**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 keyworks 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 μL in 100 μL; 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.

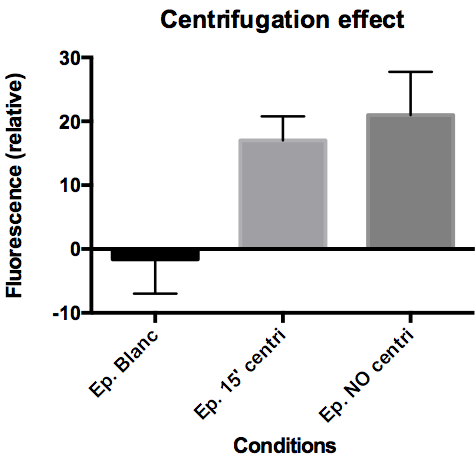
Standard cholesterol efflux assays are performed with radio-labeled cholesterol which presents more sensitivity than fluorescent-labeled cholesterol. In our method, the acceptor range comprised between 1-2 % presented a lower slope in the efflux than in the range of 2-7 % PEG-precipitated serum concentrations.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

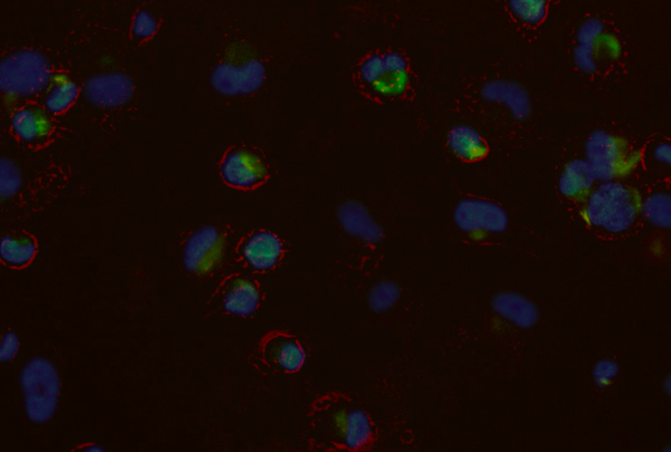
6.3 Considering this is a method based on fluorescence the emission and excitation wavelengths should be mentioned.

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 37ºC, 5% CO2.

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 is 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 follow equation: y = 20,88x + 146,7; with an

R square = 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 performed 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.* Braford 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 figured 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-through put assay. In the introduction it is compared to a radiolabeled method. However, the described method seems to involves 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 3H 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 3H-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 3H 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 3H 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](https://www.ncbi.nlm.nih.gov/pubmed/?term=Storey%20SM%5BAuthor%5D&cauthor=true&cauthor_uid=20395534) *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 reviewers 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 ºC. 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 AboB 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 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 researchers 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.  
  
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