Fluoro Cholesterol

Total Cholesterol Assay Kit

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Notes:

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I. Introduction:

Cholesterol is a lipid present in the cell membranes of eukaryotes and circulates in the blood stream. It is used in the biosynthesis of hormones, and plays an important role in cell signaling processes. Cholesterol exists as a free acid, as well as, in the esterified form as cholesteryl esters. Elevated levels of cholesterol are indicated in atherosclerosis and heart disease, and are the subject of large amount of research focused on cholesterol metabolism. Quantitative determination of cholesterol in experimental samples is central to this research.

Applications:

- Quantitation of total cholesterol in blood, plasma and serum samples.
- Testing effects of drugs on cholesterol metabolism.

II. Assay Principle:

Cell Technology's Cholesterol assay kit provides a simple, **one-step** fluorimetric or colorimetric method for determination of **total cholesterol** in serum and plasma samples. The assay is based on an enzyme-coupled reaction that detects both free cholesterol and cholesterol esters. Cholesterol esters are hydrolyzed by cholesterol esterase into cholesterol, which is then oxidized by cholesterol oxidase to yield hydrogen peroxide and cholest-4-en-3-one (ketone). The hydrogen peroxide then reacts with the cholesterol probe (detection reagent) in a 1:1 stoichiometry to produce the stable fluorescent product.

Cholesterol ester
Cholesterol esterase
↓
Cholesterol
Cholesterol Oxidase
↓
H_2O_2 + Cholest-4-en-3-one
↓
Cholesterol probe

Fluorescent Product

Lambda $_{max}$ 570nm. Lambda$_{ex/em}$ 535/585nm.

Colorimetric assay can be read on a spectrophotometer at 570nm.
Fluorescence is measured at excitation 530nm and emission 585nm.

III. Storage:

Store Part 1 of the kit frozen at -20°C upon arrival.
The Cholesterol probe (Part # 4022) should be protected from light.
To avoid repeated freeze/thaw cycles, prepare aliquots and freeze.
Allow reagents to warm to room temperature, and spin down vials briefly to ensure contents are not lost in caps.

Store Part 2 (Part # 3055: Reaction buffer and Part #7019: DMSO) refrigerated at 2-8°C.

IV. Schematic Representation of the Cholesterol Assay:

50µL of sample/cholesterol standard (Part # 7018)
+
50µL of Reaction cocktail (Enzyme mix+ Cholesterol probe+ Reaction Buffer)

Incubate at Room Temperature for 60 minutes, DARK
Read on plate reader: Excitation: 535nm, Emission: 585nm

V. Warnings and Precautions:

1. **For Research use only. Not for use in diagnostic procedures**.
2. Practice safe laboratory procedures by wearing gloves, protective clothing and eyewear.
3. The reaction is not stable in the presence of thiols (DTT or 2-mercaptoethanol). Keep these reactants below 10µM.
4. **Once the vial of Cholesterol probe(Part # 4022) is opened, it is important that low lighting conditions be used while aliquoting as well as performing the experiment. Direct and prolonged light exposure may increase the background, resulting in compromised linearity.**

VI. Catalog # FLCHOL100-2 Kit contents and Reagent Preparation:

1. **Part # 4022: Cholesterol Probe:** 1 vial dried down. Store at -20°C. Resuspend vial contents in 120µL of DMSO. Use 20µL of this solution per mL of reaction cocktail prepared. 1mL of Reaction Cocktail is sufficient for 20 tests. Aliquot and freeze as single-use portions.

2. **Part # 6023: Enzyme mix:** 1 vial dried down. Store at -20°C. Resuspend vial contents in 120µL of Reaction Buffer, Part # 3055. This makes a 50X concentrate. Use 20µL of this solution per mL of

reaction cocktail prepared. 1mL of Reaction Cocktail is sufficient for 20 tests. Aliquot and freeze as single-use portions.

3. **Part # 3055: 1X Reaction Buffer**: 20mL supplied. Store at 2-8°C

4. **Part # 7018: Cholesterol Standard:** 200µL supplied per kit. Store frozen at -20°C. Standard is supplied as a liquid at 2mg/mL=5.17mM=200mg/dL. Prepare serial dilutions ranging from 20µM to 0.3125 µM in the Reaction buffer supplied.

   Note: Standard may be cloudy when thawed out. It is recommended to heat the standard at 80°C for 20 minutes in a water bath, vortex and spin down the contents before construction of standard curve.

5. **Part # 7019: DMSO:** The vial contains 0.5ml of Dimethyl sulfoxide. Ready to use. Store at 2-8°C

VII. Materials required but not supplied:

1. Black 96-well plates (clear bottom optional for bottom reading instruments).
2. Fluorescence plate reader.
3. Deionized water.

VIII. Sample Preparation:

Cholesterol levels in human serum and plasma typically range from 2.5-7.5mmoles/L or mM.
A 1:250 to 1:500 dilution of serum or plasma samples will give RFU values that will fall within the range of the standard curve.
Cholesterol levels in serum average about 3% higher in value than in the corresponding plasma pair.[1]

IX. Assay Protocol:

Cholesterol Standard curve:

Aliquot the supplied cholesterol as 25-30µL aliquots to avoid repeated freeze/thaw.
Before using, heat one aliquot of cholesterol standard at 80°C for 20 minutes in a water bath to ensure that it is completely in solution. Vortex contents thoroughly and spin down briefly.

To a tube containing 90.4µL of reaction Buffer, add 9.6µL of the Cholesterol standard to prepare a 0.5mM cholesterol solution.
Label eight test tubes 1-8. To tube #1 containing 960µL of Reaction Buffer, add 40µL of 0.5mM cholesterol to give 20µM. Next serially dilute the 20µM cholesterol 1:2 by adding 500µL of Reaction Buffer to 500µL of 20µM cholesterol to prepare 10µM. Continue the serial dilutions through tube #8

to give a concentration of 0.3125µM in tube #8. The final concentration of cholesterol in the reaction well will be half that in the tube.

Tube #	Cholesterol Concentration in tubes µM	Final Cholesterol Concentration in wells. µM
1	20	10
2	10	5
3	5	2.5
4	2.5	1.25
5	1.25	0.625
6	0.625	0.3125
7	0.3125	0.15625
8	0	0

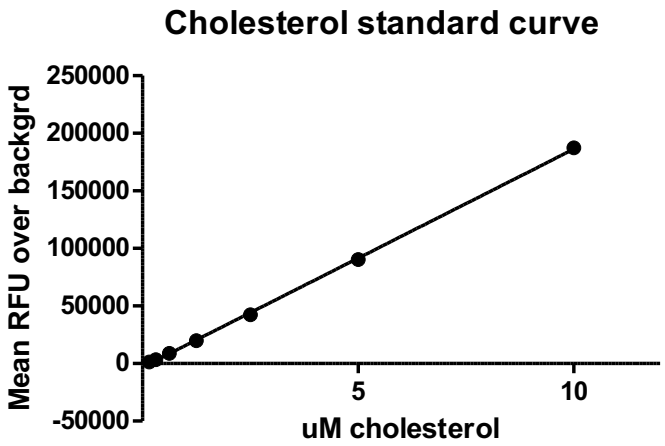

Fig.1 Cholesterol standard curve was generated using fluorimetric detection: excitation 535nm and emission 585nm. R^2 value=0.9977. Incubation time= 1 hr at Room temperature. Standard curve range 0.15625 µM to 10µM.

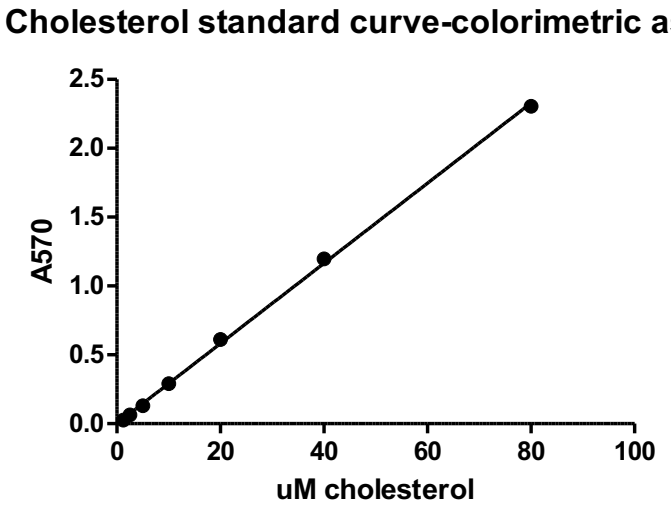

Fig.2 Cholesterol standard curve generated using colorimetric detection: absorbance read-out at 570nm. R^2 =0.9993. Incubation time=30 minutes. Standard curve range 1.25µM to 80µM.

For a colorimetric assay, prepare serial dilutions of the cholesterol standard in a range of 0- 160 µM and calculations done based on the standard curve generated. Read Absorbance at 570nm.
Note: Absorbance and fluorescent readouts should be performed on separate plates to avoid quenching effects.

Prepare Reaction Cocktail:

For 20 tests, prepare 1ml of Reaction cocktail as follows:
20 µL of resuspended Enzyme mix (Part # 6023)
20 µL of resuspended Cholesterol probe (Part # 4022)
960 µL of Reaction Buffer (Part # 3055)

Assay:

1. Plate 50 µL of cholesterol standards and samples on a 96-well microplate (clear-bottom black plate) in triplicate.
2. Add 50 µL of the reaction cocktail prepared above.
3. Cover the plate and incubate at room temperature for 1 hour, in the dark.
4. Read fluorescence at excitation 530nm and emission 585nm using a fluorescence plate reader.

Calculations:

1. Subtract the mean background from the RFU values of the standards/samples. Construct a standard curve by plotting the mean RFU over background on the Y-axis and the cholesterol concentration on the X-axis.

2. Determine the slope of the curve by using linear regression analysis. The concentration of cholesterol in the samples can be determined from the slope of the cholesterol standard curve.

3. Multiply the µM concentrations obtained for the samples by the dilution factors used to get concentration in mmoles/L or mM. Multiply this number by 2 to get the final concentration of cholesterol which is twice the concentration in the well.

4. Divide the mM concentration of cholesterol by 0.0259 to obtain readings in mg/dL. To convert mM cholesterol readings to mg/dL, multiply mM by 38.67.

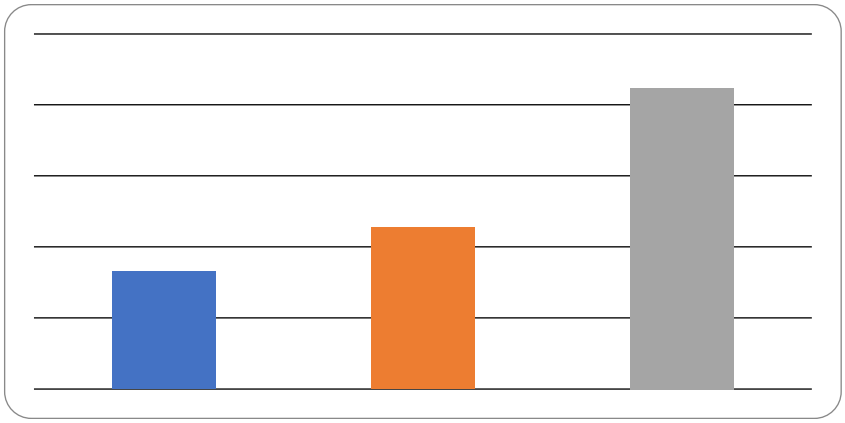

Fig.3 demonstrates the quantitation of total cholesterol in mg/dL in animal sera.

Donor 1: Type AB +ve	Donor 2: type O +ve	Donor 3: Type A -ve
214 mg/dL	210 mg/dL	241 mg/dL

Data generated from random human donors of different blood types. Total cholesterol is expressed in mg/dL.

X. Notes:

1. To adapt this as a colorimetric assay, the standard curve has to be constructed in a range of 0-160 µM and calculations done based on the standard curve generated. Absorbance readout at 570nm. Absorbance and fluorescent readouts should be performed on separate plates to avoid quenching effects.

2. The final cholesterol concentration will be **twice** the concentration in the well. It is important to remember to multiply the concentrations by 2 as well as the dilution factor used.

XI. References:

1. Cholesterol and Triglyceride concentrations in serum/plasma pairs. Clin. Chem., 23/1, 60-63 (1977)