Total Cholesterol and Cholesteryl Ester Colorimetric Assay Kit II BN00847
(Catalog #BN00847; 100 assays; Store at -20°C)

I. Introduction:
Cholesterol is an essential molecule in all animal life. It has been involved in both normal development and diseases. The Cholesterol/Cholesteryl Ester Quantitation Kit provides a simple method for sensitive quantification of free cholesterol, cholesteryl esters, or both using a colorimetric method. Most of the cholesterol in blood is in the form of cholesteryl ester. These esters can be hydrolyzed to free cholesterol under the appropriate conditions. In the assay, free cholesterol is oxidized by cholesterol dehydrogenase to generate NADH which reacts with a sensitive probe resulting in strong absorbance at 450 nm. The assay can detect free or total cholesterol depending upon whether esterase is utilized to hydrolyze cholesterol esters present. Cholesteryl ester can be determined by subtracting the value of free cholesterol from the total cholesterol (cholesterol plus cholesteryl esters). The probe in this kit is more stable, sensitive and specific. The assay can tolerate interferences from various samples significantly.

II. Kit Contents:

Component	Volume	Cap Code	Part No.
Cholesterol Assay Buffer	25 ml	WM	BN00847-1
Substrate Mix (lyophilized)	1 vial	Red	BN00847-2
Enzyme Mix (lyophilized)	1 vial	Green	BN00847-3
Esterase (lyophilized)	1 vial	Blue	BN00847-4
Cholesterol Standard (2 µg/µl)	100 µl	Yellow	BN00847-5

III. Storage and Handling:
Store kit at –20°C, protect from light. Allow Assay Buffer to warm to room temperature before use. Keep enzymes and cholesterol standard on ice while using. Read the entire protocol before perform the assay.

IV. Reagent Preparation:

Substrate Mix: Reconstitute with 220 µl of Assay Buffer and mix thoroughly. The solution is stable for 2 months at 4°C.

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

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

V. Cholesterol Assay Protocol:

1. **Standard Curve 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. Adjust volume to 50 µl/well with Cholesterol Assay Buffer to generate 0, 1, 2, 3, 4, 5 µg/well of the Cholesterol Standard.

2. **Sample Preparation:** Serum should be diluted 10X with Cholesterol Assay Buffer, then use 2-20 µl for each testing. For cells or tissue samples, extract 10^6 cells or 10 mg tissue with 200 µl CHCl$_3$: IPA:NP-40 (7:11:0.1) in a microhomogenizer. Centrifuge the extract for 5 min at 15,000xg. Transfer the liquid phase to a new tube and air dry at 50°C then place samples under vacuum for 30 min to remove any remaining solvent. Dissolve dried lipids with 200 µl of Cholesterol Assay Buffer by sonicating or vortexing until homogeneous (OK if the solution becomes cloudy). The extraction procedure can be scaled up if larger amounts of sample are desired. Use 1- 50 µl of extract per assay. Adjust volume to 50 µl/well with Cholesterol Assay Buffer. For unknown samples, we suggest testing different amounts of sample to ensure that readings are within the limits of the standard curve.

3. **Ester Hydrolysis:** Add 2 µl Esterase to each standard and samples for which the total cholesterol value is desired. (**See Notes a and b in step 4**). Incubate 30 min at 37°C.

4. **Reaction Mix Preparation:** Mix enough reagent for the number of assays performed: For each well, prepare a total 48 µl Reaction Mix containing:

   44 µl Cholesterol Assay Buffer
   2 µl Substrate Mix
   2 µl Cholesterol Enzyme Mix

   Mix. Add 48 µl of the Reaction Mix to each well containing standard or samples.

   Notes:
   a) Cholesterol Esterase hydrolyzes cholesteryl ester to cholesterol. If you want to detect free cholesterol only, omit the Cholesterol Esterase in the reaction (step 3) & adjust the volume of reaction mix to 50 µl by adding 46 µl Cholesterol Assay Buffer. In the presence of Cholesterol Esterase, the assay detects both free cholesterol and cholesteryl esters. If you want to determine Cholesteryl Ester only, subtract the value of free cholesterol from the value of total cholesterol (Cholesterol and Cholesteryl Ester).

   b) The Cholesterol Standard contains a mixture of free cholesterol and cholesterol esters in a similar ration of serum. Cholesterol Esterase must be added to the standard reaction to convert all cholesterol in the standard.

5. Incubate the reaction for 30 min at 37°C, protect from light.

6. Measure absorbance at 450 nm in a microplate reader.

7. **Calculations:** Subtract the 0 standard background reading from all readings. Plot the standard curve. Apply sample readings to the standard curve. Cholesterol concentration in samples can then be calculated:
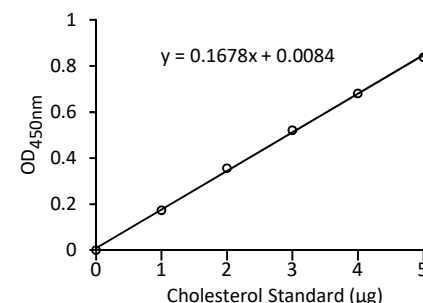
$$C = A/V \times D \quad (µg/µl)$$

Where: **A** = amount of cholesterol determined from Standard Curve (in µg).
V = volume of sample added into the reaction well (in µl).
D = Sample dilution factor

Cholesterol molecular weight: 386.65. 1µg/µl = 100 mg/dL.

y = 0.1678x + 0.0084

Figure: Cholesterol Standard Curve: Cholesterol/Cholesteryl Ester was quantified according to the kit instructions. Background from the 0 standard reading (without cholesterol) has been subtracted from all readings.

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

GENERAL TROUBLESHOOTING GUIDE:

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.		