

# Journal of Visualized Experiments

## High-throughput Nitrobenzoxadiazole-labeled Cholesterol Efflux Assay

--Manuscript Draft--

<b>Article Type:</b>	Methods Article - JoVE Produced Video
<b>Manuscript Number:</b>	JoVE58891R2
<b>Full Title:</b>	High-throughput Nitrobenzoxadiazole-labeled Cholesterol Efflux Assay
<b>Keywords:</b>	Cholesterol, HDL; ATP Binding Cassette Transporter 1; Monocyte-Derived Macrophages; 22-NBD-Cholesterol; Fluorescent Probes; Cardiovascular Diagnostic Technique; atherosclerosis; Coronary Artery Disease; Nitrobenzoxadiazole
<b>Corresponding Author:</b>	Olivia Tort, Ph.D. IDIBAPS Barcelona, Catalunya SPAIN
<b>Corresponding Author's Institution:</b>	IDIBAPS
<b>Corresponding Author E-Mail:</b>	otort@clinic.cat;otr972@gmail.com
<b>Order of Authors:</b>	Roque Pastor-Ibáñez Mireia Arnedo Olivia Tort, Ph.D.
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Barcelona, Barcelona, Spain

Barcelona, 17<sup>th</sup> September 2018

Dear Dr. DSouza,

Please find enclosed the revised manuscript by Pastor-Ibáñez et al., with reference JoVE58891 entitled "High-throughput NBD-cholesterol Efflux Assay", which we would like to publish in Journal of Visualized Experiments (JoVE).

We thank the reviewers for their valuable time and useful contribution and the editor for her advice to improve the manuscript. We highly appreciate all the inputs, which will definitely help improve the manuscript.

Overall, we have improved the protocol by adding examples and clarifying some confusions and mistakes noticed by the reviewers. We have also taken the opportunity to improve the discussion according to the editor guidelines. We have added two modifications regarding the order of the authors and the disclosure. Finally, we have attached a document containing point-by-point responses to the reviewers' comments and the concerns from the editor.

All authors concur with the submission of the revision of this work.

Thank you very much for considering our improved manuscript,

Sincerely yours,

Olivia

---

**Olivia Tort Regàs, PhD**

Laboratory of Retrovirology and Viral  
Immunopathogenesis, IDIBAPS

✉ CELLEX-IDIBAPS, 4th-A, office A4  
Casanova, 143 08036 – Barcelona, SPAIN

☎ Tel. 93.227.54.00 (Ext. 2244)

📧 [otort@clinic.ub.es](mailto:otort@clinic.ub.es)



**RETIC-RIS** | Red Española de  
Investigación en SIDA (RIS)



**Generalitat  
de Catalunya**



**Unió Europea**  
Fons Europeu de Desenvolupament Regional  
Una manera de fer Europa

**TITLE:**

High-Throughput Nitrobenzoxadiazole-labeled Cholesterol Efflux Assay

**AUTHORS AND AFFILIATIONS:**

Roque Pastor-Ibáñez, Mireia Arnedo, Olivia Tort

Group of Genomics and Pharmacogenomics in HIV, Laboratory of Retrovirology and Viral Immunopathogenesis, IDIBAPS, Hospital Clínic de Barcelona, Barcelona, Spain

**Corresponding Author:**

Olivia Tort

otort@clinic.cat

Tel: +34 93 227 5400 (ext. 2244)

**Email Addresses of Co-Authors:**

Roque Pastor (rpastor@clinic.cat)

Mireia Arnedo (marnedo@clinic.cat)

**KEYWORDS:**

NBD-cholesterol, macrophage, HDL, cholesterol efflux, fluorescence, apolipoprotein B-depleted serum, atherosclerosis, reverse cholesterol transport

**SUMMARY:**

Measurement of *in vitro* cholesterol efflux capacity to serum or plasma in macrophage cell models is a promising tool as a biomarker for atherosclerosis. In the present study, we optimize and standardize a fluorescent NBD-cholesterol efflux method and develop a high-throughput analysis using 96-well plates.

**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, [<sup>3</sup>H]-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.

**INTRODUCTION:**

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

48  
49 Atherosclerosis is a vessel wall inflammatory disease in which macrophages, T cells, mast cells,  
50 and dendritic cells infiltrate the endothelium and accumulate from the blood, eventually forming  
51 atherosclerotic plaques. Atherosclerotic plaques present a lipid core and cholesterol crystals,  
52 evidenced by high-resolution B-mode ultrasonography measurements of the carotid intima  
53 media thickness<sup>4,5</sup>. In macrophages, cholesterol efflux towards lipid acceptor particles is carried  
54 out by means of the ATP-binding cassette (ABC) receptors ABCA1, ATP binding cassette subfamily  
55 G member 1 (ABCG1), and the scavenger receptor SR-BI. The imbalance of cholesterol influx and  
56 efflux in macrophages is considered a key process in atherosclerosis initiation<sup>6</sup>. Cholesterol efflux  
57 is considered a key step in cholesterol elimination from peripheral tissue to the plasma and liver  
58 in a process called reverse cholesterol transport (RCT). Cholesterol is transferred from  
59 macrophages mainly to apolipoprotein A1 (ApoA1) found on the surface of high-density  
60 lipoprotein (HDL) particles. HDLs then transport cholesterol to the liver for excretion and re-  
61 utilization<sup>7-9</sup>.

62  
63 Traditionally, tritium (<sup>3</sup>H) radio-labelled cholesterol has been used in cholesterol efflux<sup>10</sup>. The  
64 emission signal of radioisotopes is highly sensitive<sup>10</sup>; however, radio-labelled cholesterol  
65 presents obvious handicaps such as long protocols, risk of exposure to ionizing radiation, and the  
66 need for special radioactivity facilities and equipment to ensure safe handling of radioactive  
67 emission. On the contrary, fluorescence has been successfully incorporated in diagnostic  
68 techniques due to its simplicity in fluorescent signal detection, the wide variety of fluorophores  
69 available, and its safety<sup>11</sup>. Several fluorescent-labelled sterols have been used to study  
70 cholesterol metabolism including dehydroergosterol (with intrinsic fluorescence), dansyl  
71 cholesterol, 4,4-difluoro-3a,4adiaza-s-indacene (BODIPY)-cholesterol, and 22-(N-(7-Nitrobenz-2-  
72 Oxa-1,3-Diazol-4-yl)Amino)-23,24-Bisnor-5-Cholen-3 $\beta$ -Ol (NBD-cholesterol). Particularly, NBD-  
73 cholesterol presents an efficient uptake in human cells<sup>12</sup>. Two different NBD labelled-cholesterol  
74 are currently available: 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-  
75 3b-ol (22-NBD) and 25-(N-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-methyl]amino)-27-norcholesterol  
76 (25-NBD; **Figure 1**). Cholesterol labelled with 22-NBD moiety may best suit cholesterol efflux  
77 studies, while 25-NBD-cholesterol is mainly used in cellular membrane dynamics research<sup>13,14</sup>.

78  
79 Cell lines typically used in *in vitro* cholesterol efflux assays are monocyte-like cells such as human  
80 leukemia-derived THP-1 cells, murine Raw 264.7 cells<sup>15</sup>, or J774.1. All of these cells can be  
81 differentiated into macrophages *in vitro* using phorbol 12-myristate 13-acetate (PMA), but THP-  
82 1-derived macrophages (dmTHP-1) best reflect and mimic the human macrophages<sup>16</sup>.

83  
84 In the present study, we optimize and standardize a fluorescent high-throughput method to  
85 determine the cholesterol efflux capacity of serum samples on dmTHP-1, using 22-NBD-  
86 cholesterol as an alternative to [<sup>3</sup>H]-cholesterol. In addition, we compare the optimized  
87 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

1.1. 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.

1.2. 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  $\mu$ M (*e.g.*, obtain 10 mL of NBD-cholesterol at 5  $\mu$ M final concentration) by diluting 25  $\mu$ 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)

2.1. Culture THP-1 cells in R10 medium at 37 °C, 5% CO<sub>2</sub>. Adjust to 0.3 x 10<sup>6</sup> cells/mL every 3 days.

2.2. Seed the cells in a white 96-well plate with a flat, clear bottom at 0.2 x 10<sup>6</sup> cells/well in R10 medium (100  $\mu$ L per well).

2.3. Treat the cells with 100 nM phorbol 12-myristate 13-acetate (PMA; from a 10  $\mu$ M stock) for 48-72 h, incubating at 37 °C, 5% CO<sub>2</sub> to differentiate THP-1 cells into dmTHP-1.

NOTE: It is recommended to use 5  $\mu$ L of PMA stock (10  $\mu$ M) in 500  $\mu$ L of R10 medium. Prior to this, prepare a 10  $\mu$ M PMA stock by dissolving a lyophilized 1 g vial in 10 mL of dimethyl sulfoxide (DMSO), aliquot, and stock at -20 °C.

2.4. 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  $\mu$ L of PMA stock (10  $\mu$ M), mix gently, and immediately seed 100  $\mu$ 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)

3.1. To prepare a polyethylene glycol (PEG) solution, dilute glycine in 10% PBS (with sterile H<sub>2</sub>O) 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.

3.2. 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 min<sup>17</sup> (*e.g.*, for 100  $\mu$ L of plasma or serum, add 40  $\mu$ L of 20% PEG solution).

132  
133 3.3. Centrifuge the PEG-apolipoprotein B precipitate at 13,000 x g for 15 min at 4 °C.

134  
135 3.4. Discard the precipitate. Transfer the supernatant to a new tube.

136  
137 NOTE: We suggest preparing the ABDS on day 3 (fresh). If it is not possible, prepare the ABDS on  
138 day 2 and keep it at 4 °C overnight.

#### 139 140 **4. NBD-Cholesterol Cell Loading (Day 2)**

141  
142 4.1. Discard the culture medium of the dmTHP-1 and wash the cells twice with 1x phosphate-  
143 buffered saline (PBS).

144  
145 4.2. Load the cells with 5 µM NBD-cholesterol (100 µL per well) in R10 medium and incubate  
146 overnight at 37 °C, 5% CO<sub>2</sub>.

#### 147 148 **5. Incubation with Cholesterol Acceptors (Day 3)**

149  
150 5.1. Discard the medium and wash the cells twice with PBS.

151  
152 5.2. Incubate the cells with 2-5% ABDS or the desired concentration of purified lipid acceptor  
153 (HDL, ApoA, ApoE, *etc.*) diluted in colorless RPMI 1640 medium (100 µL per well) for 4-6 h at 37  
154 °C.

155  
156 NOTE: Include a negative control (C-) consisting of colorless RPMI 1640 medium without  
157 acceptors and a positive control (C+) (*e.g.*, ABDS from a pool of healthy donors or purified HDLs).  
158 Note that the positive control applies particularly when patient samples (disease conditions) are  
159 analyzed (**Supplementary Figure 1**).

160  
161 5.3. Prepare a 200 mL stock of the cell lysis solution 1 (50 mM Tris buffer, 150 mM NaCl, H<sub>2</sub>O).  
162 Mix the cell lysis solution 1 at 1:1 (v:v) ratio with pure ethanol to obtain the lysis solution 2.

#### 163 164 **6. Fluorescent Signal Capture (Day 3)**

165  
166 6.1. Media NBD-cholesterol detection

167  
168 6.1.1. Remove the cell medium from the plates and collect it in a new white 96-well plate with  
169 an opaque flat bottom.

170  
171 6.1.2. For an optimal fluorescence signal detection in the media samples, add 100 µL of pure  
172 ethanol to 100 µL of each medium sample to obtain a 1:1 ratio in the 96-well white plate.

173  
174 6.1.3. Keep the plate with the treated media to further measure the fluorescence intensity (FI) at  
175 a 463/536 nm (excitation/emission) wavelength in the luminometer.

## 6.2. Intracellular NBD-cholesterol detection

6.2.1 Wash the cells twice with PBS.

6.2.2. To obtain the intracellular cholesterol, lyse the cells by incubating them with 100 µL of lysis solution 2 per well and shake the plate at room temperature (RT) for 25 min.

6.2.3. 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).

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

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

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

7.2. 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  $\mu$ 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 hours, 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 ( $^3\text{H}$ ) cholesterol<sup>16</sup>. 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 20%. 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 AND TABLE LEGENDS:**

**Figure 1: Molecular structures of natural cholesterol (A), 22-NBD-cholesterol (B), and 25-NBD-cholesterol (C).**



**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  $\mu$ M. 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.

**Figure 3: Time-progression NBD-cholesterol efflux in THP-1-derived macrophages.** The dmTHP-1 were loaded with 5  $\mu$ 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.

**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  $\mu$ 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.

**Figure 5: Correlation of NBD-cholesterol and [ $^3$ H]-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 [ $^3$ H]-cholesterol samples, the radioactive signal was detected using 100  $\mu$ L of medium and cell lysate mixed with the scintillation cocktail and read in the scintillation counter, following the protocol described by Low *et. al.*<sup>17</sup>. 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.

**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.

**Figure 7: Workflow diagram.**

**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 de NBD-cholesterol efflux from a pool of sera samples of 8 healthy patients. Error bars show standard deviation.

## **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, dehydroegrosterol, and 22- and 25-NBD-cholesterol<sup>12</sup>. Particularly, 22-NBD-cholesterol is appropriate for studying cholesterol dynamics with different cell types and as a substitute of radio-labeled cholesterol<sup>18</sup>. There is no consensus method to evaluate the fluorescent signal in a cholesterol efflux assay. Song *et al.*<sup>14</sup> harvested medium and cell lysates to measure FI separately; Liu *et al.*<sup>14</sup> transferred the medium to other plates, and the intracellular fluorescent signal was measured directly in the culture plate; and Zhang *et al.*<sup>15</sup> 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 signal<sup>19</sup>. 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.*<sup>14</sup> and Liu *et al.*<sup>14</sup> and unlike Zhang *et al.* who used a 1 h incubation period with the lipid acceptors<sup>19</sup>. 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<sup>20,21</sup>. As modifications, purified ApoAI 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<sup>15,22</sup>.

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 [<sup>3</sup>H]-cholesterol, by a fluorescent-labelled molecule to monitor cholesterol efflux assays<sup>14</sup>.

Cholesterol efflux may act as biomarker of atherosclerosis<sup>23</sup>, as it correlates with future cardiovascular events<sup>24,25</sup>. 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.

#### **ACKNOWLEDGMENTS:**

This work has been partially supported by the research grants FIS (PS12/00866) from Instituto de Salud Carlos III, Madrid, Spain; Fondo Europeo para el Desarrollo Regional (FEDER); Red de Investigación en SIDA (RIS), ISCIII-RETIC (RD16/0025/0002) and CERCA Programme / Generalitat de Catalunya. The authors thank the Retrovirology and Viral Immunopathology Laboratory of the Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS). We thank T. Escribà, C. Rovira, and C. Hurtado for their assistance and S. Cufí from the Knowledge and Technology Transfer Office for her guidance in protecting the invention.

#### **DISCLOSURES:**

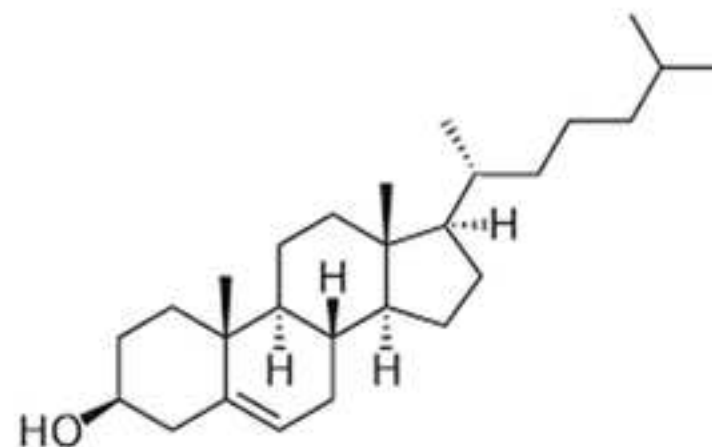
The authors wish to declare that they are inventors of the patent application (EPO; 18382337.6-1118; 17th May 2018) entitled "Method for determining cholesterol efflux" based on this method.

#### **REFERENCES:**

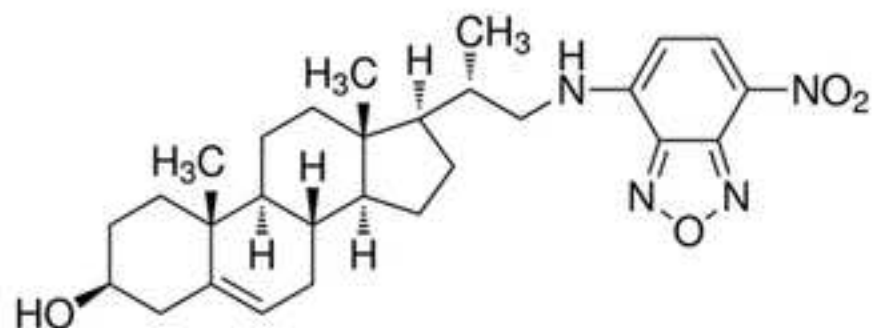
1. The top 10 causes of death. at <<http://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death>> (2018).
2. Hansson, G. K., Hermansson, A. The immune system in atherosclerosis. *Nature Immunology*. **12** (3), 204–212, doi: 10.1038/ni.2001 (2011).
3. Libby, P., Ridker, P. M., Hansson, G. K. Progress and challenges in translating the biology of atherosclerosis. *Nature*. **473** (7347), 317–325, doi: 10.1038/nature10146 (2011).
4. İlhan, F. Atherosclerosis and the role of immune cells. *World Journal of Clinical Cases*. **3** (4), 345, doi: 10.12998/wjcc.v3.i4.345 (2015).
5. Greaves, D. R., Gordon, S. Immunity, atherosclerosis and cardiovascular disease. **22**, doi: 10.1016/S1471-4906(00)01848-2 (2001).
6. Yamamoto, S., Narita, I., Kotani, K. The macrophage and its related cholesterol efflux as a HDL function index in atherosclerosis. *Clinica Chimica Acta*. **457**, 117–122, doi: 10.1016/j.cca.2016.04.012 (2016).
7. Escolà-Gil, J. C., Rotllan, N., Julve, J., Blanco-Vaca, F. In vivo macrophage-specific RCT and antioxidant and antiinflammatory HDL activity measurements: New tools for predicting HDL atheroprotection. *Atherosclerosis*. **206** (2), 321–327, doi: 10.1016/j.atherosclerosis.2008.12.044 (2009).
8. Santos-Gallego, C. G., Ibanez, B., Badimon, J. J. HDL-cholesterol: Is it really good? Differences between apoA-I and HDL. *Biochemical Pharmacology*. **76** (4), 443–452, doi: 10.1016/j.bcp.2008.04.020 (2008).
9. Rothblat, G. H., Phillips, M. C. High-density lipoprotein heterogeneity and function in reverse cholesterol transport. *Current Opinion in Lipidology*. **21** (3), 229–238, doi: 10.1097/MOL.0b013e328338472d (2010).
10. Hafiane, A., Genest, J. HDL-mediated cellular cholesterol efflux assay method. *Annals of Clinical and Laboratory Science*. **45** (6), 659–668 (2015).
11. Szalaia, R., Hadzsiev, K., Meleg, B. Cytochrome P450 Drug Metabolizing Enzymes in Roma Population Samples: Systematic Review of the Literature. *Current Medicinal Chemistry*. **23**, 1–26, doi: 10.2174/09298673236661608090 (2016).
12. McIntosh, A.L., et al. Fluorescent Sterols for the Study of Cholesterol Trafficking in Living Cells. *Probes and Tags to Study Biomolecular Function: for Proteins, RNA, and Membranes*. 1–33, doi: 10.1002/9783527623099.ch1 (2008).
13. Ostašov, P., et al. FLIM studies of 22- and 25-NBD-cholesterol in living HEK293 cells: Plasma membrane change induced by cholesterol depletion. *Chemistry and Physics of Lipids*. **167–168**, 62–69, doi: 10.1016/j.chemphyslip.2013.02.006 (2013).
14. Song, W., et al. Characterization of fluorescent NBD-cholesterol efflux in THP-1-derived macrophages. *Molecular Medicine Reports*. **12** (4), 5989–5996, doi: 10.3892/mmr.2015.4154 (2015).
15. Liu, N., Wu, C., Sun, L., Zheng, J., Guo, P. Sesamin enhances cholesterol efflux in RAW264.7 macrophages. *Molecules*. **19** (6), 7516–7527, doi: 10.3390/molecules19067516 (2014).
16. Qin, Z. The use of THP-1 cells as a model for mimicking the function and regulation of monocytes and macrophages in the vasculature. *Atherosclerosis*. **221** (1), 2–11, doi: 10.1016/j.atherosclerosis.2011.09.003 (2012).
17. Low, H., Hoang, A., Sviridov, D. Cholesterol Efflux Assay. *Journal of Visualized Experiments*. (61), 5–9, doi: 10.3791/3810 (2012).

18. Gimpl, G., Gehrig-Burger, K. Cholesterol reporter molecules. *Bioscience Reports*. **27** (6), 335–358, doi: 10.1007/s10540-007-9060-1 (2007).
19. Zhang, J., Cai, S., Peterson, B. R., Kris-Etherton, P. M., Heuvel, J. P. Vanden Development of a Cell-Based, High-Throughput Screening Assay for Cholesterol Efflux Using a Fluorescent Mimic of Cholesterol. *ASSAY and Drug Development Technologies*. **9** (2), 136–146, doi: 10.1089/adt.2010.0288 (2011).
20. Rader, D. D. J. Molecular regulation of HDL metabolism and function: implications for novel therapies. *Journal of Clinical Investigation*. **116** (12), 3090–3100, doi: 10.1172/JCI30163.3090 (2006).
21. Davidson, W. S., *et al.* The effects of apolipoprotein B depletion on HDL subspecies composition and function. *Journal of Lipid Research*. **57** (4), 674–686, doi: 10.1194/jlr.M066613 (2016).
22. Park, S. H., *et al.* Sage weed (*Salvia plebeia*) extract antagonizes foam cell formation and promotes cholesterol efflux in murine macrophages. *International Journal of Molecular Medicine*. **30** (5), 1105–1112, doi: 10.3892/ijmm.2012.1103 (2012).
23. Litvinov Y, Savushkin E. V., Garaeva E. A. D. A. Cholesterol Efflux and Reverse Cholesterol Transport: Experimental Approaches. *Current Medicinal Chemistry*. **371** (25), 2383–2393 (2016).
24. Phillips, A., Mucksavage, M. L., Wilensky, R. L., Mohler, E. R. NIH Public Access. **364** (2), 127–135, doi: 10.1056/NEJMoa1001689.Cholesterol (2011).
25. Rohatgi, A., *et al.* HDL Cholesterol Efflux Capacity and Incident Cardiovascular Events. *New England Journal of Medicine*. **371** (25), 2383–2393, doi: 10.1056/NEJMoa1409065 (2014).

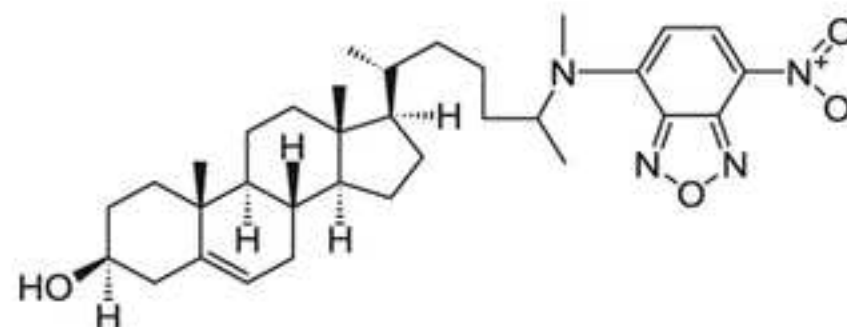
## A. Natural cholesterol

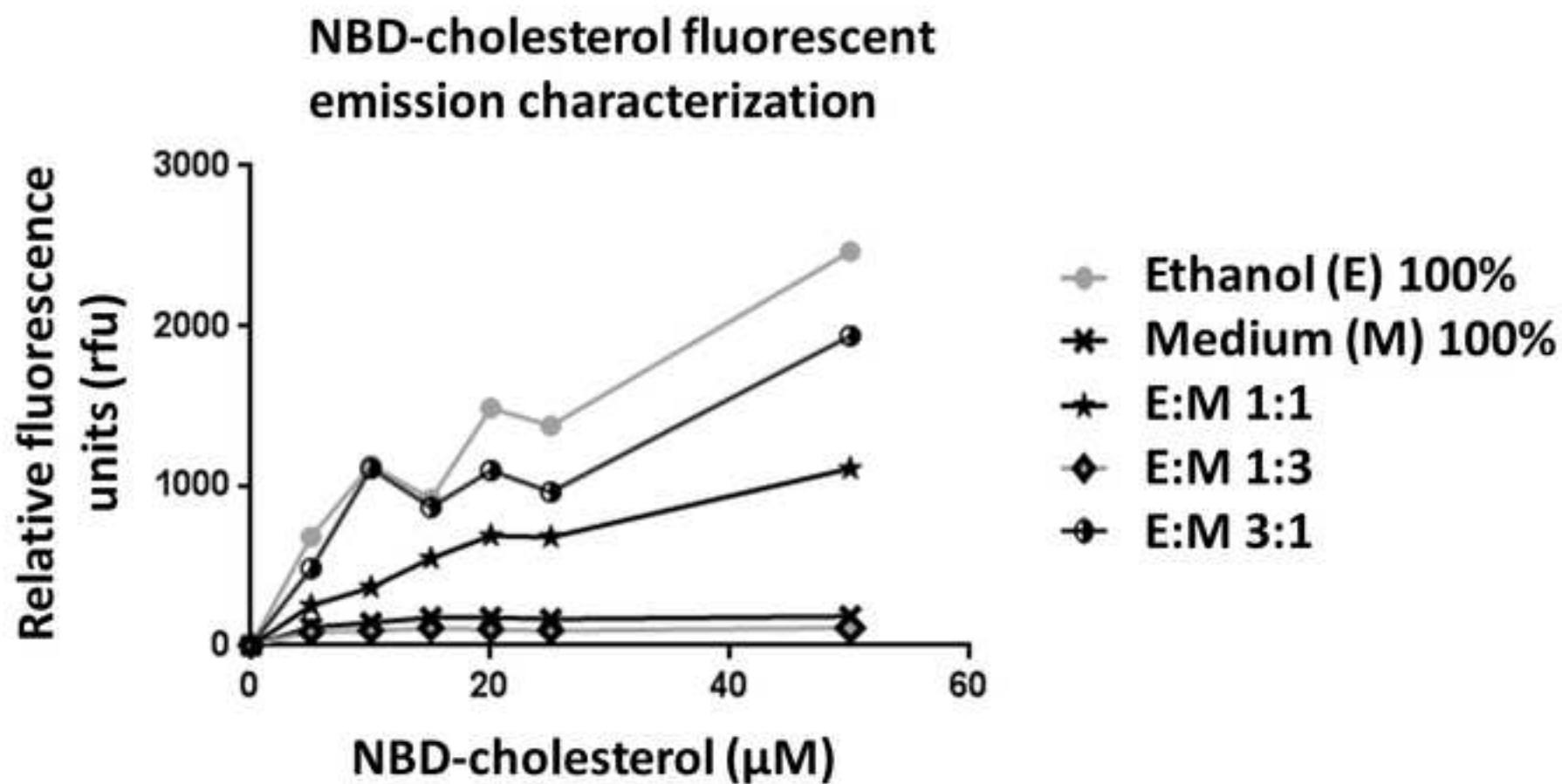


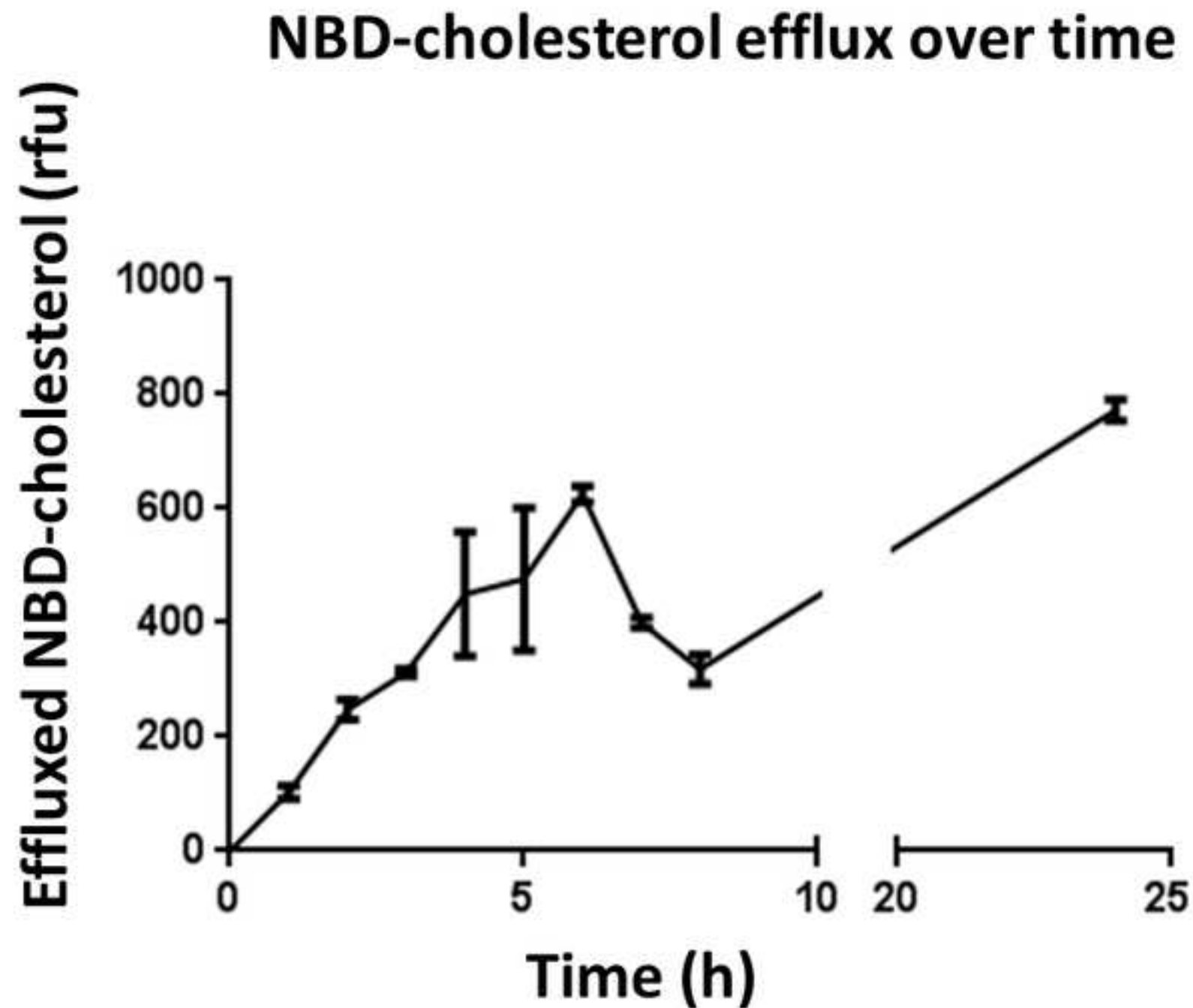
## B. 22-NBD-cholesterol



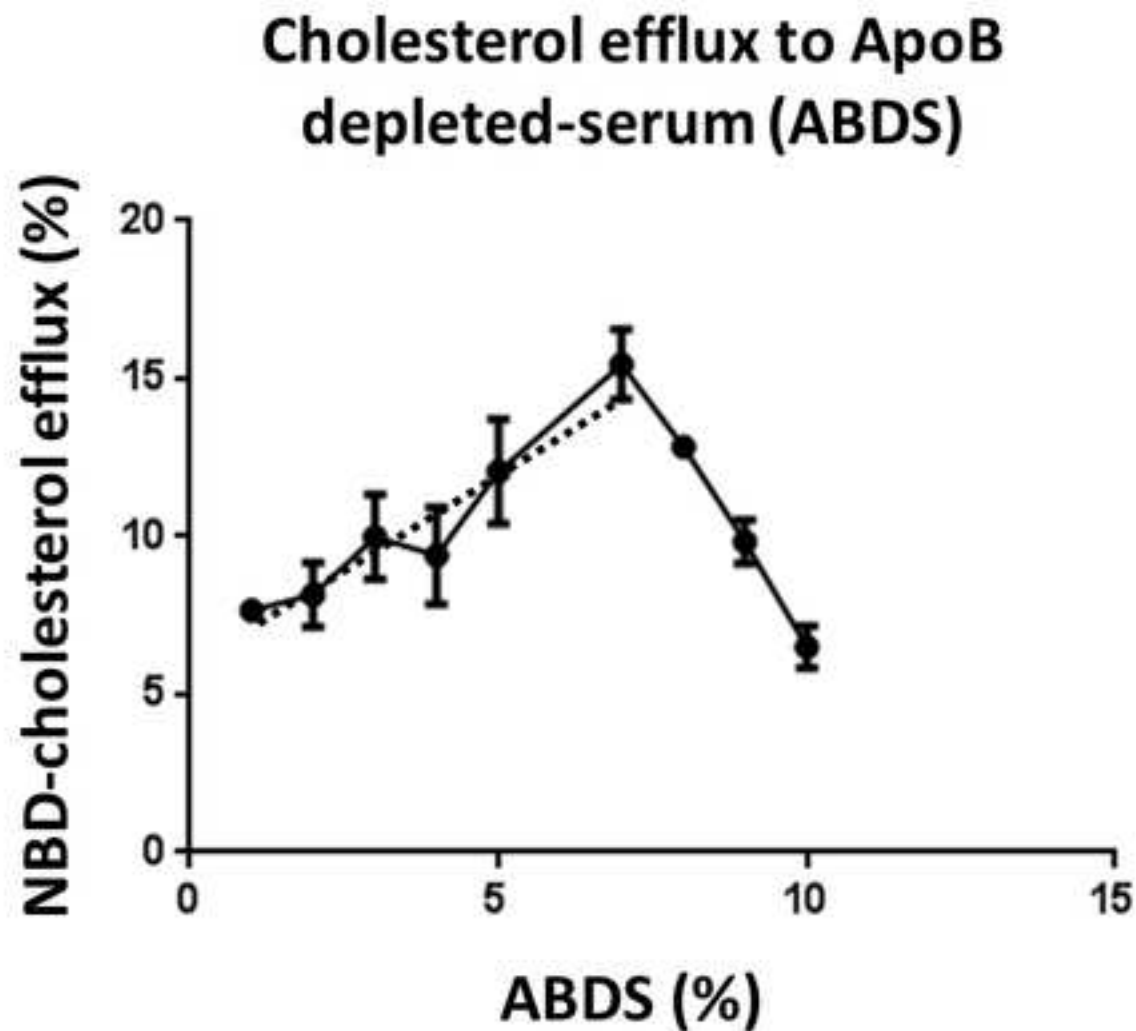
## C. 25-NBD-cholesterol











N = 9

**Correlation:**

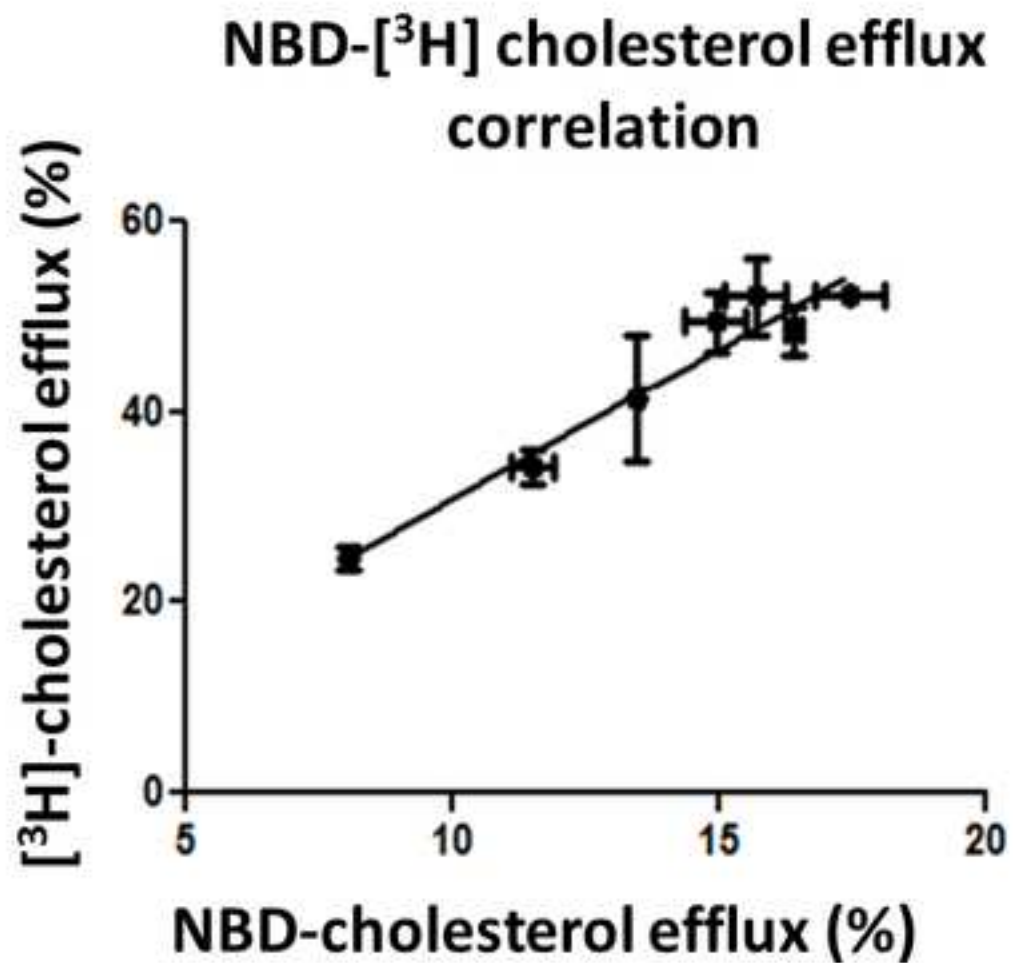
**Pearson's r: 0.96**

**p-value < 0.005 (\*\*\*)**

..... Lineal regression:

$y = 1.246x + 5.796$

**p-value < 0.001 (\*\*\*)**

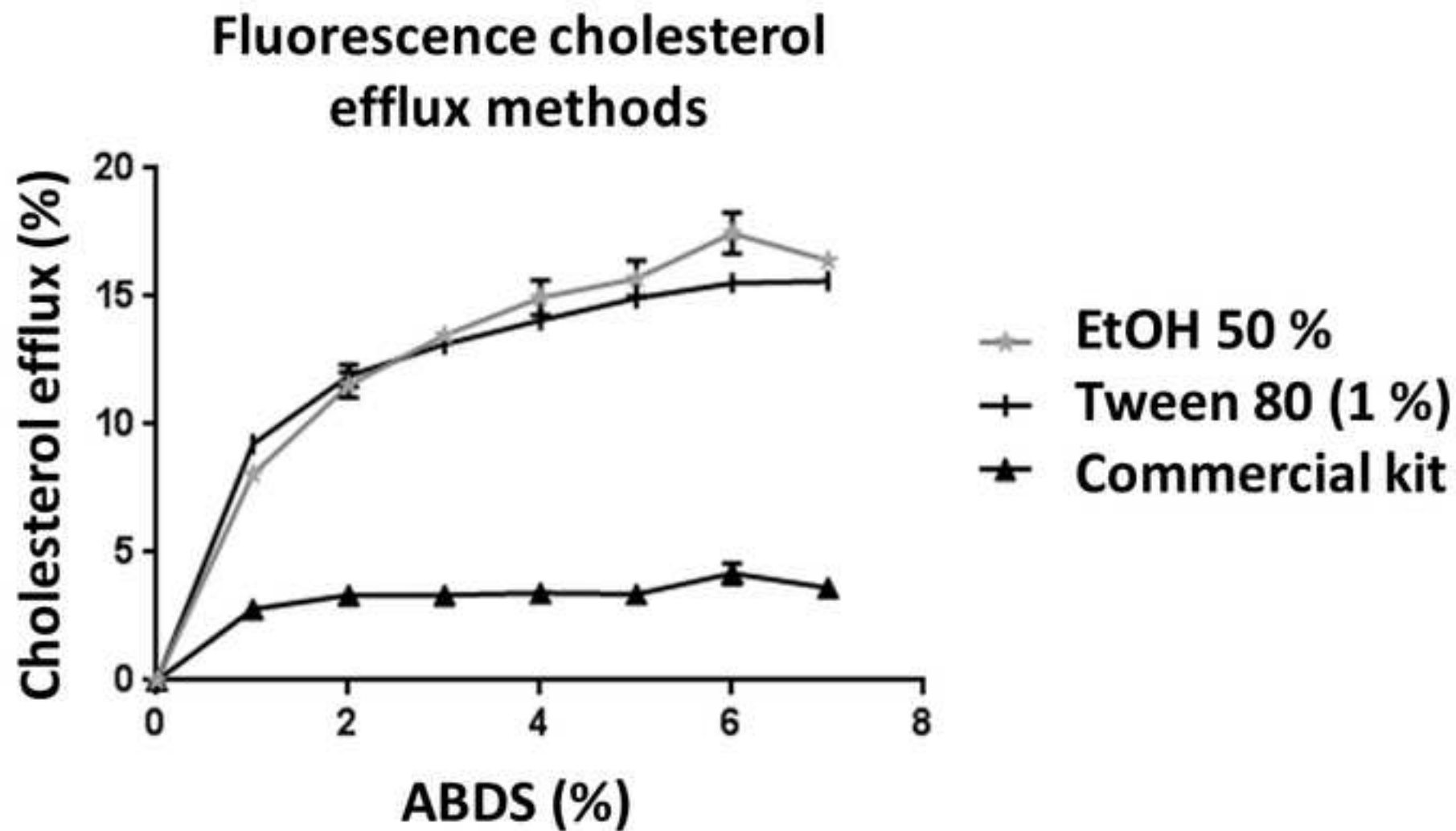


N = 8

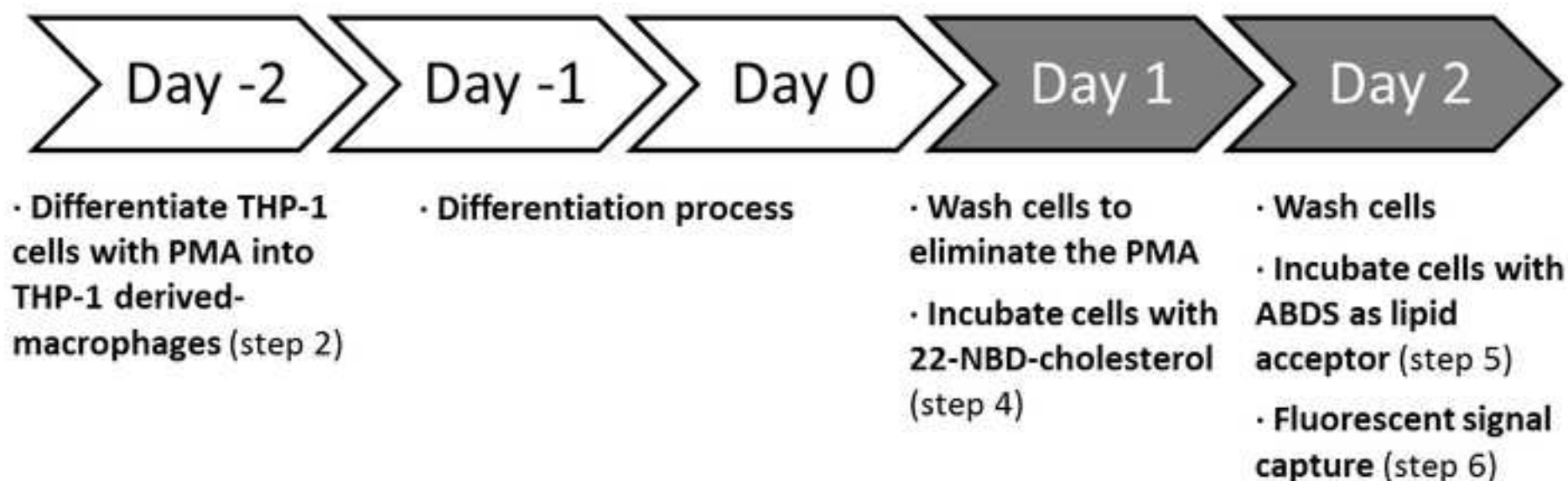
Correlation:

Pearson's  $r = 0.9746$

p-value < 0.0005 (\*\*\*)



## WORKFLOW:



<b>Name of Material/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
96-well collecting plate	Corning Inc	Costar 3912
96-well culture plate	Corning Inc	Costar 3610
<i>Cholesterol Efflux Assay Kit (Cell-based)</i>	Abcam	<i>ab196985</i>
colorless RPMI 1640	Sigma-Aldrich	R7509
Gen5 Data Analysis Software	BioTek	
Glycine	Sigma-Aldrich	G8790-100G
Luminometer	Biotek	SYNERGY HT
Lysis Solution 1	in-house	
Lysis Solution 2	in-house	
NBD-cholesterol	Thermo-Fisher	N1148
PBS	Sigma-Aldrich	P3813
PEG 8000	Sigma-Aldrich	202452-250G
PMA	Sigma-Aldrich	79346
R10	in-house	
RPMI 1640	Sigma-Aldrich	R8758
THP-1 cells	Sigma-Aldrich	ATCC, #TIB-202
Tween 80	Sigma-Aldrich	P1754

**Comments/Description**

white 96-well plate with opaque clear bottom

white 96-well plate with flat clear bottom

commercial high-throughput cell-based assay kit aimed to determine the cholesterol efflux

RPMI 1640 with no penhrol-red

Version 2.0

Glycine, non-animal use

Multi-Detection Microplate Reader

50 mM Tris Buffer, 150 mM NaCl and H<sub>2</sub>O

Pure ethanol and Cell Lysis Solution 1:1 (v:v)

22-(N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)Amino)-23,24-Bisnor-5-Cholen-3 $\beta$ -Ol

Phosphate-buffered saline

polyethylene glycol

Phorbol 12-myristate B-acetate.

RPMI 1640 supplemented with 10 % fetal bovine serum and 5 % Penicillin/Streptomycin.

RPMI 1640 with 2mM L-glutamine containing 1.5 g/L sodium bicarbonate and 4.5 g/L glucose

monocyte-like line derived from leukemia from a one year old baby





1 Alewife Center #200  
Cambridge, MA 02140  
tel. 617.945.9051  
www.jove.com

## ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:

High-throughput NBD-cholesterol Efflux Assay

Author(s):

Roque Pastor-Ibáñez, Olivia Tort, Mireia Arnedo

Item 1 (check one box): The Author elects to have the Materials be made available (as described at <http://www.jove.com/author>) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

- ☒ The Author is NOT a United States government employee.
- ☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.
- ☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

### ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: “**Agreement**” means this Article and Video License Agreement; “**Article**” means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; “**Author**” means the author who is a signatory to this Agreement; “**Collective Work**” means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; “**CRC License**” means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; “**Derivative Work**” means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; “**Institution**” means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; “**JoVE**” means MyJoVE Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; “**Materials**” means the Article and / or the Video; “**Parties**” means the Author and JoVE; “**Video**” means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4 and 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the “Open Access” box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.



## ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such

statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have



## ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's

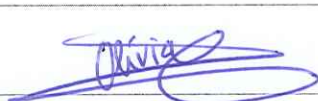
expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

### CORRESPONDING AUTHOR:

Name:	Olivia Tort	
Department:	HIV/AIDS Research Group	
Institution:	Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS)	
Article Title:	High-throughput NBD-cholesterol Efflux Assay	
Signature:		Date: 27/07/2018

Please submit a signed and dated copy of this license by one of the following three methods:

- 1) Upload a scanned copy of the document as a pdf on the JoVE submission site;
- 2) Fax the document to +1.866.381.2236;
- 3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email [submissions@jove.com](mailto:submissions@jove.com) or call +1.617.945.9051

**Editorial comments:**

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

As notified by Dr. Dsouza, this does not apply to chemical structures.

3. Please spell out each abbreviation (NBD, PEG, etc.) the first time it is used.

The full names have been included.

4. Keywords: Please provide at least 6 keywords or phrases.

The keywords have been included.

5. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

There was an error in the speed units that has been already corrected.

6. 1.1: It should be NBD instead of NDB.

Thanks to identify the mistake, this has been addressed.

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:

- 1.1 and 1.2: Listing an approximate volume to prepare would be helpful.

More detail has been included.

- 1.2: Please provide composition of R10 medium. If it is purchased, please cite the Table of Materials.

This has been included.

- 2.1: Please specify the culture temperature and other conditions.

This has been included.

- 3.1: What container is used?

We use 1.5 ml tubes, this has been added in the protocol.

- 5.3: Please specify the composition and volume prepared for the cell lysis buffer.

This has been included.

- 6.1.2: What wavelengths are measured?

This has been included.

- 6.2.3: Please provide the composition of equilibration buffer.

The composition has been included in 5.2.

- 6.3: How to adjust the sensitivity parameter at 50?

This is an internal parameter found in the software menu, as it has been detailed.

8. 7: Please write the text in the imperative tense.

This has been corrected.

9. Please include single-line spaces between all paragraphs, headings, steps, etc.

The lines have been included.

10. Lines 162-168: Please remove the embedded work flow, instead include it as Figure 1 and upload it separately to your Editorial Manager account.

This has been removed. The workflow was introduced as summary of the protocol as a final figure.

11. Lines 172-182: Please combine reagent information in relevant steps or move them to Table of Materials. Please remove commercial language from the manuscript (Sigma-Aldrich).

12. Please reference Figure 1 in the manuscript.

This has been modified.

13. Figure 4: Please use the period symbol (.) for the decimal separator, i.e., 1.246, 5.796, 0.001.

This has been modified.

14. Figures 4 and 5: Please be consistent with whether to use “Pearson r” or “Pearson R”, to use “P value” or “P-value”.

This has been modified.

15. Figure 6: Please fix the typo “Comercial”. It should be “Commercial”.

This has been modified.

16. JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

- a) Critical steps within the protocol
- b) Any modifications and troubleshooting of the technique
- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

We thank the editor for the opportunity to improve the discussion. Note that we entirely modified the discussion thus we did not include the track changes in this section as the result with the track changes was not comprehensive.

## Reviewers' comments:

*Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.*

Reviewer #1:

### Manuscript Summary:

This manuscript describes a fluorescence-based method to measure serum cholesterol efflux capacity. This is a timely manuscript as there is a general need for cholesterol efflux assays that do not involve radio-isotopes. Although the aims and results are clear the entire manuscript would greatly benefit from proof reading by a person fluent in English to correct grammar and also needs fact checking as many statements are not correct or mis-referenced. Overall the manuscript is not well written, the methods lack experimental detail and the data is missing controls. Based on the data presented it is not clear whether this is a consistent method to determine cholesterol efflux capacity.

### Major Concerns:

The major concerns are with the lack of detail in the methods and with the experiments described in the results section.

### Methods

#### 1. NBD-cholesterol preparation

The cells are exposed to quite a high concentration of ethanol. Has it been tested whether this concentration affects cell viability or cholesterol efflux? If cell viability is affected, cells may be prone to apoptose during the serum free incubation during the efflux resulting in high background fluorescence levels.

We agree with the reviewer that high concentrations of ethanol may be cytotoxic. However, the final volume in each well is 0.25  $\mu$ L in 100  $\mu$ L; representing 0.25 % (v/v) of the final volume, lower than was expected to become cytotoxic to similar cells as RAW 264.7 (Timm M *et al.*). Moreover, the two wash steps after cells incubation with NBD-cholesterol (step 5.1) takes place just before adding the ApoB-depleted serum with the objective of eliminating potential dead cells and remaining NBD-cholesterol that is adsorbed to cells and to the plate.

### Reference:

Timm M, Saaby L, Moesby L, Hansen EW. Considerations regarding use of solvents in in vitro cell based assays. *Cytotechnology*. 2013;65(5):887-894. doi:10.1007/s10616-012-9530-6.

#### 5. Incubation with cholesterol acceptors

5.2 ABDS is nowhere spelled out. As this is not a standard abbreviation it is hard to deduct what it means. Also 2-7% in what?

Standard cholesterol efflux assays are carried out with a final concentration of 1 or 2 % final PEG-precipitated serum concentrations as higher concentrations are saturating. The concentrations predominantly used in this manuscript are a lot higher than this. A time course for the standardly used concentrations (Figure 2) compared to the 5% concentration should be added.

We thank the reviewer for this observation. ABDS is now spelled out in line 113 and ApoB is now referred as Apolipoprotein B for a better understanding. ABDS was diluted in colorless RPMI 1640 medium, this has been included in the manuscript.

Standard cholesterol efflux assays are performed with radio-labeled cholesterol which presents more sensitivity than fluorescent-labeled cholesterol. In our method, the acceptor range comprised between 1-2 % presented a lower slope in the efflux than in the range of 2-7 % PEG-



precipitated serum concentrations. Thus, small changes in the acceptor generate a higher change in cholesterol efflux which means a better sensitivity of the range 2-7% in the particular case of our fluorescent technique. Moreover, saturation was not observed in concentrations lower than 8 %.

5.3 It is not clear why the buffer after the \* is called equilibration buffer. There seems to be no equilibration step in the protocol?

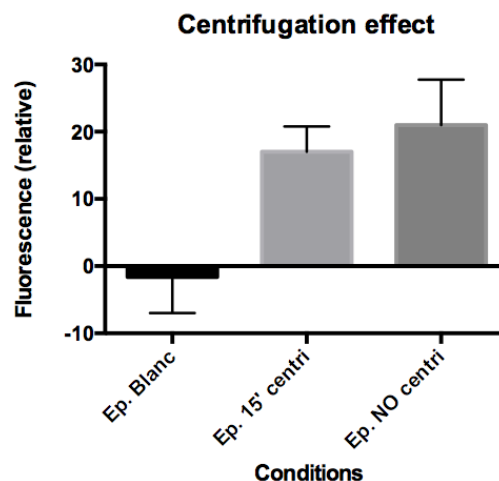
According with other cholesterol efflux assays based on radio-isotopes, we understand the confusion with the Equilibration buffer name. So we switch the 'Equilibration buffer' to colorless RPMI 1640 medium for a better understanding.

6. Fluorescent signal capture:

6.1.1 As the media is not filtered or spun down, floating cells may be present?

During the optimization process of the NBD-cholesterol efflux method we tested the effect of a centrifugation step before the fluorescence detection. We noted that the final efflux was essentially not affected after withdrawing this step (T-test; p-value = 0.4). A possible explanation is that floating cells may be removed by the two washing steps (step 5.1) just before the adding the lipid acceptors and during the relatively short period of time (4-6h) that takes the incubation, few additional cells die.

Results showed below in Figure\_X1.



Figure\_X1. Centrifugation effect on media samples. Media with the effluxed cholesterol was transferred to 1.5 mL Eppendorf, centrifuged at 10000 x g during 15 min and the fluorescence signal intensity was measured in the luminometer.

6.3 Considering this is a method based on fluorescence the emission and excitation wavelengths should be mentioned.

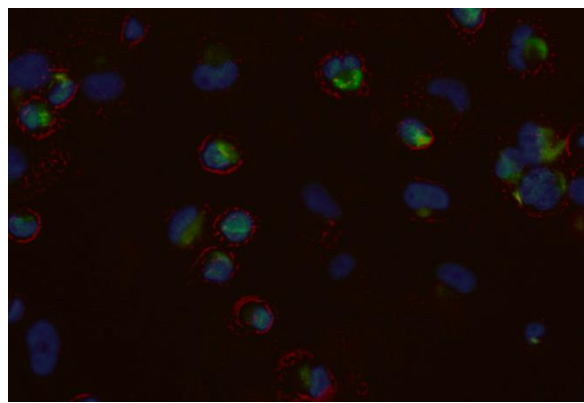
Thanks for the correction; we solve this issue in line 146.

All in all this method, is very similar than a method that uses radiation. It should be explained why there is no equilibration step, which is used in a protocol using radiation. Using the described method it would seem that a large amount of the fluorescent cholesterol will be present in the plasma membrane, which will be extracted when exposed to PEG-precipitated sera. This would not represent cholesterol removal from intracellular stores.

The base of our method was Low, H. *et al.* technique, which, as mentioned by the reviewer, includes an equilibration step consisting of incubating the Cholesterol loaded cells with media without fetal bovine serum (FBS) supplemented with 1% bovine serum albumin (BSA) for 18-20h in an incubator at 37°C, 5% CO<sub>2</sub>.

We decided to eliminate the equilibration step for 3 reasons: i) the cells in culture without FBS (in starvation) for 18-20h are likely to suffer; ii) the 2 washes with PBS in step 5.1 probably eliminates most of the excess NBD-cholesterol or weak-bound NBD-cholesterol not internalized; and iii) our protocol aims to get reduced to the minimal steps necessary but maintaining sensitivity and reproducibility, so that could eventually be applied as diagnostic test. Moreover, our fluorescent method without the equilibration step correlated with the radiolabeled method (which includes the equilibration step), consistent with an appropriate consistency.

During the optimization process we asked ourselves the same question than the reviewer: is the NDB-cholesterol located in the membrane or in the intracellular lipid reservoirs? To solve this question we acquired some images by fluorescence microscopy; it showed that NBD-cholesterol was accumulated inside the cells and only a small part of it was in the plasma membrane (Figure\_X2). In addition, L. McIntosh *et al.* propose 22- NBD-cholesterol as a suitable molecule for cholesterol uptake/trafficking.



Figures\_X2. THP-1 derived-macrophages cells. The dm-THP-1 cells were stained with DAPI (blue) and NBD-cholesterol (green); red color represent the plasma membrane observed with white light. Images were obtained by a fluorescence microscope (x40).

#### References:

- Low, H., Hoang, A., Sviridov, D. Cholesterol Efflux Assay. *Journal of Visualized Experiments*. (61), 5–9, doi: 10.3791/3810 (2012).
- McIntosh, A.L. *et al.* Fluorescent Sterols for the Study of Cholesterol Trafficking in Living Cells. *Probes and Tags to Study Biomolecular Function: for Proteins, RNA, and Membranes*. 1–33, doi: 10.1002/9783527623099.ch1 (2008).

#### Reagents:

- Why it the purpose of EDTA in the lysis solution and why is it optional.  
EDTA is a chelating agent usually used in lysis buffers. EDTA binds to metal ions with two positive charges (*e.g* magnesium and calcium) to diminish protease or DNAase action. In our case, NBD-cholesterol is a small molecule unlikely to be affected by those enzymes, thus in presence of EDTA the results are expected to remain the same we obtained.
- Stock solution for PMA should be described  
This has been included.
- Standard culturing conditions for THP-1 cells should be given.  
This has been included.

#### Results and figures

This sections needs proof reading. The grammar and statements are hard to understand.

Figure 1

The 1:1 ratio that is used is relatively flat compared to the 3:1 ratio, which would give a much more sensitive linear range. Why was the 1:1 ratio chosen?

*In Figure 1 we only show the molecular structure of cholesterol and NBD-labeled cholesterol. We believe that the referee is referring to figure 2, thus our answer is the following:*

As the reviewer said, the ratio 3:1 gives more fluorescence signal than 1:1. We assayed different concentrations of NBD-cholesterol in the different media. When the fluorophore was in a major ethanol content we observed that the increase of the fluorescence signal was irregular and non-linear with low NBD-cholesterol concentrations. This effect was not observed in the 1:1 ethanol:media ratio, so we selected that according to the linearity of the method.

The 1:1 ratio curve is represented by the following equation:  $y = 20,88x + 146,7$ ; with an  $R^2 = 0,9341$ .

#### Figure 2

It is clear that the fluorescence in the media has reached a maximum by 6 hours, but seems to go up again after 8 hours. This has not been explained.

This figure shows linearity for measuring the media-contained fluorescence. A similar experiment should have been carried out using the cell-contained fluorescence. In addition, the % efflux should be provided for this time course.

*In Figure 2 we did not perform a cholesterol efflux assay. We only tested the fluorescence progression with the increasing NBD-cholesterol concentration in presence of different media, but those media were not in contact with cells. However, we believe that the referee is referring to figure 3. If this is the case, our answer is the following:*

We hypothesize that after 6 h incubation, cholesterol may start reentering inside the cells. Once cells are loaded again, the efflux process may start again eventually reaching a homeostasis. In this experiment, we had not optimized yet the NBD-cholesterol extraction in the cell lysates, therefore we don't have this data.

#### Figure 3

What is the explanation that the efflux decreases after 6 hours? Is there any evidence that fluorescence associated with the cells increases?

We observed in the presented experiments that NBD-cholesterol effluxed from the cells during the first 6 hours of incubation in a progressive manner; the NBD-cholesterol therefore accumulates in the media during those first 6 hours. After this time, the cholesterol in the media starts to decrease, and we hypothesize that this decrease is due to the entrance of NBD-cholesterol again in the cells, as part of an homeostatic process. We did not find references about this process *in vitro*.

#### Figure 4.

It is suggested that at concentrations >7% ABDS cholesterol is re-entering the cells. Has this been tested? Was there efflux at shorter time points? If this is true it would mean that in serum samples with very high HDL levels, low efflux is observed?

This has not been tested directly. We can only suggest that cholesterol probably returned in the cells based on the decrease in the media, according with the presented results in Figure 3. We may only clarify that biochemical techniques have a linear range e.g. Bradford protein assay, ELISA. But, when the assays are dependent on complex systems such as cells, simultaneous processes may occur i.e. entrance and efflux of cholesterol.

We tested the cholesterol efflux assay with a serum sample from a pool of healthy donors (non-AIDS and any evidences of cardiovascular diseases or cardiopathies) and saturation appeared only with concentrations of ABDS higher than 7 %. The present method was suggested to use a range of 2-5 % ABDS, so saturation should not appear in this range in normal conditions applied to human serum samples. However, very high HDL levels may be in the condition where saturation of the technique occurs.

Figure 5:

Concentrations between 1-8% of ABDS are used. The previous figure showed that above 7% cholesterol efflux decreased. This is not shown in this figure?

The interpretation of the saturation of the technique from Figure 5 is difficult because it shows the cholesterol efflux absolute values obtained for the same samples measured both with the radioactive and fluorescent methods. We can observe however that the lower values present a higher distance among them than, the higher values. Precisely, those higher values have 1% ABDS between them but the difference in cholesterol efflux is low, this is consistent with saturation.

To show that this method gives reliable data, samples with low and high HDL concentrations should have been tested, preferably on different days.

We agree with the reviewer that several HDL concentrations should be tested. To this aim, and acknowledging that cholesterol efflux may show inter-individual differences, we decide to use a pool of 8 healthy donors and use a range of increasing % of their serum instead of using a single dose of different patients, to minimize the possible inter-individual variability in the optimization of the method. The dynamic range of the technique is thus for the particular application in ApoB-depleted serum

Minor Concerns:

Title:

The title claims the described method provides a high-throughput assay. In the introduction it is compared to a radiolabeled method. However, the described method seems to involve in comparison the same amount of steps and results in a similar data output?

This concern has been included into the discussion as it was shared by more than one reviewer and we think it is an important point.

The radio-labeled method involves 4 days protocol and our fluorescent-labeled technique reduces the protocol to 2 days, in both cases excluding cell seeding. An additional high-throughput improvement is that the signal capture of  $^3\text{H}$  cholesterol efflux is often manually prepared one by one in a single bottle per read with the scintillation liquid, thus two vials are needed per well (media and cell lysate). Conversely, the fluorescent method with NBD can be performed in 96-well plate and the fluorescent intensity signal is captured in the same culture plate (for cell lysates) and in a new 96-well plate for the media signal.

Introduction:

See general comment for language. The Introduction also needs proofreading to check the statements made as many statements are not entirely correct (possibly due to unintentional language mistakes).

For example:

- Line 50: "both are cardiovascular diseases that arise often by atherosclerosis" is not correct"

This has been corrected.

-Line 50: "the release of an atheroma plaque" should be the rupture of an atheroma plaque

This has been corrected.

- Line 67: "(32P) is not traditionally used in cholesterol efflux assays. Please provide references for this.

This had been corrected.

- Line 84:

J774s are missing, which is the cell line most commonly used for in vitro efflux assays

This has been included in the introduction. This was cited in the discussion.

In addition, some of the references are incorrectly referred to.

For example, ref 15 does not compare THP-1 and RAW 264.7 macrophages.

This has been corrected.



Reviewer #2:

Manuscript Summary:

In this manuscript, Pastor-Ibanez et al. detail a method for measuring cholesterol efflux that relies on a fluorescent-labelled cholesterol (NBD-cholesterol) rather than the traditional radioactive  $^3\text{H}$ -cholesterol. The protocol is mostly described in sufficient detail to allow the reader to successfully perform the experiment, and the method does seem effective. The authors optimize buffers for reading fluorescence, efflux time to ApoB-depleted serum, and concentration of ApoB-depleted serum to use for the efflux assay in the THP-1 cell line. The authors also show good correlation between NBD and  $^3\text{H}$  cholesterol, albeit with a lower sensitivity for the NBD version, and outperform a commercial fluorescent cholesterol efflux kit.

Major Concerns:

1. The authors claim their fluorescent assay is faster than the radioactive assay. The authors should explicitly state why their method is faster. Is it just using a plate reader vs. a scintillation counter?

As mentioned above to Reviewer #1:

This concern has been included into the discussion as it was shared by more than one reviewer and we think it is an important point.

The radio-labeled method involves 4 days protocol and our fluorescent-labeled technique reduces the protocol to 2 days, in both cases excluding cell seeding. An additional high-throughput improvement is that the signal capture of  $^3\text{H}$  cholesterol efflux is often manually prepared one by one in a single bottle per read with the scintillation liquid, thus two vials are needed per well (media and cell lysate). Conversely, the fluorescent method with NBD can be performed in 96-well plate and the fluorescent intensity signal is captured in the same culture plate (for cell lysates) and in a new 96-well plate for the media signal.

2. The authors state their solvent mixture reduces the variability of measurements. They also conclude they optimized cell seeding density and differentiation. I do not see any data included in this manuscript that supports these claims.

We assayed many conditions before the cholesterol efflux itself. We tested different cell seeding concentrations (50.000, 100.000 and 200.000 cells/well) and we observed that the one covering the plate at an appropriate confluence in our particular case of THP-1 cells was 200.000 cells/well. We considered that cell seeding as part of a basic lab routine that is additionally cell-dependent, plate-dependent thus we only gave the final data and did not show experimental data of this part of the process. However, we understand that it is paramount for the technique and in general for cell viability not to use an over-confluent cell culture or too few cells.

3. For the intracellular NBD-cholesterol detection protocol (6.2), the authors lyse the cells in some volume (how much?) of lysis buffer. This lysis buffer contains 50% ethanol. They then add 100uL Equilibration buffer, which will lower the ethanol percentage. This now differs from the ethanol percentage used to measure the media and used in the optimizations. The authors argue this percentage is crucial for accurate comparisons and reducing measurement-to-measurement variability. This issue needs to be addressed.

We apologize for the misspelling; this was a mistake that had been corrected in the manuscript. There is no additional dilution with the Equilibration buffer in the protocol. Cholesterol from the cells was extracted using the Solution 2 which contains a ratio 1:1 ethanol:aqueous medium. The resulting extraction is measured directly in the culture plate.

4. I am not clear why the authors used 1-8% ABDS for Fig5 when they clearly show the optimal linear range ends at 7% in Fig4.

We used 1-8 % ABDS to ensure that the linear range of the technique was entirely included in the assay.

5. In the discussion, the authors mention the detection range of cholesterol is between 3-6h after

adding acceptors. They then recommend using a 4h incubation. Fig3 does not appear to support this conclusion, and the method given by the authors uses 6h as the optimal incubation time.

The reviewer was right in saying that in Figure 3 the variability seems not to support that 4 hours incubation with the lipid acceptors was the best option. The reason why we proposed 4 hours better than 6 is because we saw that this variability did not appear when the method was applied within a cohort of patients (data not shown). Particularly in case of a high concentration of HDLs as lipid acceptor, it is safer to use 4h of incubation in order to stay further from the saturation of the technique.

The incubation time has been better suggested in the manuscript (4h changed by 4-6h range in the protocol).

6. I am unclear if all the details necessary for measuring the fluorescence are given. Is there a specific excitation and emission wavelength that should be used for this assay? Is the sensitivity parameter for their luminometer/fluorometer specific to a particular make and model? Is there a reason why the parameter setting given in the method differs from that used in Fig2? Which value was used for other figures? In additions, the authors should clarify which colour plate is needed for the assay. Clear, white, and black were all mentioned.

We appreciate the observation and we have already added the excitation/emission wavelength to the protocol according to the suggestion of the reviewer .

Moreover, Figure 2 was an early stage of the optimization process where did not involve sera samples. As we advanced in the optimization, we found that sensitivity parameter set on 70 presented measures with overflow signal. We corrected this issue adjusting this setting to 50 in the luminometer and adopted it in the final protocol

7. The authors should consider that the supernatant could be contaminated with THP-1 cells containing NBD-cholesterol, which would create inaccurate efflux readings. They should provide a strategy to counter this that works with 96-well plates.

In the development process we tested the effect of centrifugation step before the fluorescence detection. The results did not change subtracting this step.

Results showed below in Other\_Figures\_1.

We agree with the reviewer when considering that cells in the media may be responsible for increased background fluorescence signal. We already corrected for a background parameter by subtracting the CE values obtained in the negative control (cells incubated in absence of acceptor) to each medium sample.

Minor Concerns:

8. For the ApoB-depleted serum, which molecular weight of PEG is used? What is the amount used to precipitate ApoB? As this is a crucial part of the method, it should be noted in this manuscript.

This has been included.

9. It might be helpful to include a discussion on the limitations of the fluorescent assay.

This has been included.

10. I do not see why this assay cannot be used with purified lipid acceptors (i.e. ApoA1, HDL) to measure specific efflux from ABCA1 or ABCG1, as eluded to in the discussion. I think this would be an important application of the technique, and one which a lot of readers would be interested in. The authors should consider acknowledging this.

We agree with the reviewer. This method could be performed with purified lipid acceptors. We did not consider to include this part because other studies used this approach with purified lipid acceptors and we focused into a translational application. But, of course our fluorescent technique could include many types of acceptors.

11. Do the authors recommend any treatment for the cells to prevent the esterification and storage of cholesterol (i.e. ACAT inhibitor)? Perhaps this should be discussed, as it is often used in these assays.

We agree that the step to prevent esterification is often used. Before starting the study, we found in the literature that 22- NBD-cholesterol might be a more suitable probe for cholesterol uptake/trafficking (L. McIntosh et al). Other authors who performed similar studies with 22- NBD-cholesterol did not use any compound to prevent the esterification (W. Song et al). Finally, M. Storey *et al.* demonstrated that >96 % of NBD-cholesterol taken up was recovered as unesterified NBD-cholesterol.

With this evidences, and in our objective to minimize the protocol to the necessary, we decided to eliminate the treatment to prevent the esterification of the fluorocholesterol.

#### References:

- Song, W., Wang, W., Wang, Y., Dou, L., Chen, L., Yan, X. Characterization of fluorescent NBD-cholesterol efflux in THP-1-derived macrophages. *Molecular Medicine Reports*. **12** (4), 5989–5996, doi: 10.3892/mmr.2015.4154 (2015).
- McIntosh, A.L. et al. Fluorescent Sterols for the Study of Cholesterol Trafficking in Living Cells. Probes and Tags to Study Biomolecular Function: for Proteins, RNA, and Membranes. 1–33, doi: 10.1002/9783527623099.ch1 (2008).
- Storey SM, Atshaves BP, McIntosh AL, et al. Effect of sterol carrier protein-2 gene ablation on HDL-mediated cholesterol efflux from cultured primary mouse hepatocytes. *American Journal of Physiology - Gastrointestinal and Liver Physiology*. 2010;299(1):G244-G254. doi:10.1152/ajpgi.00446.2009.

12. When interpreting the results from Fig3, the authors note the max signal was captured after 5h. I think this is a typo and it should be 6h.

The reviewer is right and it should be 6h. We already correct it.

13. For Fig5, are there only 7 data points shown? Should there not be 8?

The 6 % ABDS point had not been included in this experiment.

14. Why was 1% Tween 80 used in Fig6? Was this the lysis buffer used in the commercial kit?

We optimized all the steps of the method. We aimed to find an optimal lysis buffers and tested several lysis solvents *i.e.* Ethanol, Tween 80, Tween 20, NP40 and a commercialized lysis buffer included in the Abcam cholesterol efflux kit. We tested all those buffers at different concentrations and show in figure 6 the optimal ones.

The lysis buffer used in the commercial kit is secret (the composition is not described).

Reviewer #3:

Manuscript Summary:

Here Pastor-Ibanez and colleagues describe a high-throughput assay for assessing the cholesterol efflux abilities of lipoproteins from human plasma and serum as well as cell culture supernatant. Although an undoubtedly interesting and useful technique with possible important future applications, the manuscript is poorly presented, lacks scientific quality, contains many grammatical errors and is very confusing making the protocol very hard to follow.

Major Concerns:

1. The manuscript is poorly written, figures are not well presented and the assay protocol is confusing. In this reviewer's opinion the manuscript would strongly benefit from external editorial review as the language used is non-scientific and vague in parts (i.e. Ln 65: "Cholesterol-loaded HDLs head to the liver"; Ln 112: "Leave the sera from patients on ice"; Ln 189: "wash the excess and then contact the sample to be tested" etc).

Those issues have been modified.

2. The protocol itself is confusing. Would suggest referring to each step in terms of 'days' of the experiment as shown in the workflow figure (i.e. Culture THP-1 cells in R10 on day -2... etc). Currently it is unclear on which day step 3. Preparation of ABDS is conducted. Due to the poor stability of HDL, is this performed on Day 2 or earlier? This step should be added to the workflow figure.

This has been included in the protocol.

3. As HDL, and its components, is known to degrade over time has this method been validated on long term clinical samples vs fresh samples?

No, this method was tested with frozen samples, stored at -80 °C. We take into account the reviewer consideration. It is better to perform the assay with fresh samples to minimize the HDLs degradation state.

4. The protocol is stated to be appropriate for plasma, serum and cell culture supernatant however only ApoB depleted serum is presented in the manuscript. What dilution steps are required for cell culture supernatant and plasma?

The presented protocol was standardized in sera samples, because we were wondering to correlate this technique with the radio-labelled once previously conducted in our lab. But during the optimization process we satisfactorily performed the assay also with plasma samples, with ApoB-depleted plasma.

5. Is this assay performed in singlicate or triplicate?

This assay was developed in triplicate. But it depends on the researcher's objective, so it is a particular decision.

6. Step 3.2 is missing.

This has been corrected.

7. No wavelength details are provided for reading the plates. This is a major oversight.

Thanks for the correction; we solve this issue in line 146.

8. Define CE as cholesterol efflux in step 7.

This has been included.

9. How many 'healthy donors' were used to create the pooled ABDS control and what constitutes someone being 'healthy'?

The pool of ABDS control included 8 donors (5 women and 3 men). The 'healthy donors' were selected by non-HIV-infection (our lab work with the HIV infected samples) and any evidence of cardiovascular disease or cardiopathies.

10. Figure 2. Use different line graphics (i.e. dots, dashes etc). Also what fluorimeter software is used? This is very poorly explained. What is the wavelength of the emitted light? How many experiments does this figure represent?

This has been included and corrected.

11. Figure 3 legend. According to the protocol white plates were used, not black.

This has been corrected.

12. Figure 4. Poorly graphed figure.

We have tried to improved figure 4.

13. Figure 5 legend. Pearson coefficient represents correlations, not regression. What are the 'target-shaped' error bars? This reviewer has never seen these used before. Again, black plates referred to in figure legend, but white plates listed in protocol.

The plate color has been corrected. Both are appropriate to measure fluorescence intensity in the luminometer.

The error bars represent the standard deviation for both radioactive and fluorescent cholesterol efflux experiments to each point. They are represented horizontally and vertically to show the variability of both techniques at each point, ideally they should be similar to show similar variability.

14. All Figure titles on graphs are vague and unclear

This issue has been addressed.

15. A lot of the introduction and discussion describes the difference between 22-NBD-cholesterol and 25-NBD-cholesterol and the benefits of one over the other for the measurement of cholesterol efflux from cells. However, no comparison of these different forms of NBD-cholesterol is present in the figures. This should be included to validate the method.

The reviewer is right in his sense that we did not compare the two NBD-cholesterol isoforms.

We bases our choice of 22-NBD-cholesterol on literature as this molecule is the most appropriate for cholesterol uptake/trafficking studies compared to 25-NBD. Our aim was to reflect thais in the introduction but we agree that it was too extended and we reduced it.

Minor Concerns:

1. There are many spelling mistakes in the manuscript

a. Ln 75 should read: "BODIPY-cholesterol or NBD-cholesterol".

This has been corrected.

b. Ln 98 should read: "Dissolve the NBD-cholesterol...."

This has been corrected.

c. Ln 113: State centrifuge speed as 'g' not rpm as rpm will be dependent on the centrifuge used.

This has been corrected.

d. Ln 114 should read: "Transfer the supernatant to a new tube"

This has been corrected.

e. Ln 117 should read: "phorbol 12-myristate 13-acetate (PMA). Also add concentration

This has been corrected.

f. Ln 118: Include concentration of PBS. Also that it is PBS- (I assume)

This has been corrected.

g. Ln 128: "from a pool of healthy donors".

This has been corrected.

h. Figure 6. Commercial kit

This has been corrected.

2. Ln 176: State the recommended concentration of EDTA, even if it is optional.

EDTA had been subtracted.

3. Describing the 96 well plates as "white clear (ln 105) or flat (ln 134) bottom" is confusing. Would suggest referring to the plates as "white 96 well plates with either clear or round bottom wells".

We thank the reviewer for the opportunity to improve this confusing. We have introduced "flat clear bottom" or "flat opaque bottom" to avoid confusion. We did not use plates with round bottom.

If you would like your personal information to be removed from the database, please contact the publication office.



## Cholesterol efflux of HIV-infected patients sera samples

