HDL and LDL/VLDL Quantitation Kit

Catalog Number MAK045
Storage Temperature –20 ºC

TECHNICAL BULLETIN

Product Description
Lipoproteins transport the majority of plasma lipids including cholesterol and triglycerides. High-density lipoprotein (HDL), low-density lipoprotein (LDL), and very-low-density lipoprotein (VLDL) are the lipoproteins responsible for the vast majority of cholesterol transport in the blood. High LDL levels and low HDL levels are strongly associated with increased risk of adverse cardiovascular events.

In this kit, serum HDL and LDL/VLDL are first separated and then the cholesterol concentration of each is determined by a coupled enzyme assay, which results in a colorimetric (570 nm)/fluorometric (\(\lambda_{ex} = 535/\lambda_{em} = 587\) nm) product, proportional to the cholesterol present. This kit can also be used to determine the concentration of free cholesterol and cholesteryl esters present in a sample. This kit is suitable for use with plasma and serum samples.

Components
The kit is sufficient for 100 assays in 96 well plates.

- Cholesterol Assay Buffer
  Catalog Number MAK045A
  25 mL

- 2× LDL/VLDL Precipitation Buffer
  Catalog Number MAK045B
  10 mL

- Cholesterol Probe in DMSO
  Catalog Number MAK045C
  0.2 mL

- Enzyme Mix
  Catalog Number MAK045D
  1 vl

- Cholesterol Esterase
  Catalog Number MAK045E
  1 vl

- Cholesterol Standard, 2 µg/µL
  Catalog Number MAK045F
  0.1 mL

Reagents and Equipment Required but Not Provided.
- 96 well flat-bottom plate – It is recommended to use black plates with clear bottoms for fluorescence assays and clear plates for colorimetric assays.
- Fluorescence or spectrophotometric multiwell plate reader
- Phosphate Buffered Saline (Catalog Number P5368 or equivalent)

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
Briefly centrifuge vials before opening. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

- Cholesterol Assay Buffer – Allow buffer to come to room temperature before use.
- Cholesterol Probe – Warm to room temperature to thaw the solution prior to use. Store protected from light and moisture at –20 ºC. Upon thawing, the Cholesterol Probe is ready-to-use in the colorimetric assay.

  For the fluorescence assay, dilute an aliquot of the colorimetric Cholesterol Probe Solution 5 to 10-fold with Cholesterol Assay Buffer, just prior to use. This will reduce the background of the fluorescence assay.

- Cholesterol Esterase and Enzyme Mix – Reconstitute each in 220 µL of Cholesterol Assay Buffer. Mix well by pipetting, then aliquot and store at –20 ºC. Keep cold while in use and protect from light. Use within two months of reconstitution.
Storage/Stability
The kit is shipped on wet ice. Storage at –20 °C, protected from light, is recommended.

Procedure
All samples and standards should be run in duplicate.

Cholesterol Standards for Colorimetric Detection
Dilute 20 μL of the 2 μg/μL Cholesterol Standard Solution with 140 μL of the Cholesterol Assay Buffer to prepare a 0.25 μg/μL standard solution. Add 0, 4, 8, 12, 16, and 20 μL of the 0.25 μg/μL Cholesterol Standard solution into a 96 well plate, generating 0 (blank), 1, 2, 3, 4, and 5 μg/well standards. Add Cholesterol Assay Buffer to each well to bring the volume to 50 μL.

Cholesterol Standards for Fluorometric Detection
Dilute 10 μL of the 2 μg/μL Cholesterol Standard Solution with 790 μL of the Cholesterol Assay Buffer to prepare a 25 ng/μL standard solution. Add 0, 4, 8, 12, 16, and 20 μL of the 25 ng/μL Cholesterol Standard solution into a 96 well plate, generating 0 (blank), 0.1, 0.2, 0.3, 0.4, and 0.5 μg/well standards. Add Cholesterol Assay Buffer to each well to bring the volume to 50 μL.

Sample Preparation
Both the colorimetric and fluorometric assays require 50 μL of sample for each reaction (well).

Separation of HDL and LDL/VLDL: Mix 100 μL of the 2× Precipitation Buffer with 100 μL of the serum sample in a microcentrifuge tube. Incubate for 10 minutes at room temperature and then centrifuge the samples at 2,000 × g for 10 minutes. Transfer the supernatant fraction (HDL) to a new tube. The precipitant contains the LDL/VLDL fraction. To measure LDL/VLDL, centrifuge the samples again at 2,000 × g for 10 minutes and remove any remaining trace HDL supernatant. Resuspend the precipitate in 200 μL of PBS.

Note: If the supernatant is cloudy, the sample should be recentrifuged. If the sample remains cloudy, dilute the sample 1:1 with PBS and repeat the separation procedure.

Note: Heparin is the recommended anticoagulant for blood draw and for use in this assay. EDTA is not an acceptable alternative due to interference.

Assay Reaction
1. Set up the Reaction Mixes according to the scheme in Table 1. 50 μL of the appropriate Reaction Mix is required for each reaction (well).

Table 1. Reaction Mixes

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Total Cholesterol and Standards</th>
<th>Free Cholesterol</th>
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<tbody>
<tr>
<td>Cholesterol Assay Buffer</td>
<td>44 μL</td>
<td>46 μL</td>
</tr>
<tr>
<td>Cholesterol Probe</td>
<td>2 μL</td>
<td>2 μL</td>
</tr>
<tr>
<td>Cholesterol Enzyme Mix</td>
<td>2 μL</td>
<td>2 μL</td>
</tr>
<tr>
<td>Cholesterol Esterase</td>
<td>2 μL</td>
<td>–</td>
</tr>
</tbody>
</table>

Note: Cholesterol Esterase hydrolyzes cholesteryl esters to cholesterol. In the presence of Cholesterol Esterase, the assay detects total cholesterol, both free cholesterol and cholesteryl esters. To detect free cholesterol only, omit the Cholesterol Esterase from the reaction and add 46 μL of the Cholesterol Assay Buffer to the Reaction Mix. To determine cholesteryl esters, subtract the free cholesterol value from the total cholesterol value.

The cholesterol standard contains a mixture of free cholesterol and cholesteryl esters. The Reaction Mix containing Cholesterol Esterase must be used in the reactions for the Cholesterol Standards to convert all of each standard to cholesterol.

2. Add 50 μL of the appropriate Reaction Mix to each of the wells. Mix well using a horizontal shaker or by pipetting, and incubate the reaction for 60 minutes at 37 °C. Protect the plate from light during the incubation.

3. For colorimetric assays, measure the absorbance at 570 nm (A570). For fluorometric assays, measure fluorescence intensity (λex = 535/λem = 587 nm).
Results
Calculation
The background for either assay is the value obtained for the 0 (blank) cholesterol standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate cholesterol standards to plot a standard curve. The amount of cholesterol present in the samples may be determined from the standard curve.

Note: A new standard curve must be set up each time the assay is run.

Concentration of Cholesterol

\[
(S_a/S_v) \times Df^* = C
\]

S_a = Amount of cholesterol in unknown sample (µg) from standard curve
S_v = Sample volume (µL) added into the wells
C = Concentration of cholesterol in sample
Df = The dilution factor will be 2 due to the 1:1 dilution with the 2x Precipitation buffer. If the sample requires further dilution with PBS, the factor will need to be adjusted accordingly.

Cholesterol molecular weight: 386.65 g/mole.

Sample Calculation
Amount of Cholesterol (S_a) = 5.84 µg
Sample volume (S_v) = 50 µL
Df = 2
Concentration of cholesterol in sample

\[
(5.84 \, \text{µg}/50 \, \text{µL}) \times 2 = 0.2336 \, \text{µg/µL}
\]
## Troubleshooting Guide

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<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Suggested Solution</th>
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<td>Cold assay buffer</td>
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<tr>
<td>Omission of step in procedure</td>
<td>Refer and follow Technical Bulletin precisely</td>
<td></td>
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<tr>
<td>Plate reader at incorrect wavelength</td>
<td>Check filter settings of instrument</td>
<td></td>
</tr>
<tr>
<td>Type of 96 well plate used</td>
<td>For fluorescence assays, use black plates with clear bottoms. For colorimetric assays, use clear plates</td>
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</tr>
<tr>
<td><strong>Samples with erratic readings</strong></td>
<td>Samples prepared in different buffer</td>
<td>Use the Assay Buffer provided or refer to Technical Bulletin for instructions</td>
</tr>
<tr>
<td>Cell/Tissue culture samples were incompletely homogenized</td>
<td>Repeat the sample homogenization, increasing the length and extent of homogenization step.</td>
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<tr>
<td>Samples used after multiple freeze-thaw cycles</td>
<td>Aliquot and freeze samples if samples will be used multiple times</td>
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<tr>
<td>Presence of interfering substance in the sample</td>
<td>If possible, dilute sample further</td>
<td></td>
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<tr>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples and store correctly until use</td>
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<tr>
<td><strong>Lower/higher readings in samples and standards</strong></td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
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<tr>
<td>Use of expired kit or improperly stored reagents</td>
<td>Check the expiration date and store the components appropriately</td>
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<tr>
<td>Allowing the reagents to sit for extended times on ice</td>
<td>Prepare fresh reaction mix before use</td>
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<tr>
<td>Incorrect incubation times or temperatures</td>
<td>Refer to Technical Bulletin and verify correct incubation times and temperatures</td>
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<tr>
<td>Incorrect volumes used</td>
<td>Use calibrated pipettes and aliquot correctly</td>
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<tr>
<td><strong>Non-linear standard curve</strong></td>
<td>Use of partially thawed components</td>
<td>Thaw and resuspend all components before preparing the reaction mix</td>
</tr>
<tr>
<td>Pipetting errors in preparation of standards</td>
<td>Avoid pipetting small volumes</td>
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<tr>
<td>Pipetting errors in the Reaction Mix</td>
<td>Prepare a master Reaction Mix whenever possible</td>
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<tr>
<td>Air bubbles formed in well</td>
<td>Pipette gently against the wall of the plate well</td>
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<tr>
<td>Standard stock is at incorrect concentration</td>
<td>Refer to the standard dilution instructions in the Technical Bulletin</td>
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<tr>
<td>Calculation errors</td>
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<tr>
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<td>Samples measured at incorrect wavelength</td>
<td>Check the equipment and filter settings</td>
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<td>Samples contain interfering substances</td>
<td>If possible, dilute sample further</td>
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<tr>
<td>Sample readings above/below the linear range</td>
<td>Concentrate or dilute samples so readings are in the linear range</td>
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