MANUAL

RayBio® Total Cholesterol Colorimetric Assay Kit

Catalog #: MA-TC

ISO 13485:2016

Introduction

Cholesterol plays a crucial role in various physiological processes, including the composition of cell membranes, hormone production, and cell signaling. As a vital component of animal cell structure, cholesterol is essential for maintaining membrane permeability and fluidity, as well as contributing significantly to cell signaling pathways. Monitoring cholesterol levels is critical for diagnosing and classifying hyperlipoproteinemias, as it can provide valuable insights into liver and biliary function, intestinal absorption, susceptibility to coronary artery disease, thyroid and adrenal function.

The RayBio® Total Cholesterol Colorimetric Assay Kit offers a straightforward, reliable, and highly sensitive method for measuring the concentration of total cholesterol, which encompasses both cholesteryl esters and free cholesterol, in plasma, serum, cell lysates and other biological liquid samples. This assay relies on an enzymatic reaction system that couples several essential steps. First, cholesterol esters are enzymatically hydrolyzed by cholesterol esterase, resulting in the conversion of cholesteryl esters into cholesterol and free fatty acids. Subsequently, the cholesterol, including the original content, is oxidized by cholesterol oxidase to produce cholest-4-en-3-one and hydrogen peroxide (H_2O_2). Finally, a colorimetric probe reacts with the generated hydrogen peroxide, forming a pink-colored product. The optical density measured at 500nm is directly proportional to the concentration of cholesterol within the sample.

Storage

The entire kit may be stored at 2–8 °C for up to 6 months from the date of shipment. For prepared reagent storage, see table below.

Component	Size / Description	Storage After Preparation
Microplate (Item A)	A 96-well (12 strips x 8 wells) plate	RT*
Assay Diluent	10 ml	2–8 °C
Cholesterol Standard	1 vial (100 µl of 5172 µM)	2–8 °C
Enzyme Mix Solution	20 ml	2–8 °C

RT = room temperature

**Return unused wells to the pouch containing desiccant pack, reseal along entire edge.*

Additional Materials Required

1. Microplate reader capable of measuring absorbance at 500 nm
2. Precision pipettes to deliver 2 µl to 1 ml volumes
3. Tubes to prepare sample dilutions
4. Incubator at 37°C

Sample Tips and General Considerations

NOTE: Optimal methods of sample preparation will need to be determined by each researcher empirically based on researched literature and knowledge of the samples.

- If not using fresh samples, freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot samples prior to initial storage.
- It is strongly recommended to add a protease inhibitor cocktail to cell and tissue lysate samples.
- Avoid sonication of 1 ml or less as this can quickly heat and denature proteins.
- Most samples will not need to be concentrated. If concentration is required, a spin column concentrator with a chilled centrifuge is recommended.

1. Cell lysates can be prepared as follows:

For attached cells, remove supernatant from cell culture, wash cells twice with cold 1X PBS (for suspension cells, pellet the cells by spinning down the cells at 1,000 x g for 10 min) making sure to remove any remaining PBS before adding lysis buffer. Solubilize the cells at $2x10^7$ cells/ml in lysis buffer containing protease inhibitors. Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 14,000 x g for 10 minutes. It is recommended that sample protein concentrations should be determined using a total protein assay. Lysates should be used immediately or aliquotted and stored at -70 °C. Thawed lysates should be kept on ice prior to use.
General tips for preparing lysate samples can be viewed on the online Resources page of the website: https://www.raybiotech.com/tips-on-sample-preparation/

2. Plasma samples:

Collect blood with an anticoagulant such as citrate, EDTA or oxalate and mix by inversion. Centrifuge the blood at 1000 x g at 4°C for 10 minutes. Collect plasma supernatant without disturbing the white buffy layer. Sample should be tested immediately or frozen at -80°C for storage. Typically, cholesterol levels in human plasma are in the range of 2.5-7.5 mM. Plasma samples can be diluted 1:10 with Assay Diluent.

3. Serum samples:

Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge the blood at 2500 x g for 20 minutes. Remove the yellow serum supernatant without disturbing the white buffy layer. Samples should be tested immediately or frozen at -80°C for storage. Typically, cholesterol levels in human serum are in the range of 2.5-7.5 mM. Serum samples can be diluted 1:10 with Assay Diluent.

NOTE:
Optimal experimental conditions for samples must be determined by the investigator. A set of serial dilutions is recommended for samples to achieve optimal assay results.

Standard Preparation

To prepare a dilution series of standard in the concentration range of 0 µM – 5172 µM (see Table below),

1. Label 8 microtubes #1 through 8 for standards with the following concentrations: 5172, 2586, 1293, 646.5, 323.3, 161.6, 80.8, 0 µM.
2. Pipette 40µL Assay Diluent into each tube (#2 to #8). Do not add Assay Diluent to tube #1.
3. Pipette 80 µL Cholesterol Standard (5172 µM) into tube #1.
4. Following the table below, transfer 40 µL of the 5172 µM Cholesterol Standard from tube #1 to tube #2 to make a 2586 µM cholesterol standard. Mix well and quick spin.
5. Repeat the transfer step with each successive concentration, transferring 40 µL of the prior concentration until reaching 80.8 µM. Mix each tube thoroughly before the next transfer. Tube #8 serves as the blank. Standards should be prepared fresh and used immediately.

Labeled Tubes	Cholesterol Standard (µL)	Assay Diluent (µL)	Standard Conc. (µM)
1	80 µL	0µL	5172
2	40 µL of Tube #1	40 µL	2586
3	40 µL of Tube #2	40 µL	1293
4	40 µL of Tube #3	40 µL	646.5
5	40 µL of Tube #4	40 µL	323.3
6	40 µL of Tube #5	40 µL	161.6
7	40 µL of Tube #6	40 µL	80.8
8	0µL	40 µL	0

NOTE: 5172 µM Cholesterol is equal to 200 mg/dl Cholesterol.

Assay Procedure

Each cholesterol standard and sample should be assayed in duplicate or triplicate. A standard curve should be generated each time the assay is performed.

1. Set incubator at 37°C, and pre-heat Enzyme Mix Solution right before the assay.
2. Add 10 µL of the diluted cholesterol standards or samples to the 96-well microtiter plate.
3. Add 200 µL of Enzyme Mix Solution (pre-heated at 37°C) to each well.
4. Cover with the plate cover. Carefully shake the plate for a few seconds to mix.
5. Incubate the plate for 5 minutes at 37°C.
6. Immediately measure the absorbance at 500nm using a plate reader.

Calculation of Results

Subtract the blanks

Average the absorbance value of the blank wells (Standard 0µM) and subtract this from the absorbance values of all the other wells. These are the corrected absorbance.

Plotting the standard curves

Make a plot of corrected absorbance at 500nm as a function of cholesterol concentration.

Determination of sample Cholesterol concentration

Cholesterol (µM) = $\frac{OD_{Sample} - OD_{Blank}}{Slope} \times DF$

OD_{Sample} = Optical density (OD) reading of the Sample

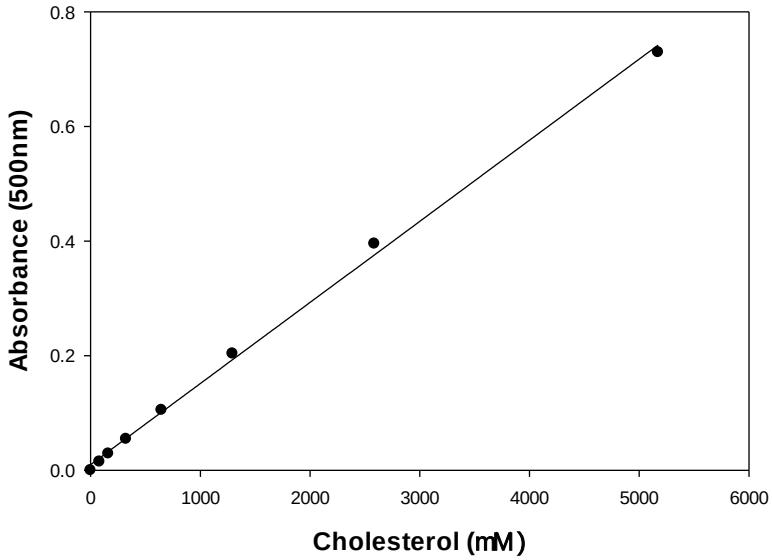
OD_{Blank} = Optical density (OD) reading of the Blank (Standard 0µM)

Slope is from the plot of cholesterol concentration vs. Absorbance, as shown in Typical Data below.

DF = Sample Dilution factor (DF = 1 for undiluted samples)

Note: If the calculated cholesterol concentration of the sample is higher than 5172µM, dilute the sample in assay diluent and repeat the assay.

A. Typical Data



These standard curves are for demonstration only. A standard curve must be run with each assay.

B. Reproducibility

Intra-assay Precision (Precision within an assay):
To assess intra-assay precision, 16 wells per sample (total of 4 samples) were tested on a single plate. The intra-assay coefficient of variation was found to be 2.9%.

Inter-assay Precision (Precision between assays):
To assess inter-assay precision, 4 samples were tested in separate assays (n=4). The inter-assay coefficient of variation was found to be 6.0%.

This product is for research use only.