HDL and LDL/VLDL Quantification Colorimetric/Fluorometric Kit

(Catalog # K613-100; 100 assays; Store at -20°C)

I. Introduction:

Regulation of HDL (high-density-lipoprotein)-cholesterol and LDL (low-density-lipoprotein)-cholesterol plays a central role in various disease developments. It is well known that low levels of HDL and high level of LDL are associated with an increased risk of cardiovascular events. BioVision's HDL and LDL/VLDL Cholesterol Quantification Kit provides a simple quantification method of HDL and LDL/VLDL after a convenient separation of HDL from LDL and VLDL (very low-density lipoprotein) in serum samples. In the assay, cholesterol oxidase specifically recognizes free cholesterol and produces products which react with probe to generate color (λ = 570 nm) and fluorescence (Ex/Em = 538/587 nm). Cholesterol esterase hydrolyzes cholesteryl ester into free cholesterol, therefore, cholesterol ester and free cholesterol can be detected separately in the presence and absence of cholesterol esterase in the reactions.

II. Kit Contents:

Components	Volume	Cap Code	Part No.
Cholesterol Assay Buffer	25 ml	WM	K613-100-1
2X LDL/VLDL Precipitation Buffer	10 ml	NM	K613-100-2
Cholesterol Probe (in DMSO, anhydrous)	200 µl	Red	K613-100-3A
Enzyme Mix (Lyophilized)	1 vial	Green	K613-100-5
Cholesterol Esterase (Lyophilized)	1 vial	Blue	K613-100-6
Cholesterol Standard (2 µg/µl)	100 µl	Yellow	K613-100-7

III. Reagent Preparation:

Cholesterol Probe: Ready to use as supplied. Warm to room temperature to thaw the DMSO solution. Store at –20°C, protect from light. Use within two months.

Cholesterol Esterase: Dissolve in 220 µl Cholesterol Assay Buffer prior to use. Aliquot and store at –20°C. Use within two months.

Enzyme Mix: Dissolve in 220 µl Cholesterol Assay Buffer prior to use. Aliquot and store at –20°C. Use within two months.

IV. Cholesterol Assay Protocol:

1. **Separation of HDL and LDL/VLDL:** Mix 100 µl of 2X Precipitation Buffer with 100 µl of serum sample in microcentrifuge tubes. Incubate 10 min at room temperature, centrifuge at 2000 x g (5000 rpm on bench-top microcentrifuge) for 10 min. Transfer the supernatant into new labeled tubes. This is the HDL fraction. The precipitates are the LDL/VLDL fraction. If you want to measure the LDL/VLDL level, the precipitate should be spun again, and the trace amount of HDL supernatant carefully removed. Resuspend the precipitate in 200 µl PBS (not provided). This is the LDL/VLDL fraction.

   Note A: If the supernatant is cloudy, the sample should be re-centrifuged. If the sample remains cloudy, dilute the sample 1:1 with PBS, and repeat the separation procedure. Multiply final results by two (2) due to the dilution with the 2X Precipitation Buffer.

   Note B: Precipitation time and temperature do not affect the results significantly.

2. **Standard Curve and Sample Preparations:** Dilute the Cholesterol Standard to 0.25 µg/µl by adding 20 µl of the Cholesterol Standard to 140 µl of Cholesterol Assay Buffer, mix well. Add 0, 4, 8, 12, 16, 20 µl into a series of wells in a 96-well plate. Adjust volume to 50 µl/well with Cholesterol Assay Buffer to generate 0, 1, 2, 3, 4, 5 µg/well of the Cholesterol Standard. (**Note:** Fluorometric assay is ~10 times more sensitive than the colorimetric assay, the cholesterol standards should be diluted 10 times further if using fluorometric assay).

   For sample testing, using 1 to 20 µl of the HDL or LDL/VLDL fraction, adjust the total volume to 50 µl/well with Cholesterol Assay Buffer. For unknown samples, we suggest testing different volumes of sample to ensure the readings are within the linear portion of the standard curve.

3. **Reaction Mix Preparation:** Mix enough reagent for the number of assays performed. For each assay, prepare a total 50 µl Reaction Mix containing:

   44 µl Cholesterol Assay Buffer
   2 µl Cholesterol Probe[1]
   2 µl Enzyme Mix
   2 µl Cholesterol Esterase[2,3]

   *Notes: 1. If using fluorometric assay, use 0.4 ul of Cholesterol Probe per reaction to reduce fluorescence background.

   2. Cholesterol Esterase hydrolyzes cholesteryl ester to free cholesterol. If you want to detect free cholesterol only, omit the Cholesterol Esterase in the reaction, and substitute with 2 µl of Assay Buffer. With the addition of Cholesterol Esterase, the assay detects total cholesterol (cholesterol and cholesteryl esters). If you want to detect cholesteryl esters only, subtract the value of free cholesterol from the value of total cholesterol.

   Cholesterol Esterase must be added to the reaction for standard curve to convert all the cholesterol in the standard solution.

4. Add 50 µl of the Reaction Mix to each well containing the Cholesterol Standard or test samples, mix well.

5. Incubate the reaction for 60 min at 37°C, protect from light. Measure O.D. at 570 nm or fluorescence at Ex/Em 538/587 nm in a micro-titer plate reader.

6. **Calculations:** Subtract 0 standard reading from readings. Plot the standard curve. Apply the sample readings to the standard curve to determine sample cholesterol amount in the reaction well. Sample cholesterol concentrations:

   C = A/V (µg/µl)

   Where: **A** is the sample cholesterol amount from the standard curve (in µg).
   V is original sample volume added to the sample reaction well (in µl).
   Cholesterol Molecular Weight: 386.6; 1 µg/µl = 100 mg/dL.

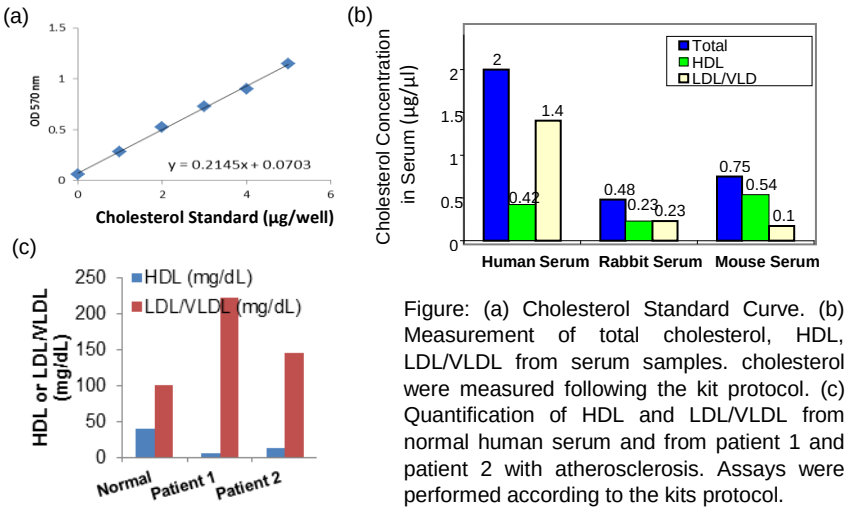

Figure: (a) Cholesterol Standard Curve. (b) Measurement of total cholesterol, HDL, LDL/VLDL from serum samples. cholesterol were measured following the kit protocol. (c) Quantification of HDL and LDL/VLDL from normal human serum and from patient 1 and patient 2 with atherosclerosis. Assays were performed according to the kits protocol.

FOR RESEARCH USE ONLY! Not to be used on humans.

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer	• Assay buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the assay buffer provided in the kit or refer data sheet for instructions
	• Samples were not deproteinized (if indicated in datasheet)	• Use the 10 kDa spin cut-off filter or PCA precipitation as indicated
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed, deproteinize samples
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the standard	• Avoid pipetting small volumes
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Standard stock is at an incorrect concentration	• Always refer the dilutions in the data sheet
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		