ab196985

Cholesterol Efflux Assay Kit (Cell-based)

Instructions for Use

A high-throughput screening assay for measuring cholesterol efflux in cells using fluorescently-labeled cholesterol.

This product is for research use only and is not intended for diagnostic use.
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1. **OVERVIEW**

Cholesterol Efflux Assay Kit (Cell-based) (ab196985) is a high-throughput screening assay for measuring cholesterol efflux in cells using fluorescently-labeled cholesterol. This assay provides a safe, sensitive and reproducible method for measuring cholesterol efflux. This product can be used to:

- Screen small molecules for their effect on cholesterol efflux as a part of drug discovery program.

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 extra hepatic cells, including macrophage-derived foam cells in arterial atherosclerotic plaque, transport excessive cholesterol back 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.
2. **ASSAY SUMMARY**

- **Label cells**
- **Treat cells**
- **Incubate**
- **Measure fluorescence (Ex/Em = 482/515 nm)**
3. **PRECAUTIONS**

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. **STORAGE AND STABILITY**

Store kit at -20ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**
5. MATERIALS SUPPLIED

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
<th>Storage Condition (After Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive Control</td>
<td>1 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Labeling Reagent</td>
<td>5 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Equilibration Buffer</td>
<td>5 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Cell Lysis Buffer</td>
<td>20 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Serum Treatment Reagent</td>
<td>1 mL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Reagent A</td>
<td>10 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
<tr>
<td>Reagent B</td>
<td>10 µL</td>
<td>-20°C</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- J774.1 Macrophage cell line
- RPMI 1640 media and Fetal Bovine Serum to grow cells
- Fluorescent microplate reader – equipped with a filter for Ex/Em = 482/515 nm
- 1X 96-well white plate with clear bottom and 1X 96-well white plate
- Pipettes and pipette tips
7. **LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.
8. **TECHNICAL HINTS**

- This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.

- Keep enzymes and heat labile components and samples on ice during the assay.

- Make sure all buffers and developing solutions are at room temperature before starting the experiment.

- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.

- Avoid foaming or bubbles when mixing or reconstituting components.

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.

- Ensure plates are properly sealed or covered during incubation steps.

- Ensure complete removal of all solutions and buffers from tubes or plates during wash steps.

- Make sure you have the appropriate type of plate for the detection method of choice.

- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.
9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.
- Open all the reagents under sterile conditions (e.g. cell culture hood).

9.1 **Labeling Reagent:**
Ready to use as supplied. Thaw Labeling Reagent on ice before adding to cells. Protect reagent from light.

9.2 **Positive Control:**
Ready to use as supplied. Thaw Positive Control on ice before adding to cells.

9.3 **Cell Lysis Buffer:**
Ready to use as supplied. Equilibrate to room temperature before adding to cells.

9.4 **Serum Treatment Reagent:**
Ready to use as supplied. Equilibrate to room temperature before adding to cells.

9.5 **Reagent A and Reagent B:**
Ready to use as supplied.

9.6 **Equilibration Media:**
Warm Equilibration Media to 37°C before use. Aliquot required amount of Equilibration Media for the experiment. Add Reagent A 2 μL/mL and Reagent B 2 μL/mL to the media just before use under sterile conditions.

9.7 **Serum Treatment Reagent:**
Ready to use as supplied. Warm to 37°C before use.
Note: Using Serum Treatment Reagent: 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.
10. ASSAY PROCEDURE

- The procedure described below is for macrophage cell line J774.1. This procedure can also be used with macrophage cells derived from THP-1 monocytes, by adding of 100 nM phorbol 12-myristate 13-acetate (PMA) for 48 hours.
- It is recommended to assay all treatments and controls in triplicate.

10.1 Label cells:

10.1.1 Grow J774.1 cells in RPMI 1640 media containing 10% FBS in cell culture flask till ~90% confluence (37°C incubator containing 5% CO₂).

10.1.2 Split cells under sterile conditions using basic cell culture techniques and plate hours approximately 1 x 10⁵ J774.1 cells/well in a 96 well plate (white plate with clear bottom) using 100 µL media/well.

10.1.3 Grow for 2 hours in a 37°C incubator containing 5% CO₂.

10.1.4 After 2 hours, when the cells are attached to the plates, wash the cell monolayer with RPMI 1640 media (no serum added).

10.1.5 Premix 50 µL of Labeling Reagent and 50 µL of Equilibration Buffer (Section 9.6) per well just before use. Prepare a Master Mix of Labeling + Equilibration buffer mix to ensure consistency.

10.1.6 Add 100 µL Labeling + Equilibration buffer mix/well to cells.

10.1.7 (OPTIONAL) Background control wells: add 100 µL Equilibration buffer mix (Section 9.6) per well to cells. **NOTE:** to test background fluorescence.

10.1.8 Incubate the plate overnight (16 hours) protected from light at 37°C in a humidified incubator with a 5% CO₂ atmosphere.

10.2 Treat Cells:

10.2.1 After overnight incubation, remove the Labeling Reagent.
10.2.2 Wash the cells gently by adding 200 µL of RPMI media (no serum) to all the wells. Remove the media.

10.2.3 Treat cells with desired cholesterol acceptors in RPMI media.

10.3 **Positive Control:**

For Positive Control, add 20 µL of Positive Control and make up the volume to 100 µL by RPMI media.

10.4 **No Treatment Control:**

10.4.1 For No Treatment Control, add 100 µL RPMI media (no serum) to no treatment control wells.

10.4.2 Incubate cells for 4-6 hours in a 37°C incubator containing 5% CO$_2$.

10.5 **Measurement:**

10.5.1 At the end of incubation, transfer supernatant to a 96-well plate (white plate).

10.5.2 Measure the fluorescence (Ex/Em = 482/515 nm).

10.5.3 Solubilize the cell monolayer by adding 100 µL of Cell Lysis Buffer and shaking on a plate shaker for 30 minutes at room temperature. Pipette up and down to dissolve any cell debris.

10.5.4 Measure the fluorescence (Ex/Em = 482/515 nm).
11. CALCULATIONS

- For statistical reasons, we recommend each sample should be assayed with a minimum of three replicates (triplicates).

Cholesterol efflux of the treatments is calculated by dividing the fluorescence intensity of the media by total fluorescence intensity of the cell lysate of the same treatment and media. This value is multiplied by 100 to obtain % Cholesterol Efflux. Subtract % Cholesterol Efflux obtained from no treatment control from the treatment groups to determine the final % Cholesterol Efflux.

\[
\% \text{ Cholesterol Efflux} = \frac{\text{Fluorescence Intensity of Media}}{\text{Fluorescence Intensity of Cell Lysate + Media}} \times 100
\]
12. TYPICAL DATA

Figure 1: Percentage (%) Cholesterol Efflux: J774.1 cells were labeled with the Labeling Media and treated with various cholesterol acceptors like Human Serum, HDL (50 µg) or Positive control known to cause cholesterol efflux. Cholesterol efflux is expressed as % efflux elicited by cells in 4 hours.
13. **QUICK ASSAY PROCEDURE**

**NOTE:** This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Thaw Labeling reagent and positive control, prepare Equilibration Media (add Reagent A + Reagent B); get equipment ready.
- Seed J774.1 cells in RPMI 1640 + 10% FBS at $1 \times 10^5$ cells/well in 100 µL media/well and incubate for 2 hours.
- Wash cells with RPMI 1640 media (no FBS).
- Prepare master mix of Labeling Reagent + Equilibration Buffer mix (50 µL/well + 50 µL/well) (Number samples + 1).
- Add 100 µL/well Labeling Reagent + Equilibration Buffer mix to cells. **OPTIONAL:** background control – 100 µL Equilibration buffer.
- Incubate cells 16 hours (overnight) at 37°C.
- Wash cells with RPMI (no FBS) and remove media.
- Treat cells with desired cholesterol acceptors in RPMI media.
- Prepare Positive Control and No Treatment Control.
- Incubate cells 4 – 6 hours at 37°C.
- Transfer **supernatant** to a new 96-well white plate and measure fluorescence (Ex/Em = 482/515 nm).
- Solubilize cell monolayer in 100 µL Cell Lysis Buffer and measure fluorescence (Ex/Em = 482/515 nm).
## 14. TROUBLESHOOTING

<table>
<thead>
<tr>
<th>Problem</th>
<th>Cause</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay not working</td>
<td>Use of ice-cold buffer</td>
<td>Buffers must be at room temperature</td>
</tr>
<tr>
<td></td>
<td>Plate read at incorrect wavelength</td>
<td>Check the wavelength and filter settings of instrument</td>
</tr>
<tr>
<td></td>
<td>Use of a different 96-well plate</td>
<td>Specifically for this assay: 96-well white plate with clear bottom + 96-well white plate</td>
</tr>
<tr>
<td>Sample with erratic readings</td>
<td>Samples used after multiple free/ thaw cycles</td>
<td>Aliquot and freeze samples if needed to use multiple times</td>
</tr>
<tr>
<td></td>
<td>Use of old or inappropriately stored samples</td>
<td>Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use</td>
</tr>
<tr>
<td></td>
<td>Presence of interfering substance in the sample</td>
<td>Check protocol for interfering substances; deproteinize samples</td>
</tr>
<tr>
<td></td>
<td>Incorrect incubation times or temperatures</td>
<td>Verify correct incubation times and temperatures in protocol</td>
</tr>
<tr>
<td></td>
<td>Improperly thawed components</td>
<td>Thaw all components completely and mix gently before use</td>
</tr>
<tr>
<td>Unanticipated results</td>
<td>Measured at incorrect wavelength</td>
<td>Check equipment and filter setting</td>
</tr>
<tr>
<td></td>
<td>Samples contain interfering substances</td>
<td>Troubleshoot if it interferes with the kit</td>
</tr>
<tr>
<td></td>
<td>Overgrowth of cells</td>
<td>Ensure to plate cells at specified density before starting treatment</td>
</tr>
</tbody>
</table>
15. FAQ
16. INTERFERENCES
17. NOTES