ab65390 – Cholesterol Assay Kit - HDL and LDL/VLDL

For the rapid, sensitive and accurate measurement of HDL and LDL/VLDL cholesterol in serum and tissue lysates.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab65390 (use www.abcam.cn/ab65390 for China, or www.abcam.co.jp/ab65390 for Japan)

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.
Δ **Note:** Reconstituted components are stable for 2 months.

Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
Assay Buffer 2	25 mL	-20°C	-20°C
2X LDL/VLDL Precipitation Buffer	10 mL	-20°C	-20°C
Cholesterol Standard	100 µL	-20°C	-20°C
OxiRed™ Probe	0.2 mL	-20°C	-20°C
Cholesterol Esterase	1 vial	-20°C	-20°C
Enzyme Mix I	1 vial	-20°C	-20°C

PLEASE NOTE: Assay Buffer 2 was previously labelled as Assay Buffer II and Cholesterol Assay Buffer, and OxiRed™ Probe as OxiRed Probe and Cholesterol Probe. The composition has not changed.

Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully utilize this assay:
- Microplate reader capable of measuring absorbance at OD 570 nm (colorimetric) or fluorescence at Ex/Em = 535/587 nm (fluorometric)
- Double distilled water (ddH$_2$O)
- PBS
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- 96 well plate with clear flat bottom (for colorimetric assay) / 96 well plate with clear flat bottom, preferably black (for fluorometric assay)
- Dounce homogenizer (if using tissue)

Reagent Preparation

- Briefly centrifuge small vials at low speed prior to opening.

Assay Buffer 2, 2X LDL/VLDL Precipitation Buffer, and **Cholesterol Standard:** Ready to use as supplied. Equilibrate to room temperature before use.

OxiRed™ Probe: Warm by placing in a 37°C bath for 1 – 5 min to thaw the DMSO solution before use. Repeat this step every time probe is needed. Store at -20°C protected from light. Once the probe is thawed, use within two months.

Cholesterol Esterase and **Enzyme Mix I:** Dissolve each in 220 µL Assay Buffer 2. Keep on ice during the assay. Use within two months.

Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.

For Colorimetric Assay: Prepare 200 µL 0.25 µg/µL Cholesterol Working Standard by diluting 25 µL of the provided Cholesterol Standard (2 µg/µL solution) with 175 µL of Assay Buffer 2.
For Fluorometric Assay: Prepare a 0.25 µg/µL Cholesterol standard by diluting 5 µL of the provided Cholesterol Standard with 35 µL of Assay Buffer 2. Prepare 250 µL of 0.025 µg/µL Cholesterol Working Standard by diluting 25 µL of 0.25 µg/µL Cholesterol standard with 225 µL of Cholesterol Buffer.
Δ **Note:** Detection sensitivity of fluorometric assay is 10-fold higher than colorimetric assay.
Using the Cholesterol Working Standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes. Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL):

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

Sample Preparation

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- Use fresh samples or snap freeze samples in liquid nitrogen upon extraction and store them immediately at -80°C. Thaw samples on ice before use. Be aware that this might affect the stability of your samples and the readings can be lower than expected.
- Do not use EDTA when collecting plasma or serum. We recommend using heparin for collection.
- For **Tissue lysate** preparation start from step 1.

Δ **Note:** this kit is primarily designed for use with serum and plasma samples. Tissue sample preparation may need optimization and modification by the user for successful use.
- For **Plasma and Serum (and other biological fluids)** start from step 6.
- For Quantification of **Total Cholesterol** use samples directly; no preparation step is required. Proceed to Assay Procedure Section.

Tissue lysates:
1. Harvest tissue (initial recommendation: 10 mg).
2. Wash tissue in cold PBS to remove any trace of blood.
3. Cut in small pieces and homogenize tissue in 100 µL of Assay Buffer 2 with a Dounce homogenizer or pestle sitting on ice, with 10-15 passes.
4. Centrifuge sample for 5-10 minutes at 4°C at 13,000 x*g* using a cold microcentrifuge to remove any insoluble material.
5. Collect supernatant and transfer to a new tube. This fraction can be used to measure **Total Cholesterol**.

Separation of HDL and LDL/VLDL (all sample types):
Mix 100 µL sample with 100 µL 2X LDL/VLDL Precipitation Buffer in microcentrifuge tubes.
Δ **Note:** You can use < 100 µL sample if you don't have enough sample. Simply add the same volume of 2X LDL/VLDL Precipitation Buffer to your sample and proceed with the sample preparation process.

6. Incubate 10 minutes at room temperature.
7. Centrifuge sample for 10 minutes at RT at 2000 x*g* (5,000 rpm on a bench microcentrifuge).
8. Transfer supernatant into a new tube. This is the **HDL fraction**.

Δ **Note:** If the supernatant is cloudy, sample should be re-centrifuged. If the sample remains cloudy, dilute the sample 1:1 with PBS and repeat the separation procedure from Step 6.

9. Centrifuge the precipitate once more for 10 minutes at RT at 2000 x*g* to remove any HDL left in the sample.
10. Remove trace amount of supernatant carefully.
11. Resuspend precipitate in 200 µL PBS. This is the **LDL/VLDL fraction**.

Δ **Note**: We suggest using different volumes of sample to ensure readings are within the standard curve range.

Assay Procedure

- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.
- For fluorometric assay, using 5x less probe decreases background reading and increases detection sensitivity.

Plate Loading:

- Standard wells = 50 µL standard dilutions.
- Sample wells:
  For TOTAL cholesterol = 2-50 µL samples (adjust volume to 50 µL/well with Assay Buffer 2).
  For FREE cholesterol = 2-50 µL samples (adjust volume to 50 µL/well with Assay Buffer 2).

Δ **Note:** For plasma and serum we recommend using 1-20 µL in sample wells.

Cholesterol reaction mix:

12. Prepare 50 µL of Total Cholesterol Reaction Mix and 50 µL Free Cholesterol Reaction Mix for each reaction. Mix enough reagents for the number of assays to be performed. Prepare a master mix to ensure consistency.

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

Δ **Note:** Cholesterol esterase hydrolyzes cholesteryl ester to free cholesterol. If no esterase is added to the reaction, the assay detects only free cholesterol. With the addition of cholesterol esterase, the assay detects total cholesterol (cholesterol and cholesteryl esters).

13. Add 50 µL of Total Cholesterol Reaction Mix into each standard and Total Cholesterol sample wells.
14. Add 50 µL of Free Cholesterol Reaction Mix into the Free Cholesterol sample wells.
    Cholesterol Esterase must be added to the reaction for standard curve to convert all the cholesterol in the standard solution.

Measurement:

15. Mix and incubate at 37°C for 60 min protected from light.
16. Measure output immediately on a microplate reader at OD 570 nm (Colorimetric Assay), or Ex/Em = 535/587 nm (Fluorometric Assay).

Calculations:

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

Standard curve calculation:

17. Subtract the mean absorbance/fluorescence value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance/fluorescence.
18. Average the duplicate reading for each standard and sample.
19. Plot the corrected standard curve reading (absorbance/fluorescence values) and draw the line of the best fit to construct the standard curve (most plate reader software or Excel can do this step). Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

Measure of Cholesterol in the sample:

20. Apply the corrected sample absorbance/fluorescence reading to the standard curve to get Cholesterol (A) amount in the sample wells.
21. Concentration of Cholesterol (µg/µL) in the test samples is calculated as:

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

Where:
A = amount of cholesterol in the sample well calculated from standard curve (µg).
V = sample volume added in the sample wells (µL).
D = sample dilution factor: for total cholesterol = 1; for HDL and LDL/VLDL fractions = 2 (addition of 2X LDL/VLDL Precipitation Buffer).
DF = additional dilution factor if sample has been diluted further to fit within standard curve range.

Total Cholesterol, Free Cholesterol and Cholesteryl Ester calculation:

22. Calculate Total Cholesterol, Free Cholesterol and Cholesteryl esters present in the sample as follows:

Total Cholesterol = Total Cholesterol Sample value
Free Cholesterol = Free Cholesterol Sample value
Cholesteryl esters = Total Cholesterol – Free Cholesterol

Cholesterol Molecular Weight: 386.6 g/mol
1 µg/µL = 100 mg/dL.

Interferences
These chemical or biological materials will cause interferences in this assay causing compromised results or complete failure:

- Avoid EDTA and other chelators when preparing plasma or serum samples.
- Sodium Fluoride (NaF)

Technical Support
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