

Video Article

High-throughput Nitrobenzoxadiazole-labeled Cholesterol Efflux Assay

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Abstract

Atherosclerosis leads to cardiovascular disease (CVD). It is still unclear whether cholesterol-HDL (cHDL) concentration plays a causal role in atherosclerosis development. However, an important factor in early stages of atheroma plaque formation is cholesterol efflux capacity to HDL (the ability of HDL particles to accept cholesterol from macrophages) in order to avoid foam cell formation. This is a key step in avoiding the accumulation of cholesterol in the endothelium and a part of reverse cholesterol transport (RCT) to eliminate cholesterol through the liver. Cholesterol efflux capacity to serum or plasma in macrophage cell models is a promising tool that can be used as biomarker for atherosclerosis. Traditionally, [³H]-cholesterol has been used in cholesterol efflux assays. In this study, we aim to develop a safer and faster strategy using fluorescent labelled-cholesterol (NBD-cholesterol) in a cellular assay to trace the cholesterol uptake and efflux process in THP-1-derived macrophages. Finally, we optimize and standardize the NBD-cholesterol efflux method and develop a high-throughput analysis using 96-well plates.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58891/>

Introduction

According to the World Health Organization, the current principal causes of death worldwide are ischemic heart disease and stroke (accounting for a total of 15.2 million deaths)¹. Both are cardiovascular diseases (CVD) that can be preceded by atherosclerosis and the rupture of atheroma plaques in the blood vessels^{2,3}.

Atherosclerosis is a vessel wall inflammatory disease in which macrophages, T cells, mast cells, and dendritic cells infiltrate the endothelium and accumulate from the blood, eventually forming atherosclerotic plaques. Atherosclerotic plaques present a lipid core and cholesterol crystals, evidenced by high-resolution B-mode ultrasonography measurements of the carotid intima media thickness^{4,5}. In macrophages, cholesterol efflux towards lipid acceptor particles is carried out by means of the ATP-binding cassette (ABC) receptors ABCA1, ATP binding cassette subfamily G member 1 (ABCG1), and the scavenger receptor SR-BI. The imbalance of cholesterol influx and efflux in macrophages is considered a key process in atherosclerosis initiation⁶. Cholesterol efflux is considered a key step in cholesterol elimination from peripheral tissue to the plasma and liver in a process called reverse cholesterol transport (RCT). Cholesterol is transferred from macrophages mainly to apolipoprotein A1 (ApoA1) found on the surface of high-density lipoprotein (HDL) particles. HDLs then transport cholesterol to the liver for excretion and re-utilization^{7,8,9}.

Traditionally, tritium (³H) radio-labelled cholesterol has been used in cholesterol efflux¹⁰. The emission signal of radioisotopes is highly sensitive¹⁰; however, radio-labelled cholesterol presents obvious handicaps such as long protocols, risk of exposure to ionizing radiation, and the need for special radioactivity facilities and equipment to ensure safe handling of radioactive emission. On the contrary, fluorescence has been successfully incorporated in diagnostic techniques due to its simplicity in fluorescent signal detection, the wide variety of fluorophores available, and its safety¹¹. Several fluorescent-labelled sterols have been used to study cholesterol metabolism including dehydroergosterol (with intrinsic fluorescence), dansyl cholesterol, 4,4-difluoro-3a,4adiaza-s-indacene (BODIPY)-cholesterol, and 22-(N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino)-23,24-Bisnor-5-Cholen-3β-OI (NBD-cholesterol). Particularly, NBD-cholesterol presents an efficient uptake in human cells¹². Two different NBD labelled-cholesterol are currently available: 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3b-ol (22-NBD) and 25-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl)amino)-27-norcholesterol (25-NBD; **Figure 1**). Cholesterol labelled with 22-NBD moiety may best suit cholesterol efflux studies, while 25-NBD-cholesterol is mainly used in cellular membrane dynamics research^{13,14}.

Cell lines typically used in in vitro cholesterol efflux assays are monocyte-like cells such as human leukemia-derived THP-1 cells, murine Raw 264.7 cells¹⁵, or J774.1. All of these cells can be differentiated into macrophages in vitro using phorbol 12-myristate 13-acetate (PMA), but THP-1-derived macrophages (dmTHP-1) best reflect and mimic the human macrophages¹⁶.

In the present study, we optimize and standardize a fluorescent high-throughput method to determine the cholesterol efflux capacity of serum samples on dmTHP-1, using 22-NBD-cholesterol as an alternative to [3 H]-cholesterol. In addition, we compare the optimized fluorescent technique with the standard radioactive analog.

Protocol

For this study, ethical committee approval (Comitè Ètic d'Investigació Clínica, Hospital Clinic, Barcelona; approval number HCB/2014/0756) and written, informed consent from all subjects were obtained.

1. NBD-cholesterol Preparation

1. Dissolve the NBD-cholesterol (MW 494.63; see **Table of Materials**) in pure ethanol to obtain the stock (2 mM). For a 10 mg vial, dissolve the entire vial contents in a 10.1 mL volume of ethanol to obtain a 2 mM stock.
2. Dilute the NBD-cholesterol from the stock in RPMI 1640 supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin (R10 medium) to reach a final concentration of 5 μ M (e.g., obtain 10 mL of NBD-cholesterol at 5 μ M final concentration) by diluting 25 μ L of NBD-cholesterol stock (2 mM) in R10 medium. This volume is sufficient for a 96-well plate.

2. Cell Culture and Seeding (Day-2)

1. Culture THP-1 cells in R10 medium at 37 °C, 5% CO₂. Adjust to 0.3 x 10⁶ cells/mL every 3 days.
2. Seed the cells in a white 96-well plate with a flat, clear bottom at 0.2 x 10⁶ cells/well in R10 medium (100 μ L per well).
3. Treat the cells with 100 nM phorbol 12-myristate 13-acetate (PMA; from a 10 μ M stock) for 48–72 h, incubating at 37 °C, 5% CO₂ to differentiate THP-1 cells into dmTHP-1.
NOTE: It is recommended to use 5 μ L of PMA stock (10 μ M) in 500 μ L of R10 medium. Prior to this, prepare a 10 μ M PMA stock by dissolving a lyophilized 1 g vial in 10 mL of dimethyl sulfoxide (DMSO), aliquot, and stock at -20 °C.
4. Alternatively (suggestion) combine steps 2.2 and 2.3 for better homogenization. Prepare 10 mL of THP-1 cells in a 15 mL tube, add 200 μ L of PMA stock (10 μ M), mix gently, and immediately seed 100 μ L per well onto the plate. Incubate the cells as described in step 2.3.

3. Apolipoprotein B Depleted Serum (ABDS) Preparation (Day 2 or 3)

1. To prepare a polyethylene glycol (PEG) solution, dilute glycine in 10% PBS (with sterile H₂O) to a concentration of 200 mM at pH 7.4. Add 40 mL of 200 mM glycine to 10 g of PEG 8000 to obtain PEG 20% (w/v). Mix the solution vigorously to homogenize.
2. Apply 4 parts of 20% PEG per 10 parts of serum/plasma on each serum/plasma sample in a 1.5 mL tube and leave the mixture on ice for 25 min¹⁷ (e.g., for 100 μ L of plasma or serum, add 40 μ L of 20% PEG solution).
3. Centrifuge the PEG-apolipoprotein B precipitate at 13,000 x g for 15 min at 4 °C.
4. Discard the precipitate. Transfer the supernatant to a new tube.
NOTE: We suggest preparing the ABDS on day 3 (fresh). If it is not possible, prepare the ABDS on day 2 and keep it at 4 °C overnight.

4. NBD-cholesterol Cell Loading (Day 2)

1. Discard the culture medium of the dmTHP-1 and wash the cells twice with 1x phosphate-buffered saline (PBS).
2. Load the cells with 5 μ M NBD-cholesterol (100 μ L per well) in R10 medium and incubate overnight at 37 °C, 5% CO₂.

5. Incubation with Cholesterol Acceptors (Day 3)

1. Discard the medium and wash the cells twice with PBS.
2. Incubate the cells with 2–5% ABDS or the desired concentration of purified lipid acceptor (HDL, ApoA, ApoE, etc.) diluted in colorless RPMI 1640 medium (100 μ L per well) for 4–6 h at 37 °C.
NOTE: Include a negative control (C-) consisting of colorless RPMI 1640 medium without acceptors and a positive control (C+) (e.g., ABDS from a pool of healthy donors or purified HDLs). Note that the positive control applies particularly when patient samples (disease conditions) are analyzed (**Supplementary Figure 1**).
3. Prepare a 200 mL stock of the cell lysis solution 1 (50 mM Tris buffer, 150 mM NaCl, H₂O). Mix the cell lysis solution 1 at 1:1 (v:v) ratio with pure ethanol to obtain the lysis solution 2.

6. Fluorescent Signal Capture (Day 3)

1. **Media NBD-cholesterol detection**
 1. Remove the cell medium from the plates and collect it in a new white 96-well plate with an opaque flat bottom.
 2. For an optimal fluorescence signal detection in the media samples, add 100 μ L of pure ethanol to 100 μ L of each medium sample to obtain a 1:1 ratio in the 96-well white plate.
 3. Keep the plate with the treated media to further measure the fluorescence intensity (FI) at a 463/536 nm (excitation/emission) wavelength in the luminometer.
2. **Intracellular NBD-cholesterol detection**
 1. Wash the cells twice with PBS.

2. To obtain the intracellular cholesterol, lyse the cells by incubating them with 100 μ L of lysis solution 2 per well and shake the plate at room temperature (RT) for 25 min.
3. For an optimal fluorescence signal detection in the cell lysate samples, capture the fluorescence intensity of the cell lysates in the same white 96-well plate with clear flat bottom (the same plate in which cells were seeded in step 2.2).
3. 6.3 Measure the fluorescence intensity (FI) in the luminometer while adjusting the sensitivity parameter to 50 in the software (see **Table of Materials**).

7. Results Analysis

1. Express the cholesterol efflux rate of a sample as a percentage calculated by the formula:

$$\text{CE sample} = \frac{\text{Medium fluorescence}}{\text{Medium + Cell lysate fluorescence}} \times 100$$

2. Obtain the final measure of cholesterol efflux (CE) by subtracting the CE of the negative control (sample loaded with NBD-cholesterol but incubated with colorless RPMI 1640 medium, without cholesterol acceptors) from the CE of a given samples:

$$\text{CE} = \text{CE sample} - \text{CE neg. control sample}$$

Representative Results

The aim of the cholesterol efflux assay is to determine in vitro the cholesterol efflux capacity of a given serum, plasma, or supernatant containing HDL particles. The method consists of loading labelled-cholesterol into a culture of a standard macrophage cell line and inducing contact with the testing sample diluted into FBS-free media with the cells. Finally, the fluorescent levels from the NBD are measured in the media and cell lysate. To optimize measurements, the effluxed cholesterol from the cells to media is mixed with one volume of a solvent containing ethanol. The NBD-cholesterol remaining in the cells is resuspended into a solvent containing ethanol or detergent before measurements. Finally, the ratio of effluxed/total labelled-cholesterol is determined. To set up the technique, apolipoprotein B-depleted serum (ABDS) from a pool of healthy donors was used.

The best dilution conditions for the NBD-cholesterol in both medium and ethanol were investigated. We tested the fluorescent behavior of free NBD-cholesterol in several solvent environments, including different ratios of ethanol and colorless RPMI 1640. The fluorescence intensity (FI) of NBD-cholesterol diluted in the aqueous solution showed the lowest FI values, while NBD-cholesterol within pure ethanol emitted the highest FI. This suggests that the fluorescence emission of the 22-NBD-cholesterol molecule is highly dependent on the medium in which it is contained. Accordingly, the fluorescence signal of the 22-NBD-cholesterol is significantly higher when placed in an ethanol containing media. We observed that at a ratio of 1:1 media:ethanol, the fluorescence intensity signal increases proportionally with the concentration of the cholesterol analog in the range of 0.5–50 μ M, suggesting an appropriate behavior of the probe in this case (**Figure 2**). The linear behavior in the 1:1 condition (media:ethanol) is represented by the follow equation: $y = 20.88x + 146.7$ with $R^2 = 0.9341$.

A key step in this method is the time spent incubating the loaded cells with cholesterol acceptors so the cholesterol is able to efflux. In order to determine the required incubation time, cell media was harvested at different timepoints (1 to 24 h; **Figure 3**). The cholesterol efflux in the range of 0 to 6 h evolved linearly ($p < 0.0001$; $y = 18.2x + 127.3$) in a time-dependent manner. The maximum signal captured was at 6 h after ABDS was added to cells. After 6 h, the cholesterol lost regularity in the efflux, so variability increased after 6 h of incubation with the ABDS (**Figure 3**).

We additionally tested the saturation threshold and dynamic range as applied to human ABDS by measuring CE at different percentages of HDL-containing media (ABDS). The NBD-cholesterol efflux in the high throughput assay (96-well plate) evolved linearly within 1–7% ABDS, where FI increased in a concentration-dependent manner with the ABDS, reaching the peak of cholesterol efflux capacity at 7% ABDS (**Figure 4**). At concentrations higher than 7%, FI decreased in an inverse relationship with the ABDS percentage. This may have occurred because the cholesterol was reentering the cells from the NBD-cholesterol loaded HDL present in the media.

The standard method to measure cholesterol efflux uses radio-labeled (^3H) cholesterol¹⁶. To evaluate the performance of our fluorescence-based method, we performed and compared both fluorescent and radio-labeled techniques. We found that the traditional radioactivity technique and our NBD-cholesterol method were highly correlated (Pearson's $r = 0.97$; $p < 0.001$) using different concentrations of ABDS (1–8%; **Figure 5**). Overall, this suggests that our fluorescent method may be a safer substitute than the radioactive technique.

Finally, we compared our method to fluorescent probe different from NBD. We compared our method to a commercial high-throughput cell-based assay kit aimed to determine the cholesterol efflux in cells (cholesterol efflux assay kit; cell-based). The method developed in our group was sensitive to an increase of acceptor concentration (% ABDS) within CE values between 5 and 15%. However, the commercial kit, performed following the manufacturer's instructions, resulted in CE measures below 5% along the range of ABDS assayed; thus, the resulting CE was essentially unaffected when ABDS was increased (**Figure 6**).

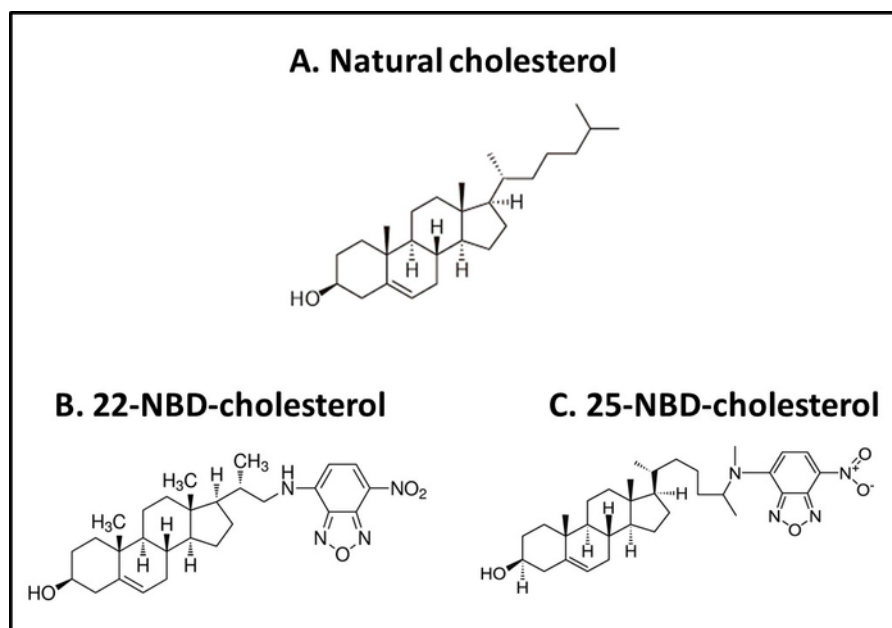


Figure 1: Molecular structures of natural cholesterol (A), 22-NBD-cholesterol (B), and 25-NBD-cholesterol (C). [Please click here to view a larger version of this figure.](#)

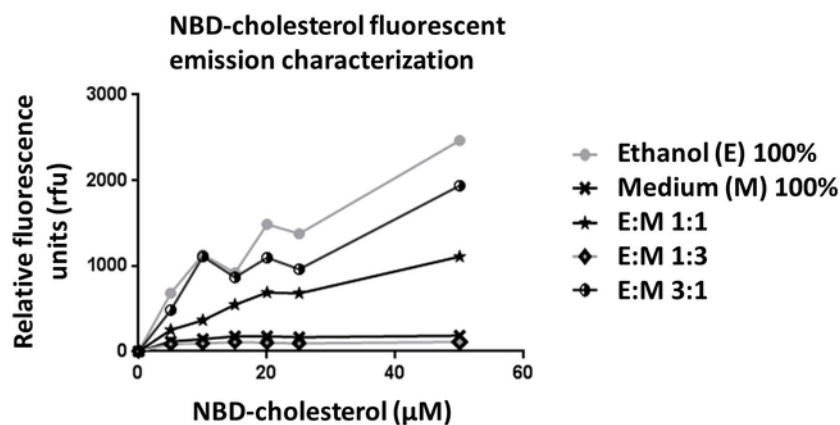


Figure 2: NBD-cholesterol fluorescence intensity (FI) with different ratios of ethanol and colorless RPMI 1640 medium. NBD-cholesterol was diluted in 1:0, 3:1, 1:1, 1:3, and 0:1 ethanol:aqueous medium ratios at NBD-cholesterol concentrations between 0.5 and 50 μM. FI signal was detected by the luminometer with the sensitivity parameter set at 70 in the fluorimeter software. The FI signal of media and cell lysate was measured using white 96-well plates. [Please click here to view a larger version of this figure.](#)

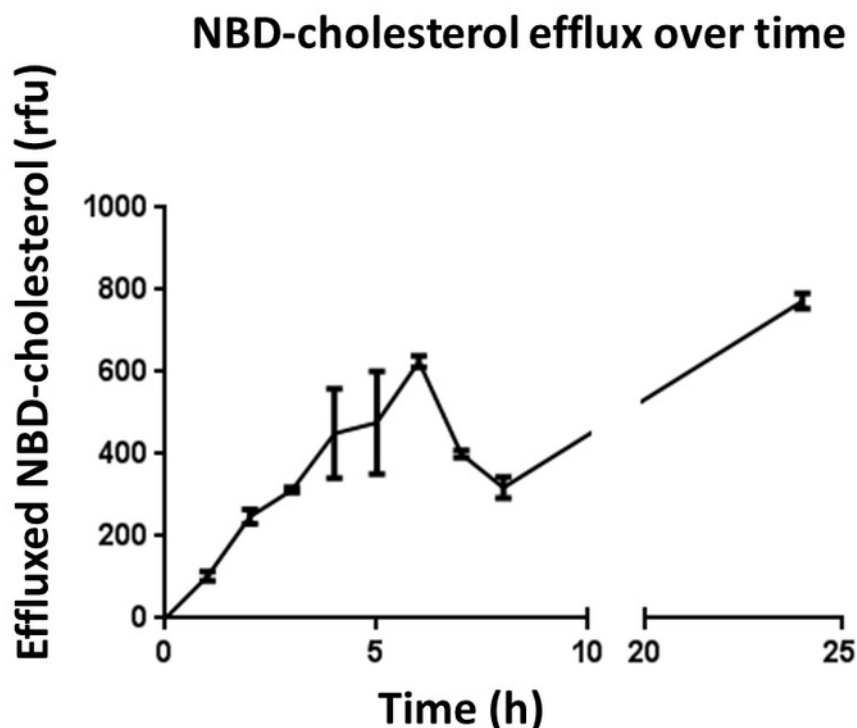


Figure 3: Time-progression NBD-cholesterol efflux in THP-1-derived macrophages. The dmTHP-1 were loaded with 5 μ M NBD-cholesterol and incubated with 5% ABDS. Cell media were measured in a white 96-well plate at different timepoints (1–24 h). The values are presented as the mean $\pm$ standard deviation of quadruplicate wells. FI signal was detected by the luminometer with the sensitivity parameter set at 70 in the fluorimeter software. [Please click here to view a larger version of this figure.](#)

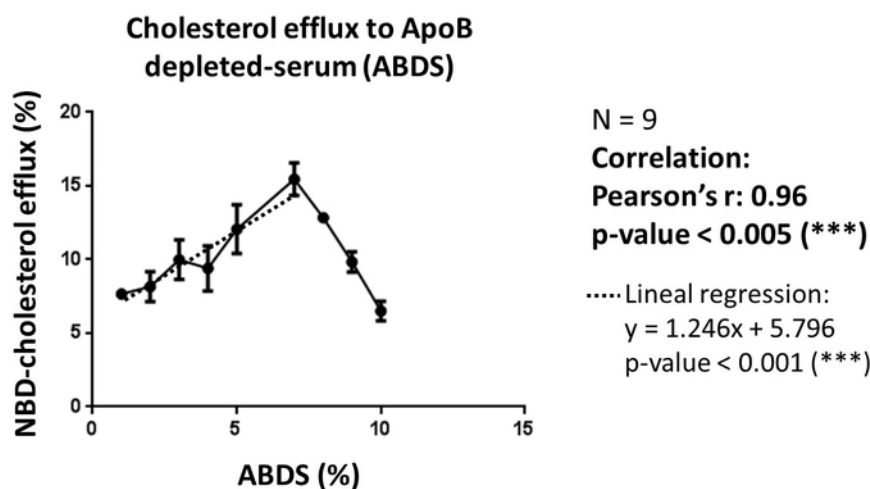


Figure 4: NBD-cholesterol efflux in THP-1-derived macrophages in 96-well plate with different concentrations of ABDS. The dmTHP-1 cells were treated with 5 μ M NBD-cholesterol, incubated with different percentages of ABDS, and lysed with ethanol. The FI signal of media and cell lysate was measured using a white 96-well plate in the fluorimeter at 1:1 (ethanol:lysis solution 1). The measures of negative controls (ABDS untreated media) were subtracted at the levels provided. The values are presented as the mean $\pm$ standard deviation of triplicate wells. FI signal was detected by the luminometer with the sensitivity parameter set at 50 in the fluorimeter software. [Please click here to view a larger version of this figure.](#)

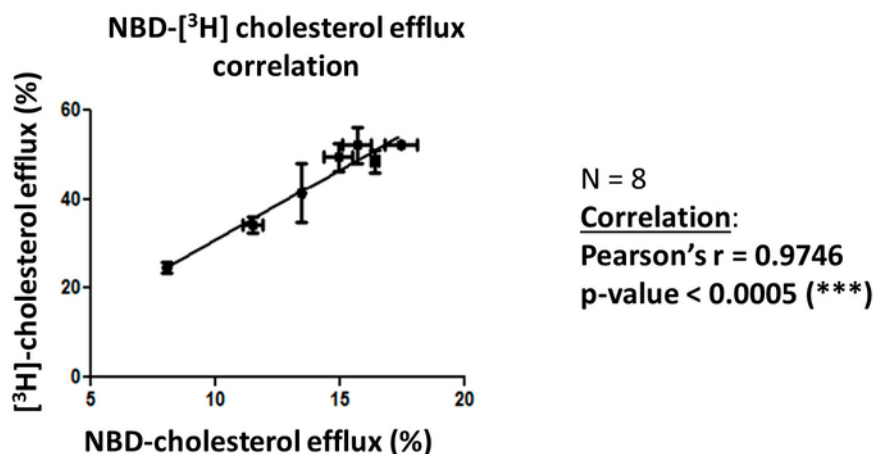


Figure 5: Correlation of NBD-cholesterol and [³H]-cholesterol efflux in THP-1-derived macrophages. Following dmTHP-1 with the corresponding labelled-cholesterol for 6 h. The cholesterol efflux was measured using 1-8% ABDS. For NBD-cholesterol samples, the fluorescent signal was detected using white 96-well plates in the fluorimeter at 1:1 ethanol:lysis solution 1. FI signal was detected by the luminometer with the sensitivity parameter set at 50 in the fluorimeter software. For [³H]-cholesterol samples, the radioactive signal was detected using 100 μ L of medium and cell lysate mixed with the scintillation cocktail and red in the scintillation counter, following the protocol described by Low et al.¹⁷. Correlation efficiency was determined using Pearson's r correlation coefficient. The measures of negative controls (ABDS untreated media) were subtracted from both fluorescence and radioactive levels provided. The values are presented as the mean $\pm$ standard deviation of triplicate wells. [Please click here to view a larger version of this figure.](#)

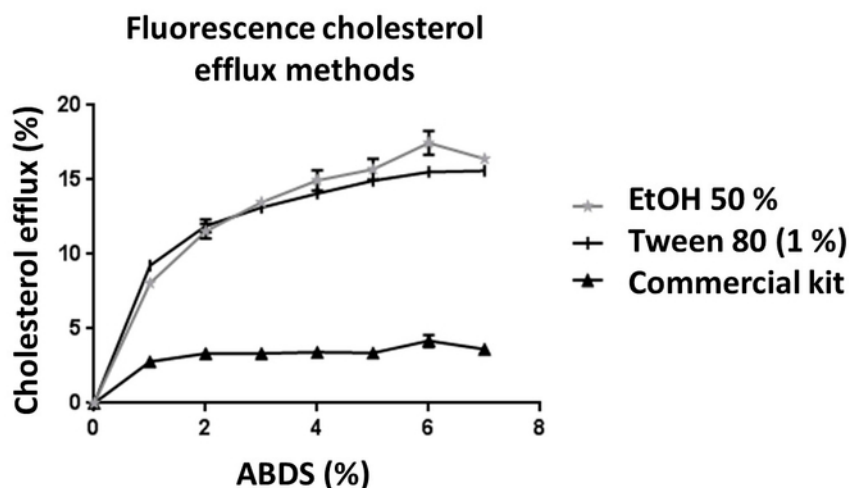


Figure 6: Comparison between high-throughput NBD-cholesterol efflux assay and a commercialized fluorescent cell-based assay kit. Cholesterol efflux from ABDS was assessed following the described protocol, using as lysis solvent ethanol (50%) or Tween 80 (1%). The fluorescent cell-based assay kit was assessed according to the manufacturer's protocol in the kit. The values are presented as the mean $\pm$ standard deviation of triplicate wells. FI signal was detected by the luminometer with the sensitivity parameter set at 50 in the fluorimeter software. [Please click here to view a larger version of this figure.](#)

WORKFLOW:

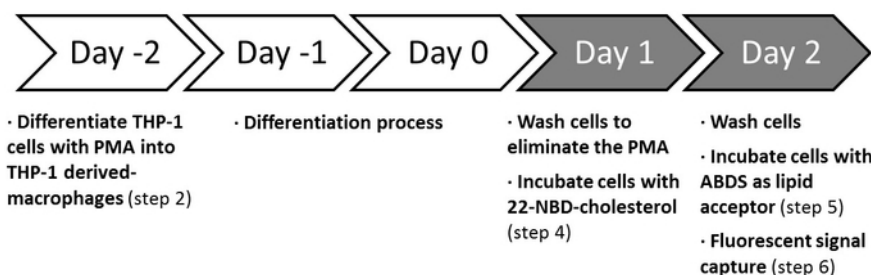
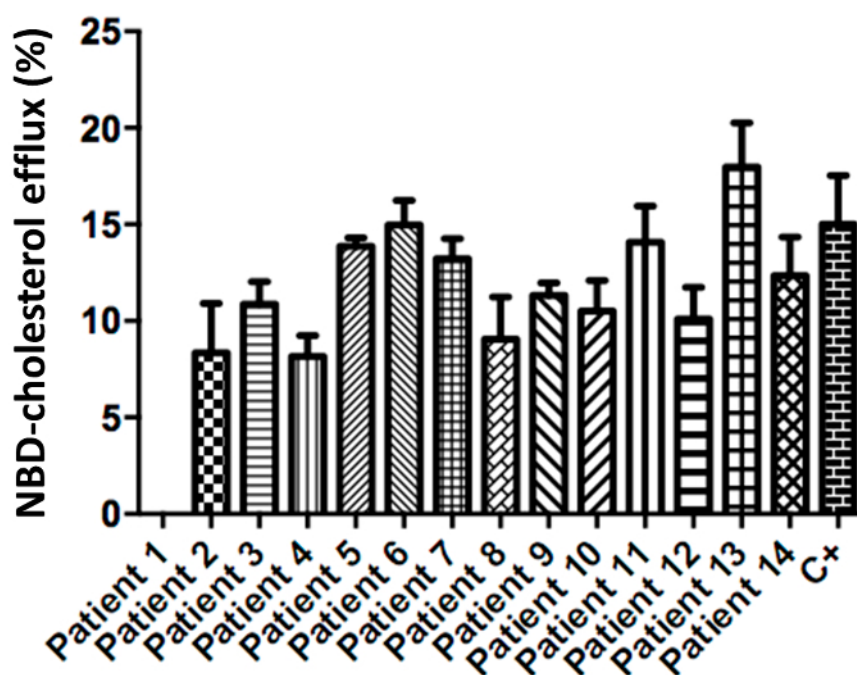


Figure 7: Workflow diagram. [Please click here to view a larger version of this figure.](#)

Cholesterol efflux of HIV-infected patients sera samples



Supplementary Figure 1: NBD-cholesterol efflux of serum samples from HIV-infected patients and a standard positive control (C+). The inter-individual variation of the NBD-cholesterol efflux method applied to a set of HIV-infected patients is shown. A positive internal control (C+) represents the NBD-cholesterol efflux from a pool of sera samples of 8 healthy patients. Error bars show standard deviation. [Please click here to view a larger version of this figure.](#)

Discussion

Fluorescent-labelled cholesterol is a promising strategy to analyze and investigate the properties and metabolism of natural cholesterol *in vitro*. Its main advantages are that it can be taken up by cells, allows for intracellular and membrane distribution studies, and can be applied to cholesterol efflux assays such as in this protocol (**Figure 7**). Some fluorescent-labelled sterols allow cholesterol tracking *in vitro* including BODIPY-cholesterol, dansyl-cholesterol, dehydroergosterol, and 22- and 25-NBD-cholesterol¹². Particularly, 22-NBD-cholesterol is appropriate for studying cholesterol dynamics with different cell types and as a substitute of radio-labeled cholesterol¹⁸. There is no consensus method to evaluate the fluorescent signal in a cholesterol efflux assay. Song et al.¹⁴ harvested medium and cell lysates to measure FI separately; Liu et al.¹⁴ transferred the medium to other plates, and the intracellular fluorescent signal was measured directly in the culture plate; and Zhang et al.¹⁵ used a lysis buffer to lyse the cells, followed by purification of the fluorescent cholesterol to dilute it in pure ethanol and detect the fluorescence intensity signal¹⁹. In our cholesterol efflux assay, use of the same final solvent composition in media and cells permitted the homogenization of the cell media and measurements of lysate efficiently.

The present study profiled the NBD-cholesterol efflux over time and found a time-dependent progression until 6 h of incubation with the acceptor ABDS, similar to Song et al.¹⁴ and Liu et al.¹⁴ and unlike Zhang et al. who used a 1 h incubation period with the lipid acceptors¹⁹. We found that after 6 h of incubation, the effluxed cholesterol lost linearity; therefore, it is paramount not to exceed 6 h. Our optimal range of cholesterol efflux was between 3 to 6 h after adding the acceptors. We suggest incubating cells for 4 h with the cholesterol acceptors. Additional critical steps are use of the colorless media to apply the acceptors in order to avoid background, seeding a semi-confluent cell culture layer, and ensuring a correct adherence of those to the plate. It should be noted that the clear bottom of the white plate is useful to follow the cells under an inverse light microscope.

Most cholesterol efflux assays are carried out using purified lipid acceptors in the cholesterol efflux test. The present technique was developed to obtain the cholesterol efflux capacity from human serum or plasma samples. We use the intrinsic cholesterol acceptors present in the serum, but it is critical to remove the particles containing apolipoprotein B, which would recirculate the cholesterol molecules inside cells^{20,21}. As modifications, purified ApoA1 and HDL can also be used as acceptors. Also, cell lines other than human THP-1 have been successfully used in fluorescent cholesterol efflux assays, such as Raw 264.7 or J774A1 macrophages and would thus likely work in our method^{15,22}.

The main limitations of this method is that results depend on the specific cell line, a standard cell line but cell-free method would be advantageous to use as a biomarker. Also, the sera used to standardize this method was frozen; the NBD moiety on cholesterol is less physiological than the radio-labeled one, and its uptake differs from the endogenous cholesterol; and we tested the final outcome of a set of processes (i.e., involving serum particles, a cell line, possible esterification of cholesterol, possible diffusion of cholesterol or simultaneous efflux

and influx processes). However, as an advantage, fluorescence-based techniques have shown to have high potential as substitutes for traditional radio-labelled techniques and in the substitution of [^3H]-cholesterol, by a fluorescent-labelled molecule to monitor cholesterol efflux assays¹⁴.

Cholesterol efflux may act as biomarker of atherosclerosis²³, as it correlates with future cardiovascular events^{24,25}. A possibility to study the role of macrophages in atherosclerosis is through purified HDLs and its composition; however, HDLs purification is an arduous and time-consuming process. Besides, during the purification, other serum components with atherogenic effect may be underestimated or lost. For that reason, ABDS was chosen as the lipid acceptor. The high-throughput scale and time reduction were made possible by substituting the scintillation counter for a plate reader, measuring the fluorescent signal in the same culture plate (in the case of the cell lysate), and reducing the protocol by two days. Moreover, this technique shows higher sensitivity when cells are exposed to different concentrations of serum than the measures obtained from a commercialized kit to assess cholesterol efflux.

In conclusion, a NBD-cholesterol efflux assay that correlates with the traditional radioactive method has been described. We determined the optimal time to incubate lipid acceptors from human samples (serum or plasma) in the form of ABDS. We optimized the cell seeding, differentiation, dynamic range, and saturation point of the technique. As for its main novelty, we optimized a solvent combination used in measurements for obtaining high intensities and an improved linear range.

Disclosures

The authors wish to declare that they are inventors of the patent application (EPO; 18382337.6-1118; 17th May 2018) entitled "Method for determining cholesterol efflux" based on this method.

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