

## Product Information

### Cholesterol Quantitation Kit

Catalog Number **MAK043**  
Storage Temperature  $-20^{\circ}\text{C}$

## TECHNICAL BULLETIN

### Product Description

Cholesterol is an important component of mammalian cell membranes where it functions in intracellular transport, cell signaling, and maintaining membrane fluidity. Within the blood, cholesterol circulates as both the free acid and as cholesterol esters. Controlling serum cholesterol has an important therapeutic role as elevated cholesterol levels are associated with the development of atherosclerosis and cardiovascular pathologies. Recent evidence suggests a disturbance of cholesterol homeostasis contributes to the development of a chronic inflammatory state.

The Cholesterol Quantitation Kit can also be used to determine the concentration of free cholesterol, cholesteryl esters, or both (total) present in a sample. In this kit, total cholesterol concentration is determined by a coupled enzyme assay, which results in a colorimetric (570 nm)/fluorometric ( $\lambda_{\text{ex}} = 535/\lambda_{\text{em}} = 587 \text{ nm}$ ) product, proportional to the cholesterol present. This kit is suitable for use with cell and tissue culture samples, urine, plasma, serum, and other biological samples.

### Components

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

Cholesterol Assay Buffer Catalog Number MAK043A	25 mL
Cholesterol Probe in DMSO Catalog Number MAK043B	0.2 mL
Enzyme Mix Catalog Number MAK043C	1 vL
Cholesterol Esterase Catalog Number MAK043D	1 vL
Cholesterol Standard, 2 $\mu\text{g}/\mu\text{L}$ Catalog Number MAK043E	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
- Chloroform (Catalog Number C2432 or equivalent), Isopropanol (Catalog Number 278475 or equivalent), and NP-40/IGEPAL® CA-630 (Catalog Number I8896 or equivalent)

### Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the 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  $-20^{\circ}\text{C}$ . 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  $\mu\text{L}$  of Cholesterol Assay Buffer. Mix well by pipetting, then aliquot and store at  $-20^{\circ}\text{C}$ . 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  $-20^{\circ}\text{C}$ , protected from light, is recommended.

### Procedure

All samples and standards should be run in duplicate.

#### Cholesterol Standards for Colorimetric Detection

Dilute 20  $\mu\text{L}$  of the 2  $\mu\text{g}/\mu\text{L}$  Cholesterol Standard solution with 140  $\mu\text{L}$  of the Cholesterol Assay Buffer to prepare a 0.25  $\mu\text{g}/\mu\text{L}$  standard solution. Add 0, 4, 8, 12, 16, and 20  $\mu\text{L}$  of the 0.25  $\mu\text{g}/\mu\text{L}$  Cholesterol Standard solution into a 96 well plate, generating 0 (blank), 1, 2, 3, 4, and 5  $\mu\text{g}/\text{well}$  standards. Add Cholesterol Assay Buffer to each well to bring the volume to 50  $\mu\text{L}$ .

#### Cholesterol Standards for Fluorometric Detection

Dilute 10  $\mu\text{L}$  of the 2  $\mu\text{g}/\mu\text{L}$  Cholesterol Standard Solution with 790  $\mu\text{L}$  of the Cholesterol Assay Buffer to prepare a 25  $\text{ng}/\mu\text{L}$  standard solution. Add 0, 4, 8, 12, 16, and 20  $\mu\text{L}$  of the 25  $\text{ng}/\mu\text{L}$  Cholesterol Standard solution into a 96 well plate, generating 0 (blank), 0.1, 0.2, 0.3, 0.4, and 0.5  $\mu\text{g}/\text{well}$  standards. Add Cholesterol Assay Buffer to each well to bring the volume to 50  $\mu\text{L}$ .

#### Sample Preparation

Both the colorimetric and fluorometric assays require 50  $\mu\text{L}$  of sample for each reaction (well).

Tissue (10 mg) or cells ( $1 \times 10^6$ ) can be extracted with 200  $\mu\text{L}$  of chloroform:isopropanol:IGEPAL CA-630 (7:11:0.1) in a microhomogenizer. Centrifuge the samples at  $13,000 \times g$  for 10 minutes to remove insoluble material. Transfer the organic phase to a new tube and air dry at  $50^{\circ}\text{C}$  to remove chloroform. Discard the pellet. Put the samples under vacuum for 30 minutes to remove any residue organic solvent. Dissolve dried lipids with 200  $\mu\text{L}$  of the Cholesterol Assay Buffer and sonicate or vortex until mixture is homogenous. The lipid solution may be cloudy.

Serum samples (0.5–5  $\mu\text{L}/\text{well}$ ) can be added directly to well.

For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

Bring samples to a final volume of 50  $\mu\text{L}$  with Cholesterol Assay Buffer.

### Assay Reaction

1. Set up the Reaction Mixes according to the scheme in Table 1. 50  $\mu\text{L}$  of the appropriate Reaction Mix is required for each reaction (well).

**Table 1.**

Reaction Mixes

Reagent	Total Cholesterol and Standards	Free Cholesterol
Cholesterol Assay Buffer	44 $\mu\text{L}$	46 $\mu\text{L}$
Cholesterol Probe	2 $\mu\text{L}$	2 $\mu\text{L}$
Cholesterol Enzyme Mix	2 $\mu\text{L}$	2 $\mu\text{L}$
Cholesterol Esterase	2 $\mu\text{L}$	–

**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  $\mu\text{L}$  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  $\mu\text{L}$  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  $37^{\circ}\text{C}$ . Protect the plate from light during the incubation.
3. For colorimetric assays, measure the absorbance at 570 nm ( $A_{570}$ ). For fluorometric assays, measure fluorescence intensity ( $\lambda_{\text{ex}} = 535/\lambda_{\text{em}} = 587 \text{ nm}$ ).

## Results

### Calculations

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

$$S_a/S_v = C$$

$S_a$  = Amount of cholesterol in unknown sample ( $\mu\text{g}$ )  
from standard curve

$S_v$  = Sample volume ( $\mu\text{L}$ ) added into the wells

$C$  = Concentration of cholesterol in sample

Cholesterol molecular weight: 386.65 g/mole.

### Sample Calculation

Amount of Cholesterol ( $S_a$ ) = 5.84  $\mu\text{g}$

Sample volume ( $S_v$ ) = 50  $\mu\text{L}$

Concentration of cholesterol in sample

$$5.84 \mu\text{g}/50 \mu\text{L} = 0.1168 \mu\text{g}/\mu\text{L}$$

**Troubleshooting Guide**

<b>Problem</b>	<b>Possible Cause</b>	<b>Suggested Solution</b>
Assay not working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	For fluorescence assays, use black plates with clear bottoms. For colorimetric assays, use clear plates
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if samples will be used multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until 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	Check the expiration date and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	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 plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

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