Cholesterol efflux capacity of high-density lipoprotein (HDL) is a new biomarker that characterizes a key step in reverse cholesterol transport. Excessive cholesterol in the cell is either converted to cholesteryl esters (CE) or effluxed out of the cell by a number of intracellular transporters, such as ATP binding cassette transporter proteins A1 (ABCA1) and G1 (ABCG1) and scavenger receptor type BI (SR-BI). The natural acceptor of cholesterol in serum is HDL and Apolipoprotein A-I. A strong negative correlation exists between cholesterol efflux capacity of serum and the incidence of atherosclerosis. BioIntersect’s Cholesterol Efflux Assay is designed to quantitate the rate of cholesterol efflux from cultured cells and/or the capacity of plasma/serum acceptors to accept cholesterol released from the cells. Our assay uses fluorescently labeled cholesterol and provides a safe, highly sensitive, high-throughput and reproducible method for measuring cholesterol efflux.

This assay can be performed on any cell type containing intracellular transporters of cholesterol efflux. For reverse cholesterol transport studies, we recommend using macrophage J774A.1 cell line. This assay can also be performed on endothelial, HepG2, RAW, differentiated THP-1 and adipocyte cell lines.

### Samples
- Human Serum, Purified and Recombinant lipoproteins.

### Applications
- High-throughput screening of human serum samples for clinical studies.
- Small molecules screening to increase cholesterol efflux capacity.

### User Supplied Material
- Cell line with appropriate media.
- Fluorescence Plate reader.
- One 96-well white clear bottom culture plate and one 96-well white plate.

### Pre-Assay Preparations

### Assay Protocol

**Cell Plating:** Harvest cells of interest. Plate 1-5x10⁴ cells/well in a 96-well white clear bottom plate, containing complete media with serum, according to the protocol. Within 4-6 hrs, adherent cells are attached to the plate (check under microscope to confirm). Wash the cells with serum-free media.

**Labeling Cells with Fluorescent cholesterol:** Mix Labeled Cholesterol and Equilibration Solution (containing Solution 1 and 2) in a ratio of 1:1 (e.g. mix 1 ml of Labeled Cholesterol with 1 ml of Equilibration Solution). Add 100 µl of mix to each well. Cover the plate with aluminum foil and incubate for 16 hrs or overnight in a 37°C incubator containing 5% CO₂.

**Serum Treatment:** To test the cholesterol efflux capacity of serum, pre-treat of human serum is required before use. Add 2 parts of Serum Treatment Solution to 5 parts of human serum (Ratio 2:5). Incubate for 20 min on ice. Centrifuge the mixture at ~9,000 x g, for 10 min at 4°C. Use the supernatant to treat the cells as desired and make up the volume to 100 µl by serum-free media.

**Treatments with Efflux Mediators:** After 16 hrs incubation, remove the labeling solution from cultured cells wells and wash the plate gently three times with 200 µl of serum-free media, without disturbing the cell monolayer. Treat the cells with desired amount of serum/compound of interest in 100 µl of serum-free media. For background control, use media alone (no serum). For Positive Control, add 10-20 µl of Positive Control per well. Make up the volume to 100 µl by media. Incubate cells for 4-6 hrs in a 37°C incubator with 5% CO₂.

**Reading:** After the incubation, transfer the media containing the efflux mediator to a 96-well white plate. To the cell monolayer, add 100 µl of Lysis Buffer to each well and incubate the plate on an orbital shaker for 15 min. Pipette up and down to dissolve any cell debris, if needed. Transfer the clear cell lysate to same 96-well white plate. Measure the fluorescence (RFU) at Ex/Em = 482/515 nm using a fluorescence plate reader.

**Calculations:** Cholesterol efflux capacity of different treatments is calculated by dividing the fluorescence (RFU) of the media by total fluorescence (RFU) of the cell lysate plus media. Multiply by 100 to obtain % Cholesterol Efflux. Subtract % Cholesterol Efflux of background control from the treatment groups to determine the final % Cholesterol Efflux.

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\% \text{ Cholesterol Efflux} = \frac{\text{RFU of the Media}}{\text{Total RFU of Media + Cell Lysate}} \times 100
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Express percentage (%) Cholesterol Efflux/time (hrs)
Figure 1: Cholesterol Efflux Quantitation in Macrophage Cells: J774A.1 cells were fluorescently labeled with cholesterol and treated with various cholesterol mediators like Treated Serum (human), HDL (50 µg/ml), recombinant Apo AI, Patients with known atherosclerotic issues (Patient 1-3) or with Positive Cntrl. Cholesterol efflux is expressed as percentage (%) efflux elicited by cells in 4hrs.

Figure 2: Cholesterol Efflux Quantitation in Endothelial Cells: Endothelial cells were fluorescently labeled with cholesterol and treated with various cholesterol mediators like Treated Serum (human), Treated Serum with BLT-1 (SR-BI inhibitor), or with Positive Cntrl. Cholesterol efflux is expressed as percentage (%) efflux elicited by cells in 4hrs.

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