ab65359 Cholesterol/Cholesteryl Ester Quantitation Assay kit (Colorimetric/Fluorometric)

For the rapid, sensitive and accurate measurement of free cholesterol, cholesteryl esters, or both in various samples.

This product is for research use only and is not intended for diagnostic use

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

Storage and Stability:

Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Aliquot components in working volumes before storing at the recommended temperature.

Reconstituted components are stable for 2 months.

Materials Supplied

Item	Amount	Storage (Before Prep)	Storage (After Prep)
Assay Buffer II/Cholesterol Assay Buffer	25 mL	-20°C	-20°C
OxiRed Probe/Cholesterol Probe (in DMSO; Anhydrous)	0.2 mL	-20°C	-20°C
Enzyme Mix I/Enzyme Mix (Lyophilized)	1 vial	-20°C	-20°C
Cholesterol Esterase (Lyophilized)	1 vial	-20°C	-20°C
Cholesterol Standard (2 µg/µL)	100 µL	-20°C	-20°C

Materials required, not supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:

- MilliQ water or other type of double distilled water (ddH_2O)
- PBS
- Microcentrifuge
- Microhomogenizer
- Pipettes and pipette tips
- Colorimetric or fluorescent microplate reader – equipped with filter for OD570 nm or Ex/Em = 535/587 nm (respectively)
- 96 well plate: clear plates for colorimetric assay; black plates (clear bottoms) for fluorometric assay
- Orbital shaker
- Chloroform: isopropanol: NP-40 (7:11:0.1) preparation
- Vacuum drier

Reagent preparation: Briefly centrifuge small vials at low speed prior to opening.

OxiRed Probe/Cholesterol Probe:

Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 min to thaw the DMSO solution before use. **NOTE: DMSO tends to be a solid when stored at -20°C, even when left at room temperature so it needs to melt for a few minutes at 37°C.** Aliquot OxiRed Probe/probe so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light. Once the OxiRed Probe/probe is thawed, use within two months.

Assay Buffer II/Cholesterol Assay Buffer:

Ready to use as supplied. Equilibrate to room temperature before use. Store aliquots at -20°C.

Cholesterol Esterase: Dissolve in 220 µL Assay Buffer II/Cholesterol Assay Buffer. Keep on ice during the assay. Aliquot esterase so that there is enough to perform the desired number of assays. Store aliquots at -20°C.

Enzyme Mix I/Enzyme Mix: Dissolve in 220 µL Assay Buffer II/Cholesterol Assay Buffer. Keep on ice during the assay .Aliquot mix so that there is enough to perform the desired number of assays. Store aliquots at -20°C.

Cholesterol Standard: Ready to use as supplied. Equilibrate to room temperature before use. Aliquot mix so that there is enough to perform the desired number of assays. Store at -20°C.

Standard preparation

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.

For the colorimetric assay:

1. Prepare a 0.25 µg/µL standard by diluting 25 µL of Cholesterol Standard in 175 µL of Assay Buffer II/Cholesterol Assay Buffer.
2. Using 0.25 µg/µL standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

For the fluorometric assay:

1. Prepare a 25 ng/µL Cholesterol standard by diluting 10 µL Cholesterol Standard in 790 µL of Assay Buffer II/Cholesterol Assay Buffer.
2. Using 25 ng/µL standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Cholesterol Standard (µL)	Assay Buffer II/Assay Buffer (µL)	Final Volume standard in well (µL)	End [cholesterol] in well	
				Colorimetric	Fluorometric
1	0	150	50	0 µg/well	0 µg/well
2	12	138	50	1 µg/well	0.1 µg/well
3	24	126	50	2 µg/well	0.2 µg/well
4	36	114	50	3 µg/well	0.3 µg/well
5	48	102	50	4 µg/well	0.4 µg/well
6	60	90	50	5 µg/well	0.5 µg/well

Each dilution has enough amount of standard to set up duplicate reading (2 x 50 µL).

NOTE: *If your sample readings fall out the range of your fluorometric standard curve, you might need to adjust the dilutions and create a new standard curve.*

Sample preparation

General Sample information:

We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.

We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

Cell (adherent or suspension) samples:

1. Harvest the amount of cells necessary for each assay (initial recommendation = 1x 10^6 cells).
2. Wash cells with cold PBS.
3. Extract lipids by resuspending the sample in 200 µL of Chloroform: Isopropanol: NP-40 (7:11:0.1) in a micro-homogenizer.

4. Spin the extract 5 – 10 minutes at 15,000 x g in a centrifuge.
5. Transfer all of the liquid (organic phase), avoiding the pellet, to a new tube.
6. Air dry at 50°C to remove chloroform.
7. Vacuum for 30 minutes to remove trace organic solvent.
8. Dissolve dried lipids (by sonicating or vortexing) with 200 µL of Assay Buffer II/Assay Buffer.

Tissue samples:

1. Harvest the amount of tissue necessary for each assay (initial recommendation = 10 mg tissue).
2. Wash with cold PBS.
3. Extract lipids by resuspending the sample in 200 µL of Chloroform: Isopropanol: NP-40 (7:11:0.1) in a micro-homogenizer.
4. Spin the extract 5 – 10 minutes at 15,000 x g in a centrifuge.
5. Transfer all of the liquid (organic phase) avoiding the pellet, to a new tube.
6. Air dry at 50°C to remove chloroform.
7. Vacuum for 30 min to remove trace organic solvent.
8. Dissolve (by sonicating or vortexing) with 200 µL of Assay Buffer II/Assay Buffer.

Serum samples:

Dilute samples 10-fold in the Assay Buffer II/Cholesterol Assay Buffer.
Recommendation: 0.5 – 5 µL serum/assay.
NOTE: *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*
Endogenous compounds in the sample may interfere with the reaction. To ensure accurate determination of cholesterol in the test samples, we recommend spiking samples with a known amount of Standard (2 µg).

Assay Procedure and Detecton

Equilibrate all materials and prepared reagents to room temperature prior to use.
It is recommended to assay all standards, controls and samples in duplicate.

1 **Set up Reaction wells:**

- Standard wells = 50 µL Standard dilutions.
- Sample wells for TOTAL cholesterol = 2 – 50 µL samples (adjust volume to 50 µL/well with Assay Buffer II/Cholesterol Assay Buffer).
- Sample wells for FREE cholesterol= 2 – 50 µL samples (adjust volume to 50 µL with Assay Buffer II/Cholesterol Assay Buffer).

2 **Reaction Mix:**

Prepare Reaction Mix for each reaction:

Component	Colorimetric		Fluorometric	
	Total Cholesterol Reaction Mix (µL)	Free Cholesterol Reaction Mix (µL)	Total Cholesterol Reaction Mix (µL)	Free Cholesterol Reaction Mix (µL)
Assay Buffer II/Cholesterol Assay Buffer	44	46	45.6	47.6
Cholesterol Probe	2	2	0.4	0.4
Enzyme Mix I/Cholesterol Enzyme Mix	2	2	2	2
Cholesterol Esterase *	2	0	2	0

NOTE:** Cholesterol Esterase hydrolyzes cholesteryl ester to free cholesterol. To detect free cholesterol only, omit the Cholesterol Esterase in the reaction, and substitute with 2 µL of Assay Buffer II/Cholesterol Assay Buffer. With the addition of Cholesterol Esterase, the assay detects total cholesterol (cholesterol and cholesteryl esters).* ***Cholesterol Esterase must be added to the standard curve wells to convert all the cholesterol in the standard solution.
NOTE*: For the fluorometric assay, use 0.4 µL/well of the OxiRed Probe/Probe to decrease the background readings, therefore increasing detection sensitivity.*
Mix enough reagents for the number of assays to be performed. Prepare a Master Mix of the Reaction Mix to ensure consistency.
We recommend the calculation: X µL component x (Number samples + standards +1)

3 Add 50 µL of Total Cholesterol Reaction Mix into Standard wells.
4 Add 50 µL of Total Cholesterol Reaction Mix to Total Cholesterol sample well.
5 Add 50 µL of Free Cholesterol Reaction Mix to Free Cholesterol sample wells.
6 Mix and incubate at 37°C for 60 min protected for light.
7 Measure output on a microplate reader.
Colorimetric assay: measure OD570 nm.
Fluorometric assay: measure Ex/Em = 535/587 nm.

Calculations

Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

1 Average the duplicate reading for each standard and sample.
2 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.
3 Plot the corrected absorbance values for each standard as a function of the final concentration of Cholesterol.
4 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).
Extrapolate sample readings from the standard curve plotted using the following equation:

$$A = \left(\frac{Corrected\ absorbance - (y - intercept)}{Slope}\right)$$

5 Concentration of samples in the test samples is calculated as:

$$Cholesterol\ Concentration = \left(\frac{A}{V}\right) * D$$

Where:
A = amount of cholesterol (µg) determined from Standard Curve.
V = volume of sample (µL) added into the reaction well.
D = Dilution Factor.
Cholesterol Molecular Weight: 386.6 g/mol
1 µg/µL = 100 mg/dL.
Total Cholesterol (Free Cholesterol + Cholesteryl esters): use Total Cholesterol Reaction Mix.
Free Colesterol: use Free Cholesterol Reaction Mix.
Cholesteryl esters: Total Cholesterol value – Free Cholesterol value.

Troubleshooting

Problem	Cause	Solution

Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples

FAQs

The enzyme does not fully dissolve in 220µL of Assay Buffer II/Cholesterol Assay Buffer. Will this affect the results?

The enzyme solution is likely super-saturated and hence some material is insoluble. We have tested the assay with 100% solubilized enzyme and 50% solubilized enzyme and the results were identical. The enzyme solution can be warmed up briefly for 1-2 minutes at 37C to help solubilize more enzyme.

The OxiRed Probe/probe changes to red color after dissolving. Does this affect the assay?

The OxiRed Probe/probe changing to red color after dissolving in DMSO should not affect the quality of the assay. The OxiRed Probe/probe will always be pink to red and the extent of redness depends on the level of oxidation. However, a pinkish/mild red color should not affect the assay significantly.

To measure free cholesterol and total cholesterol, are two separate sets of standard curves needed?

No. Only one standard curve after adding the cholesterol esterase should be produced. 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 curve reaction to convert all cholesterol.

In serum both esterified and non-esterified cholesterol is detectable. From tissue homogenates only the non-esterified version is detected. Why?

When tissues are homogenized, cellular enzymes including esterases are released. These enzymes hydrolyze the esterified cholesterol to non-esterified cholesterol.

Can EDTA be used for blood collection for this assay?

Yes, EDTA will be fine.

Can this kit measure free or protein-bound cholesterol?

This kit can measure both free and apolipoprotein-bound forms of cholesterol.

Can this kit be used with tissue samples, e.g. liver?

Yes, tissue samples can be used with this kit. It is critical to homogenize the tissue in Chloroform: Isopropanol: NP-40 for effective extraction of lipids from the sample. Please follow the instructions on the sample preparation section.

Interferences

These chemicals or biological will cause interferences in this assay causing compromised results or complete failure:
Triton X-100

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)