Assay Kit
- **RUO:** Research-Use-Only

Revised: 01/2026

1. Assay summary

- Reagent preparation
- Standard curve preparation
- Sample preparation
- Add standards and samples
- Measure fluorescence intensity
- ![img-0.jpeg](img-0.jpeg)

2. 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.

3. 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.

4. 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°C, 12 months
Reagent 2	Substrate	0.06 mL × 1 vial	0.12 mL × 1 vial	-20°C, 12 months, shading light
Reagent 3	Enzyme Reagent 1	0.3 mL × 1 vial	0.3 mL × 1 vial	-20°C, 12 months, shading light
Reagent 4	Enzyme Reagent 2	0.3 mL × 1 vial	0.3 mL × 1 vial	-20°C, 12 months

Item	Component	Size 1(48 T)	Size 2(96 T)	Storage
Reagent 5	5.17 mmol/L Cholesterol Standard Solution	0.2 mL × 1 vial	0.2 mL × 1 vial	-20°C, 12 months
Reagent 6	Extracting Solution	30 mL × 1 vial	60 mL × 1 vial	-20°C, 12 months, shading light
	Black Microplate	96 wells	96 wells	No requirement
	Plate Sealer	2 pieces	2 pieces	

5. Materials prepared by users

Instruments:

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

6. Reagent preparation

1. Equilibrate all the reagents to room temperature before use.
2. **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°C for 30 min if it doesn't dissolve completely.)
3. **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 fresh.
4. **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 fresh.
5. **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.

7. Sample preparation

Sample preparation

Serum and plasma: detect directly. If not detected on the same day, the serum or plasma can be stored at -80°C 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°C.

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 (MAES0177).

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°C. Collect supernatant and keep it on ice for detection.

⑤ Meanwhile, determine the protein concentration of supernatant (MAES0177).

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

Jurkat cells - 1

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

8. 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 formation 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.

9. 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°C 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°C 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.

10. Calculation

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 absolute fluorescence value.

3. Plot the standard curve by using absolute fluorescence value of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve (y = a1x + b1) with graph software (or EXCEL).

1) Serum (plasma) sample:

TC content (μmol/L) = (ΔF − b1) ÷ a1 × f

2) Tissue and Cell sample:

TC content (μmol/g_{prot}) = (ΔF1 − b1) ÷ a1 × f ÷ C_{pr}

[Note]

ΔF: Absolute fluorescence intensity of serum (plasma) sample (F_{sample} – F_{blank})

ΔF1: 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, mg_{prot}/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 absolute fluorescence value.

3. Plot the standard curve by using absolute fluorescence value of standard and correspondent concentration as y-axis and x-axis respectively. Create the standard curve (y = a2x + b2) with graph software (or EXCEL).

1) Serum (plasma) sample:

FC content (μmol/L) = (ΔF2 − b2) ÷ a2 × f

2) Tissue and Cell sample:

FC content (μmol/g_{prot}) = (ΔF3 − b2) ÷ a2 × f ÷ C_{pr}

[Note]:

ΔF2: Absolute fluorescence intensity of serum (plasma) (F_{sample} – F_{blank})

ΔF3: 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, mg_{prot}/mL

3. Calculation of cholesterol esters:

CE content = TC content − FC content

11. Appendix I Performance Characteristics

Parameter:

Intra-assay Precision

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

Intra-assay Precision

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.

Inter-assay Precision

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

Recovery

Three samples of high concentration, middle concentration and low concentration were tested with each concentration measured 6 times in parallel to get an average recovery rate of 96%.

Recovery

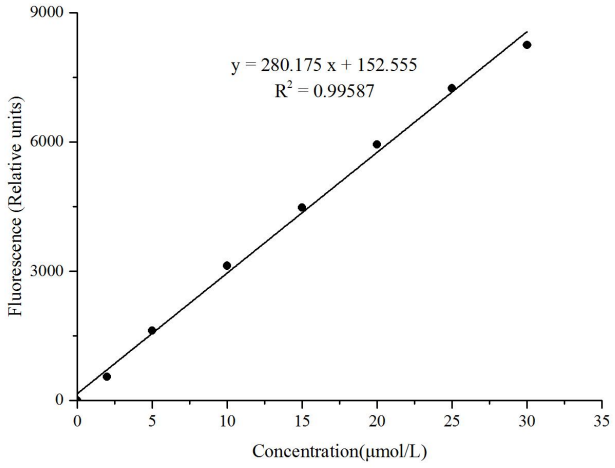
Standard	Expected Conc.(µmol/L)	Observed Conc. (µmol/L)	Recovery rate (%)
Standard 1	2.5	2.5	98
Standard 2	12	11.4	95
Standard 3	23	21.9	95

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.

Standard curve data

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), the standard curve and data are provided below for reference only:



Standard curve data

Concentration (µmol/L)	Fluorescence value		Average fluorescence value	Absolute fluorescence value
0	210	216	213	0

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

12. Appendix II Example Analysis

Example analysis:

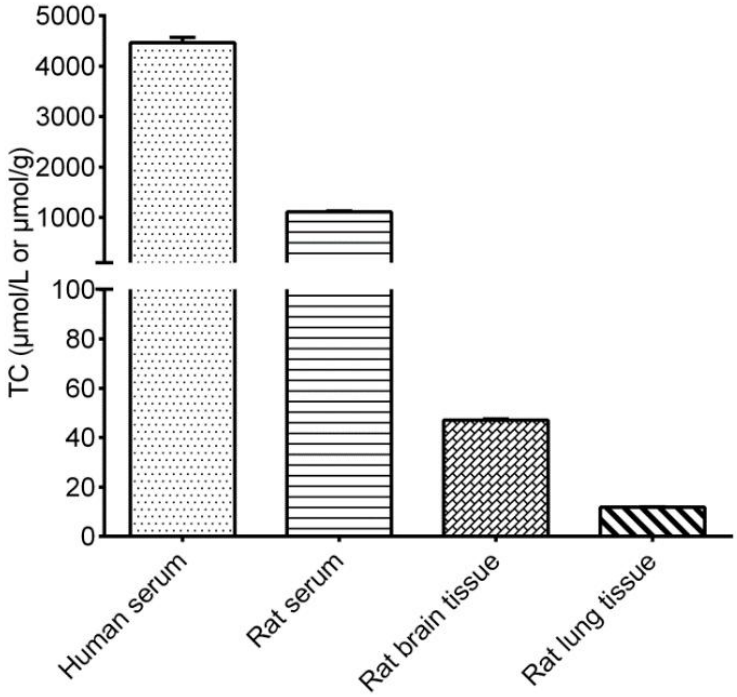
1. Determination of total cholesterol

Dilute human serum with buffer solution 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 = 284.28x + 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

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



2. Determination of cholesterol ester

Dilute human serum with buffer solution 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 = 274.85x + 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:

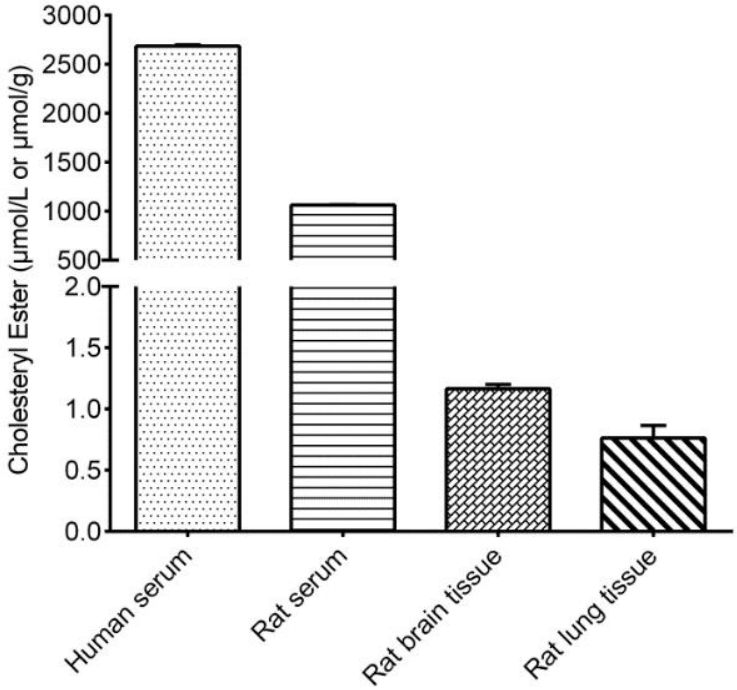
FC content (μmol/L) = (830 - 125 - 280.99) ÷ 274.85 × 300 = 463 μmol/L

Standard curve of total cholesterol: y = 273.49x + 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

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



13. Statement

1. This assay kit is for Research Use Only. Assay Genie assumes no responsibility for any problems or legal liabilities arising from the use of this kit for clinical diagnosis or any other purpose.

2. Please read the instructions carefully and calibrate the instruments before performing the experiments. Follow the instructions strictly throughout the procedure.

3. Appropriate protective measures must be taken, including wearing a lab coat and latex gloves.

4. If the concentration of the substance falls outside the detection range, perform an additional dilution or concentration step on the sample.

5. It is recommended to perform a pre-test if your sample type is not listed in the instruction manual.

6. Experimental results are closely related to reagent quality, operator technique, environmental conditions, and other factors. Assay Genie guarantees the quality of the kits only and is NOT responsible for sample consumption resulting from use of the assay kits. It is advisable to estimate the expected sample usage and reserve sufficient samples before starting the experiment.

Assay Genie 100% money-back guarantee!

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.

Manufacturers Statement: This final kit system is assembled and quality-released by Assay Genie Limited.