MA-0119

Cholesterol/Cholesterol Ester Assay

(100 wells, Colorimetric, OD 570 nm or Fluorometric, Ex/Em = 535/587 nm, Store at -20°C)

Background Information:

Cholesterol is essential to all animal life. It is a precursor to steroid hormones and vitamin D and makes up about 30% of cell membranes. It is transported via lipoproteins (HDL, LDL, VLDL) in the blood, as free cholesterol and as cholesterol esters. AkrivisBio's Cholesterol/Cholesterol Ester Assay provides a simple, sensitive method of quantifying free, esterified or total cholesterol using absorbance or fluorescence. In the assay, cholesterol esters are optionally hydrolyzed. Free cholesterol thus formed is oxidized by cholesterol oxidase to yield hydrogen peroxide, which is utilized by peroxidase to oxidize ADHP, forming resorufin providing color (OD 570 nm) and fluorescence (Excitation/Emission = 535/587 nm). The assay detects total cholesterol (cholesterol + cholesterol esters) in the presence of cholesterol esterase. It measures free cholesterol only, in the absence of cholesterol esterase. Cholesterol ester content is determined differentially by subtracting free cholesterol from the total (cholesterol + cholesterol esters).

Assay Principle:

1 - Cholesterol esters are hydrolyzed forming free cholesterol (optional)
2 - Cholesterol is oxidized by cholesterol oxidase forming hydrogen peroxide
3 - Peroxidase utilizes the hydrogen peroxide to convert ADHP to resorufin (λ_{max} 570 nm; Excitation/Emission 535/580 nm).

Kit Contents:

Assay Buffer	25 ml	WM	MA-0119A
ADHP Solution	200 µl	Red	MA-0119B
Cholesterol Oxidase/HRP	lyoph	Green	MA-0119C
Cholesterol Esterase	lyoph	Blue	MA-0119D
Cholesterol Standard	100 µl	Yellow	MA-0119E

Storage and Handling:

Store the unopened kit at -20°C. Warm all components to room temperature before using. Centrifuge small vials for a few seconds prior to opening.
Assay Buffer: Ready to use as supplied. Store at 4°C.
ADHP Solution: DMSO freezes just below room temperature. It must be brought to room temperature before use. Store at -20°C.
Cholesterol Oxidase/HRP, Cholesterol Esterase: Add 220 µl Assay Buffer to each and dissolve. Once the enzymes have been reconstituted, store at -20°C. If you plan to use the Assay several times over a period of time, aliquot the enzyme solutions into convenient portions and freeze to prevent multiple freeze/thaw cycles which accelerates enzyme denaturation.
Cholesterol Standard: 2 mg/ml total cholesterol. Keep on ice while in use.

Assay Protocol:

1. Standard Curve: For an absorbance-based assay, add 10 µl of the Standard to 90 µl of Assay Buffer and mix to give a 0.2 mg/ml solution. Transfer 0 – 5 – 10 – 15 – 20 - 25 µl to a 96-well plate. Adjust all wells to 50 µl with Assay Buffer giving 0 – 1 – 2 – 3 – 4 – 5 µg per well, respectively, of the Cholesterol Standard.
For a fluorescence-based assay, dilute the Cholesterol Standard to 0.02 mg/ml by adding 10 µl of the Cholesterol Standard to 990 µl of Assay Buffer. Transfer the same volumes as in the absorbance-based assay, giving 0 - 0.1 - 0.2 - 0.3 - 0.4 - 0.5 µg per well of the Cholesterol Standard.

2. Sample Preparation: Extract cells (10^6) or tissue (10 mg) with 200 µl of chloroform **:** Isopropanol **:** Tergitol 15-S-9 (7**:**11**:**0.1) in a small homogenizer. Centrifuge the extract at 16,000 X g for 5 minutes at room temperature, then carefully transfer all the liquid, without disturbing the pellet, to a new tube, dry in a Speed-Vac or under a nitrogen stream until dry. Place the samples under vacuum for 30 min. to remove trace organic solvent. Dissolve the dried lipids with 200 µl of Assay Buffer. Sonicating or vortexing helps the dissolution/suspension. Dilute serum samples 10X with Assay Buffer. Use 1- 50 µl of sample per assay. Adjust all well volumes to 50 µl with Assay Buffer.
Notes: Sample values must fall into the range of the standard curve. If a sample exceeds this range, dilute and rerun. Some samples exhibit a significant background. Run samples in pairs with one of the pair used as a background control. Some samples exhibit a matrix effect where endogenous compounds interfere with the reaction giving lower OD or RFU/µg than the standards. To correct for this, run each sample as a triad, using one as a background control and adding a known amount (2 µg) to the second. The third is the uncorrected test sample. The difference between the test samples with and without the internal calibrant allows for accurate determination of cholesterol in the unspiked sample.

2. Initiate Reaction: Prepare sufficient Reaction Mix for the number of samples and standards to be run. Each well requires 50 µl of Reaction Mix containing:

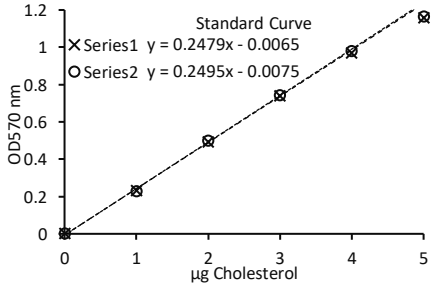
	Reaction Mix	*Background Control Mix
Assay Buffer	44 µl	46 µl
ADHP Solution[1]	2 µl	2 µl
Cholesterol Oxidase/HRP	2 µl	-----
Cholesterol Esterase[2,3]	2 µl	2 µl

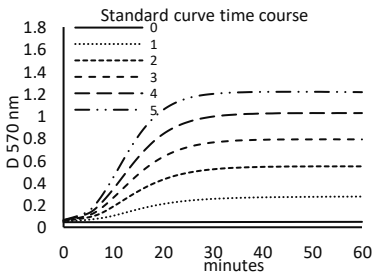
Add 50 µl of the Reaction Mix to each well containing standard or test samples.
Note: For the fluorescence-based assay, use 0.2-0.3 µl of the ADHP for each reaction to decrease fluorescence background. To detect free cholesterol only, omit cholesterol esterase in the reaction. In the presence of cholesterol esterase, the assay detects both free cholesterol and esterified cholesterol. To determine esterified cholesterol only, subtract the value of free cholesterol from the total cholesterol. The cholesterol standard contains a mixture of free and esterified cholesterol in a ratio similar to serum. Cholesterol esterase must be added to the standards to detect all cholesterol.

3. Measurement: Monitor the reaction in kinetic mode at 37°C for 30-60 minutes. When the signal from the standards levels off at a constant value, data acquisition can be stopped.

Typical Results:

µg Standard	Standard Raw Values OD		Background Corrected Values OD	
0	0.0477	0.0486	0	0
1	0.2784	0.2758	0.2307	0.2272
2	0.5392	0.5475	0.4915	0.4989
3	0.7872	0.7897	0.7395	0.7411
4	1.0174	1.0265	0.9697	0.9779
5	1.2057	1.2145	1.1580	1.1659





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4. Calculation: Subtract the 0 cholesterol standard reading from all other standards readings. Plot the standard curve. Determine the slope of the standard curve. This defines the OD/µg of the assay. If the higher standards deviate from a straight line causing a bend, disregard those when determining the slope (See 5 µg standard in the standard curve above). If a background control has been included, subtract it from the paired sample reading. Divide the test sample OD by the slope of the standard curve to get the µg of cholesterol in the test sample well.
If the test samples have been run as a triad with a spike added to one of the wells, subtract the background control value from the other two wells. The difference between the two wells is the signal from 2 µg of cholesterol (OD/2 µg). Divide the background corrected value for the unspiked sample by the OD/2 µg to get the amount of cholesterol in the test sample well.

To get µg cholesterol in the original sample:
Cholesterol in well / sample volume added to well = µg cholesterol/µl sample
(µg cholesterol/µl sample) X 200 µl (volume sample was redissolved in) = total cholesterol from sample
Total cholesterol from sample / mass (or volume or # of cells) = cholesterol/ mg tissue (or volume or # of cells).

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