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Cholesterol Efflux Fluorometric Assay Kit (Cell-Based)

rev 3/19

(Catalog # K582-100; 100 assays; Store at -20°C)

I. Introduction:

Cholesterol efflux from the peripheral tissues and cells in atherosclerotic plaque is an initial and critical step in Reverse Cholesterol Transport (RCT). RCT is the process by which extrahepatic cells, including macrophage-derived foam cells in arterial atherosclerotic plaque, export cholesterol to plasma high-density lipoprotein (HDL) particles via the action of transmembrane lipid transporters such as ABCA1 and ABCG1. This sequestered cholesterol is eventually transported from HDL to the liver for bile acid synthesis and excretion, thus lowering the peripheral lipid burden. A negative correlation has been established between the in vitro efflux of cholesterol from macrophages and atherosclerosis, making enhancement of macrophage RCT a potential therapeutic approach to preventing or regressing atherosclerosis and lowering the risk of death from cardiovascular disease. BioVision's Cholesterol Efflux Assay is a high-throughput screening assay for measuring cholesterol efflux from cells using a fluorescently-labeled cholesterol analogue. The kit includes a reagent to remove apolipoprotein B-containing lipoprotein particles (LDL and VLDL) from serum samples, enabling quantification of the efflux/uptake capacity of serum HDL without potential interference from LDL. This assay provides a safe (non-radioactive), sensitive and reproducible method for measuring cholesterol efflux and screening of drugs or other test compounds that may modulate RCT.

II. Application:

- Screen serum samples or lipoproteins (isolated or recombinant) for ability to take up cholesterol.
- Screen small molecules for their effect on cholesterol efflux and RCT (a valuable tool for drug discovery).

III. Sample Type:

Human serum, purified HDL particles, isolated or recombinant lipoproteins

IV. Kit Contents:

Components	K582-100	Cap Code	Part Number
Labeling Reagent	5 ml	Crimp Cap	K582-100-1
Equilibration Buffer	5 ml	NM	K582-100-2
Reagent A	10 µl	Green	K582-100-3
Reagent B	50 µl	Yellow	K582-100-4
Cell Lysis Buffer	20 ml	WM	K582-100-5
Positive Control	1 ml	Blue	K582-100-6
Serum Treatment Reagent	1 ml	Purple	K582-100-7

V. User Supplied Reagents and Equipment:

- Macrophage cell line (*e.g.* J774A.1 cells)
- Phenol red-free RPMI-1640 culture medium, Fetal Bovine Serum (FBS) and 5% CO_2 cell culture incubator
- Multiwell fluorescence microplate reader
- Multi-channel pipette
- Clear 96-well tissue-culture plate (one) and white 96-well plate with opaque flat-bottom wells (two)

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Read the entire protocol before performing the assay. Open all the reagents under sterile conditions (*e.g.* a cell culture hood) only.

- **Labeling Reagent:** Store at -20°C, **protected from light**. Thaw to room temperature before use and open under sterile conditions.
- **Equilibration Buffer:** Store at -20°C, **protected from light**. For each experiment, aliquot required amount of equilibration buffer for the experiment. Immediately prior to use, add Reagent A (2 µl/ml) and Reagent B (10 µl/ml) to aliquoted equilibration buffer under sterile conditions and warm to 37°C before adding to the cells.
- **Cell Lysis Buffer:** Store at -20°C.Warm to room temperature before adding to the cells.
- **Positive Control:** Store at -20°C. Thaw at room temperature and warm to 37°C before adding to the cells.
- **Serum Treatment Reagent:** Store at -20°C. Thaw at room temperature prior to use.

VII. Cholesterol Efflux Assay Protocol:

The procedure described below is for murine macrophage cell line J774.1. This procedure can also be used with macrophage cells derived from THP-1 monocytes, which can be differentiated into cells with a macrophage-like phenotype upon treatment with 100 nM phorbol 12-myristate-13-acetate (PMA) for 48-72 hours.

1. Load Cells with Fluorescently-Labeled Cholesterol:

a. Grow J774.1 macrophage cells in growth medium (RPMI 1640 with 10% FBS) until cells reach ~90% confluency. Split cells under sterile conditions (**do not use trypsin for detachment when splitting/seeding cells,** see note below) and seed approximately 1 x 10^5 J774.1 cells/well in a 96-well tissue culture plate using 100 µl growth medium per well. When planning the assay, remember to seed additional wells for assay validation (positive and negative (no cholesterol acceptor) control conditions). Incubate cells for 4-6 hours at 37°C (in a humidified cell culture incubator with a 5% CO_2 atmosphere) to allow cells to fully adhere to the plate.

Notes:

- Treatment of macrophage (J774.1 or THP-1) cells with trypsin or other cell dissociation solutions can destroy the extracellular matrix scaffolding required for cholesterol acceptor binding and will inhibit or ablate cholesterol efflux. We recommend using manual scraping with a rubber cell scraper to split and seed macrophage cultures.
- To ensure accuracy, we recommend each planned treatment condition (including each test cholesterol acceptor, positive control and negative (no cholesterol acceptor) controls) to be performed in duplicate or triplicate wells.

b. For each test well (including positive and negative cholesterol acceptor control wells), prepare 100 µl of labeling medium by mixing 50 µl of Labeling Reagent and 50 µl of serum-free RPMI medium. Gently aspirate growth medium from wells and wash the cell monolayer with serum-free RPMI medium. Add 100 µl of the labeling medium mix per well. Incubate the plate for 1 hour, **protected from light** at 37°C in a humidified incubator with a 5% CO_2 atmosphere.

c. Following labeling, prepare 100 µl of equilibration medium for each well by mixing 50 µl Equilibration Buffer (**with appropriate concentrations of Reagent A and B added immediately before use,** see Section VI) and 50 µl of serum-free RPMI medium. Aspirate labeling medium from wells and add 100 µl of the equilibration medium mix per well.

d. Incubate the plate overnight (12-16 hours), **protected from light** at 37°C in a humidified incubator with a 5% CO_2 atmosphere.

2. Treat Cells with Cholesterol Acceptor:

a. If using human serum as a cholesterol acceptor, prepare LDL/VLDL-depleted serum using the Serum Treatment Reagent prior to addition to cells. Add 2 parts of Serum Treatment Reagent to 5 parts of human serum (a 2:5 v/v ratio; for example, mix 40 µl Serum Treatment Reagent with 100 µl human serum). Incubate mixture for 20 min on ice. Centrifuge the mixture at 9000 x *g* for 10 min at 4°C and transfer supernatant to a clean microfuge tube. Keep on ice until used.

b. After overnight incubation, gently aspirate the equilibration medium from cells and wash by adding 200 µl of phenol red-free, serum-free RPMI medium to each well. Aspirate the wash medium and add desired cholesterol acceptors diluted in phenol red-free, serum-free RPMI medium, according to the table below. If using pretreated human serum (prepared above), we recommend using 2 µl supernatant per well (2% of the total well volume). However 1-4 µl of LDL/VLDL-depleted serum (1-4% of the total well volume) may be used if desired. For cholesterol acceptor positive control, add 20 µl of Positive Control and bring the volume up to 100 µl with serum-free RPMI. For negative control (no cholesterol acceptor), add 100 µl serum-free RPMI to negative control well(s).

	Pretreated Serum	Purified HDL	Positive Control	Negative Control
Sample	2 µl	2.5 – 20 µg	20 µl	—
Serum-Free RMPI-1640	to 100 µl	to 100 µl	80 µl	100 µl

Note: If desired, test compounds (*i.e.* potential stimulants or inhibitors of cholesterol efflux) may be added during the efflux incubation period. Test compound stock solutions should be diluted in phenol red-free, serum-free RPMI to 10X desired final concentration. For measurement of cholesterol efflux to a given acceptor in the presence of test compounds, bring the well volume up to 90 µl with serum-free RMPI (instead of 100 µl) and add 10 µl of the 10X test compound solution. If an organic solvent is used to dissolve test compound, we recommend performing a vehicle condition (with the same final concentration of solvent) to control for solvent effects.

c. Return plate to 5% CO_2 incubator and incubate cells treated with cholesterol acceptors at 37°C for 4 hours (or desired time based upon your specific treatment conditions and protocol), **protected from light**.

3. Measurement: At the end of incubation, transfer supernatant (medium containing cholesterol acceptor) of each well (including control wells) to a white 96-well plate (with opaque, flat-bottom wells) and measure the fluorescence (Ex/Em = 485/523 nm) in endpoint mode. Solubilize the adherent cell monolayer by adding 100 µl of Cell Lysis Buffer to each well and incubating the plate (protected from light) with orbital shaking at room temperature for 30 min. Pipette up and down to dissolve any cell debris and transfer cell lysate to another white 96-well plate (with opaque, flat-bottom wells) of the same type used for the supernatant. Measure the fluorescence (Ex/Em = 485/523 nm) of the cell lysate.

4. Calculations: Cholesterol efflux from the labeled macrophage cells to a particular cholesterol acceptor/treatment condition is calculated by dividing the fluorescence intensity (RFU) obtained for the supernatant by the sum of the fluorescence intensity of the supernatant and cell lysate of the same treatment. This value is multiplied by 100 to obtain %Cholesterol Efflux in 4 hours. Subtract %Cholesterol Efflux obtained from the negative (no treatment) control from the treatment groups to determine the final net %Cholesterol Efflux.

$$\text{\% Cholesterol Efflux} = \frac{\text{RFU of Supernatant}}{\text{RFU of Cell Lysate + RFU of Supernatant}} \times 100$$

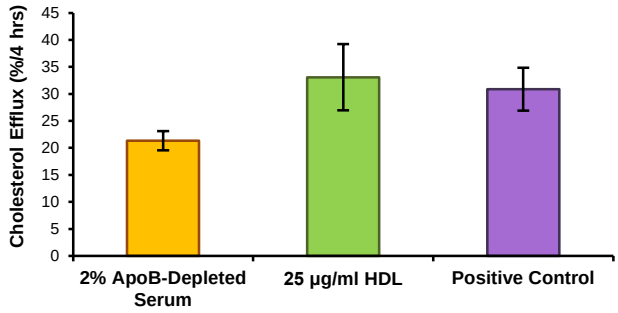

Figure: Percentage (%) Labeled Cholesterol Efflux. J774.1 cells were labeled with the fluorescent cholesterol Labeling Media and treated with various cholesterol acceptors such as 2% LDL/VLDL-depleted Human Serum (2 µl/well), purified human HDL (25 µg/ml, 2.5 µg/well) or Positive Control reagent. Cholesterol efflux is expressed as percentage of fluorescently-labeled cholesterol transferred from labeled adherent macrophages to designated cholesterol acceptor present in supernatant over a 4 hour time period, with no treatment control (efflux to medium containing no acceptor) percentage subtracted (mean ± SD of *N* = 5-6 wells).

VIII. RELATED PRODUCTS:

Cholesterol/Cholesteryl Ester Assay Kit (K603) LDL Uptake (Cell-Based) Assay Kit (K436) CETP Activity Assay Kit II (K595)
HDL and LDL/VLDL Quantification Kit (K613) HDL Uptake (Cell-Based) Assay Kit (K586) Human High-Density Lipoproteins (7853)

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