Technical Bulletin

Cholesterol Efflux Assay Kit

Catalogue number MAK192

Product Description

Reverse Cholesterol Transport (RCT) is the mechanism by which the peripheral lipid burden is reduced by transport to liver and its fecal excretion. The first important step in RCT is the cholesterol efflux from macrophage-derived foam cells present in atherosclerotic plaques. The cholesterol efflux is critical for the maintenance of net cholesterol balance in arterial walls and in the reduction of proinflammatory responses triggered by lipid-laden macrophages.

The Cholesterol Efflux Assay Kit is a high-throughput screening assay that measures cholesterol efflux in cells using fluorescent-labeled cholesterol (λ_{Ex} = 485/ λ_{Em} = 523 nm). It is suitable for serum, isolated, or recombinant lipoprotein samples. It may also be used to screen small molecules for their effect on cholesterol efflux and therefore, may be a valuable tool in drug discovery programs.

Components

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

- Labeling Reagent 5 mL
  Catalogue Number MAK192A
- Equilibration Buffer 5 mL
  Catalogue Number MAK192B
- Reagent A 10 µL
  Catalogue Number MAK192C
- Reagent B 50 µL
  Catalogue Number MAK192D
- Cell Lysis Buffer 20 mL
  Catalogue Number MAK192E
- Positive Control 1 mL
  Catalogue Number MAK192F
- Serum Treatment Reagent 1 mL
  Catalogue Number MAK192G

Reagents and Equipment Required but Not Provided

- J774.1 or other macrophagic cell line
- Phenol red-free, FBS-free RPMI 1640 medium (Catalogue Number R7509 or equivalent)
- Phenol red-free RPMI 1640 medium with 10% fetal bovine serum (FBS)
- Purified HDL or human serum as cholesterol acceptor
- Clear 96-well tissue culture plate
- 96-well flat-bottom plates – It is recommended to use white plates with clear bottoms for fluorescence readings in this assay
- Fluorescence multiwell plate reader
- Microcentrifuge capable of 9,000 × g at 2-8 °C
- Microcentrifuge tubes
- Humidified incubator

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.

Storage/Stability

The kit is shipped on wet ice. Store components at -20 °C, protected from light, is recommended. Open all the reagents under sterile conditions.

Preparation Instructions

Briefly centrifuge small vials prior to opening. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

Labeling Reagent - Thaw on ice before use and open under sterile conditions. Store at -20 °C. Protect from light.

Equilibration Buffer – Warm to 37 °C before use. Mix well by pipetting, then aliquot and store at -20 °C protected from light. Just before use, add 2 µL/mL of Reagent A and 10 µl/ml of Reagent B to an aliquot under sterile conditions and warm to 37 °C before adding to the cells.

Cell Lysis Buffer and Serum Treatment Reagent – Store at -20 °C. Thaw before adding to cells.

Positive Control – Store at -20 °C. Thaw on ice before adding to cells.

Procedure

The procedure described is for the J774.1 macrophage cell line. It can also be used with other macrophagic cell lines. Additional optimization may be required.

Preparation of duplicate or triplicate wells for each condition (test sample, cholesterol acceptor, positive control, and negative control) is recommended to ensure accuracy.

Sample Preparation

Using sterile technique, make a uniform suspension of J774.1 cells in phenol red-free RPMI 1640 medium with 10% FBS. Plate ~1×10^5 cells/well (100 µL/well) in a 96-well tissue culture plate. Seed additional wells as necessary for assay validation. Incubate the plate for4-6 hours in a humidified incubator maintained at 37 °C with 5% CO_2 to allow the cells to adhere. After incubation, wash the cells with phenol red-free, FBS-free RPMI 1640 medium.

To correct for background in Samples, include a Sample Blank by omitting the Labeling Reagent. The Sample Blank readings can then be subtracted from the Sample readings.

Assay Reaction

1. Prepare a Labeling Mix according to the scheme in Table 1. 100 µL of Labeling Mix is required for each reaction (well).

Table 1.

Labeling Mix

Reagent	Amount
Labeling Reagent	50 µL
RPMI medium (Phenol red-free and FBS-free)	50 µL

2. Aspirate the RPI 1640 medium from each well of cells. Add 100 µL of the Labeling Mix to each well. Incubate the plate for 1 hour protected from light in a humidified incubator maintained at 37 °C with 5% CO_2.

   Following labeling, prepare an Equilibration Mix according to the scheme in Table 2 at 37 °C.100 µL of Equilibration Mix is required for each reaction (well).

Table 2.

Equilibration Mix

Reagent	Amount
37 °C Equilibration Buffer (with 2 µL/mL of Reagent A and 10 µl/ml of Reagent B)	50 µL
RPMI medium (Phenol red-free and FBS-free)	50 µL

Aspirate Labeling Mix from wells and add 100 µl of the Equilibration Mix per well.

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

Treating cells with Cholesterol Acceptor

Note: If human serum is being used as the cholesterol acceptor, pretreat the serum. Add 2 parts of Serum Treatment Reagent to 5 parts of human serum and incubate for 20 minutes on ice. Centrifuge for 10 minutes at 9,000 × g at 2-8 °C. Transfer supernatant to a clean microcentrifuge tube and keep on ice until used.

6. After overnight incubation, gently aspirate the Equilibration Mix from cells and wash by adding 200 µl of RPMI medium (Phenol red-free and FBS-free) to each well.

7. Aspirate the wash medium and add desired cholesterol acceptors diluted in RPMI medium (Phenol red-free and FBS-free), according to Table 3 below.

Table 3.

Reagent	Pretreated Serum (cholesterol acceptor)	Purified HDL (cholesterol acceptor)	Positive Control	Negative Control
Sample	2 µl	2.5-20 µg	20 µl	--
RPMI medium (Phenol red-free and FBS-free)	98 µl	Adjust to 100 µl	80 µl	100 µl

8. Incubate the plate for 4 hours protected from light in an incubator maintained at 37 °C with 5% CO_2.

Measurement

9. At the end of incubation, transfer supernatant (medium containing cholesterol acceptor) of each well to a white 96-well plate (with opaque, flat-bottom wells) and measure the fluorescence (F_m, λ_{Ex} = 485/ λ_{Em} = 523 nm) in endpoint mode.

10. Solubilize the cell monolayer in the wells of the original plate with 100 µL/well of Cell Lysis Buffer. Shake the plate for 30 minutes at room temperature.

11. Pipette up and down to dissolve any cell debris. Transfer the cell lysate to a fresh white 96-well plate and measure the fluorescence (F_c, λ_{Ex} = 485/λ_{Em} = 523 nm).

Results

Subtract the F_m value for the Sample Blank from each sample F_m reading and the F_c value for the Sample Blank from each sample F_c reading to obtain corrected measurements.

Using the corrected measurements, determine the percent cholesterol efflux present in the samples (C).

$$C = [F_m/(F_m + F_c)] \times 100\%$$

Where:
F_m = fluorescent intensity of supernatant
F_c = fluorescent intensity of cell lysate

Sample Calculation:

$F_m = 8.72$
$F_c = 2.06$
$F_m + F_c = 10.78$
$C = 8.72/10.78 \times 100\% = 80.9\%$

References

1. Glomset, J.A., and Wright, J.L., Some properties of a cholesterol esterifying enzyme in human plasma. Biochim. Biophys. Acta, **89**, 266–276 (1964).
2. Glomset, J.A., The plasma lecithins: cholesterol acyltransferase reaction. J. Lipid Res., **9**, 155–167 (1968).
3. Ono, K., Current concept of reverse cholesterol transport and novel strategy for atheroprotection. J. Cardiol., **60**, 339–343 (2012).

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