Video Article

Cell-free Biochemical Fluorometric Enzymatic Assay for High-throughput Measurement of Lipid Peroxidation in High Density Lipoprotein

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Abstract

Low high-density lipoprotein cholesterol (HDL-C) levels are one of the most powerful independent negative predictors of atherosclerotic cardiovascular disease (CVD). The structure and function of HDL rather than HDL-C may more accurately predict atherosclerosis. Several HDL protein and lipid compositional changes that impair HDL function occur in inflammatory states such as atherosclerosis. HDL function is usually determined by cell based assays such as cholesterol efflux assay but these assays have numerous drawbacks lack of standardization. Cell-free assays may give more robust measures of HDL function compared to cell-based assays. HDL oxidation impairs HDL function. HDL has a major role in lipid peroxide transport and high amount of lipid peroxides is related to abnormal HDL function. Lipid-probe interactions should be considered when interpreting the results of non-enzymatic fluorescence assays for measuring the lipid oxidative state. This motivated us to develop a cell-free biochemical enzymatic method to assess HDL lipid peroxide content (HDLox) that contributes to HDL dysfunction. This method is based on the enzyme horseradish peroxidase (HRP) and the fluorochrome Amplex Red that can quantify (without cholesterol oxidase) the lipid peroxide content per mg of HDL-C. Here a protocol is describedfor determination of HDL-lipid peroxidation using the fluorochrome reagent. Assay variability can be reduced by strict standardization of experimental conditions. Higher HDLox values are associated with reduced HDL antioxidant function. The readout of this assay is associated with readouts of validated cell-based assays, surrogate measures of cardiovascular disease, systemic inflammation, immune dysfunction, and associated cardiovascular and metabolic risk phenotypes. This technical approach is a robust method to assess HDL function in human disease where systemic inflammation, oxidative stress and oxidized lipids have a key role (such as atherosclerosis).

Video Link

The video component of this article can be found at https://www.jove.com/video/56325/

Introduction

Atherosclerotic cardiovascular disease (CVD) is the leading cause of death worldwide[1,2]. Epidemiological studies have shown that low levels of high-density lipoprotein (HDL) cholesterol are generally inversely associated with the risk for the development of atherosclerosis[1,2]. Although several studies support an atheroprotective role for HDL[1,2], the mechanism by which HDL attenuates the initiation and progression of atherosclerosis is complex [3,4]. Thus, it has been suggested that the complex structure and function of HDL rather than absolute level may more accurately predict atherosclerosis [5,6,7,8]. Several HDL protein and lipid compositional changes that impair HDL function occur in inflammatory states such as atherosclerosis. These i) reduce its cholesterol efflux potential [9], ii) decrease anti-inflammatory and increase HDL-associated pro-inflammatory proteins [6,7], iii) decrease antioxidant factor levels and activity and HDLs ability to inhibit oxidation of Low Density Lipoprotein (LDLox)[10] and iv) increase lipid hydroperoxide content and redox activity (HDLox)[9,11]. Robust assays that evaluate the pleotropic functions of HDL (such as cholesterol efflux, antioxidant function) may complement determination of HDL-HDL-C in the clinic.

HDL function is usually assessed by cell-based methods such as the cholesterol efflux assay[8,12,13,14]. These methods have major limitations including significant heterogeneity with regards to types of cells used, type of readout reported, lack of standardization and confounding effects of triglycerides [7,15]. These drawbacks pose difficulties for large clinical studies[16]. Cell-free assays may give more robust measures of HDL function compared to cell-based assays. The cholesterol efflux is one of the most important functions of HDL but it can only be determined by cell-based assays. Other approaches to determine HDL function such as proteomics[17,18,19,20,21,22,23,24] and cell-based monocyte chemotaxis assays of HDL function [17,22,25] have not been standardized and cannot be used in large scale human studies.

HDL has significant antioxidant atheroprotective effect[5,6,7,8]. The antioxidant function of HDL has been determined in the presence of LDL in previous cell free fluorometric assays [26]. These biochemical fluorometric methods of HDL antioxidant function were originally developed by Mohamad Navab and Alan Fogelman and their colleagues[26]. Although many human studies have used these methods to determine HDL function [17,18,19,20,21,22,23,24], lipid (HDL)-lipid (LDL) and lipid-fluorochrome interactions may limit reproducibility of these cell free non-enzymatic biochemical assays of HDL function[27,28].

Recent interest has focused on the functional consequences of HDL oxidation that is the result of oxidation of both lipids and proteins within HDL [27,29,30]. Prior studies have shown that oxidation of HDL impairs HDL function [27,29,30]. HDL has a major role in lipid peroxide transport and high amount of lipid peroxides is related to abnormal HDL function. Thus HDL lipid peroxide content can be used to determine HDL function [9,17,20,31] and given the known limitations of prior assays of HDL function[7,15,27,32], we developed an alternative fluorometric method that quantifies HDL lipid peroxide content (HDLox) [32]. This method is based on the enzyme horseradish peroxidase (HRP) and the fluorochrome Amplex Red that can quantify (without cholesterol oxidase) the lipid peroxide content per mg of HDL-C [32]. The biochemical principle of the assay is shown in **Figure 1**. We have shown that this fluorescence-based approach does not have the limitations of prior HDL function assays[27,28]. This assay has been further refined and standardized in our laboratory so that it can reliably be used in large scale human studies even with cryopreserved plasma [32,33,34,35,36,37,38,39,40,41,42]. The readout of this assay is associated with readouts of validated cell-based assays, surrogate measures of cardiovascular disease, systemic inflammation, immune dysfunction and associated cardiovascular and metabolic risk phenotypes[32,33,34,35,36,37,38,39]. Here, we describe this simple, yet robust method to measure HDL lipid peroxide content (HDLox). This assay can be used as a tool to answer important research questions regarding the role of HDL function in human disease where systemic inflammation, oxidative stress and oxidized lipids have a key role (such as atherosclerosis)[32].

Protocol

All experiments using human biological samples were performed with ethics approval from the University of California Los Angeles, Los Angeles and the Alfred Hospital Human Ethics committee, Melbourne.

NOTE: There are many variations of the fluorochrome HDL function Assay (see discussion)[32]. Below we will describe the protocol that gives the most consistent and reproducible results. An overview of the assay is shown in **Figure 2**.

1. Specimen Processing

1. Use fresh, non-hemolyzed serum (can be collected in serum separator tubes) or citrate plasma obtained after 12-14 hours fasting. If using cryopreserved samples, include appropriate control as described in this Protocol.

2. Day 0-Preparation for the assay

1. Prepare working layout of experiment with the appropriate controls, samples and replicates. Run each sample at least in triplicates. A representative 96 well layout is shown in **Figure 3**.
2. Calculate all volumes of reagents that will be used for the assay based on number of samples and replicates.
   NOTE: Include an additional 10% volume in each working stock to make sure there is enough volume for each reagent for the specific experiment.
3. Label all required tubes for all different steps of the assay e.g. for the separation of HDL, measurement of HDL-C (optional) and the fluorochromeassay.

3. Day 1-Preparation of controls

1. **Preparation of study-specific pooled control**
   1. Create a control sample from pooled plasma or serum of all study samples. If running 20 samples in each plate in triplicate, combine 10 µL from each sample into a 200 µL pooled control. Give a separate aliquot of this pooled control to the clinical laboratory to determine the HDL-C value of this control.
      NOTE: Use this pooled control to standardize the assay and account for all possible known (e.g. type of matrix serum versus plasma, freeze-thaw cycles, cryopreservation) and unknown confounders that may affect experimental variability[27,28].

2. **Preparation of laboratory-specific quality control (QCs)**
   1. Prepare a large stock of HDL in each laboratory (for example HDL isolated from 5 mL of plasma from 10 healthy donors) and cryopreserve several aliquots of this stock to minimize freeze-thaw cycles.
   2. Use at least 10 different replicates from this stock to determine the average value of HDLox. An acceptable range of measured HDLox values for each QC sample included in each assay is within <15% of the coefficient of variation of this average value.
   3. Give a separate aliquot of this pooled control to the clinical laboratory to determine the HDL-C value of this control (e.g. 40 mg/dL).
      NOTE: A detailed approach how to use blood from blood banks to create these controls has previously been described [27,28,32]. Use additional QCs as needed (e.g. one from a healthy donor known to have normal HDL function and one from a donor known to have largely abnormal HDL function.

3. **Optimization of background signal and blank values (optional)**
   1. To minimize background, add the appropriate amount of catalase (1-4 U/mL) in the incubation medium to rapidly remove the formed H_2O_2 due to spontaneous air oxidation of the buffers.

NOTE: Since the values from the blank wells (no HDL) are being subtracted from the values of the HDL samples and the results are being reported relative to a pooled control (that is included in the same 96 well plate), we have found that this step does not practically affect the results. Thus, optimization of the background signal with catalase can be omitted, if needed.

4. Day 1-Separation of HDL Cholesterol using HDL precipitation

NOTE: Use a commercially available standardized HDL Cholesterol precipitating reagent to isolate apoB depleted serum according to the manufacturer's instructions. These reagents are widely used in colorimetric assays to determine HDL cholesterol levels.

1. Use fresh (or thaw) plasma or serum sample.
2. Mix equal volumes (e.g. 80 µL) of plasma and HDL Cholesterol Precipitating Reagent (20% w/v polyethylene glycol in glycine buffer at pH 10.0 (25 °C)).
3. Mix well by pipetting up and down.
4. Centrifuge at 1000-2000 x g for 10 min.
5. Aspirate the supernatant (HDL fraction).
6. Ideally use immediately the isolated HDL for the fluorochromeassay of HDL lipid peroxidation. However, if several samples are run in the same day and other steps such as measurement of HDL-cholesterol concentration are also done, then store the isolated HDL at 4 °C and use it the next day for the fluorochromeHDL function Assay.

5. Day 1-Determination of HDL-C in isolated HDL

NOTE: This is optional if the value of HDL-C from the clinical laboratory is used to normalize HDLox by HDL-C amount.

1. Quantify the HDL-cholesterol from plasma, using standard colorimetric assays[27]. Add 50 µL of Cholesterol Reagent in each well and determine cholesterol concentration using a colorimetric plate reader and a cholesterol standard provided in each kit.

6. Day 2-Preparation of reagents

1. Prepare HRP and cholesterol solutions of HRP 5 U/mL solution (Range 1-10 U/mL).
2. Prepare 20 mM fluorochrome (e.g., Amplex Red) solutions: Thaw a vial of fluorochrome reagent and DMSO to room temperature. Prior to use, dissolve fluorochrome reagent (1 mg) in 200 µL of DMSO. Store stock solution frozen at ≤-20 °C, protected from light.
3. Prepare positive and negative controls: Use 1X 'reaction buffer' without cholesterol and 20 mM H_2O_2 working solution as a negative and positive control, respectively.

7. Day 2-Fluorochrome Assay

1. Add 50 µL of 1x Reaction buffer as blank (negative control).
2. Optional: Add 20 mM working solution of H_2O_2 as positive control in each plate.
3. To minimize experimental variability and ensure that addition of reagents and samples are done consistently and in a timely manner, perform all additions of samples in a separate 96 well round-bottom, clear, polystyrene or polypropylene plate (Plate 1). Then use a multichannel pipette to transfer specific volumes into 3 96-well plates (Plates 2-4: polypropylene, flat bottom, black) (that have identical layout) (**Figure 2**, **Figure 3**).
    1. For example, first add 160 µL of isolated HDL into each well/sample of plate 1 and then with the use of multichannel pipette transfer 50 µL of HDL-cholesterol from each well/sample into the 96-well black plates. Change tips between each row/column and use non-filtered tips for all the transfers.
4. Leave samples in the wells. Do not discard. There are no wash steps between addition of reagents.
5. Add 50 µL of HRP solution 5 U/mL (0.25 U) to each well.
   NOTE: Use HRP before the addition of the fluorochrome reagent.
6. Incubate for 30 min at 37 °C. Do not discard samples after incubation (no wash steps between additions of reagents).
7. Add 50 µL of fluorochromereagent for a final concentration of 300 µM. At this point, total reaction volume is 150 µL. Mix well and protect from light.
8. Assess the fluorescent readout (in the dark) every minute over 120 min at 37 °C with a fluorescent plate reader (530/590 nm filters).
   NOTE: Use a shorter 60-minute interval with fresh samples. We have found that 120-minute assay gives more reproducible data with the use of cryopreserved samples.
9. Record data using appropriate software.

8. Day 3-Data analysis

1. Record fluorescence units (arbitrary units) at 120 minutes after addition of the fluorochrome Reagent for all the samples including blank wells and all controls.
2. Calculate the mean value of fluorescence units (based on at least triplicates) (HDL_{ox_sample}). Do not take outliers (> 2 SDs from the average value) into consideration.
3. Subtract the background fluorescence by using the following equation:

$$Fluorescence\ in\ HDL\ sample\ (FU) - Fluorescence\ in\ blank(no\ HDL)\ (FU) = HDLox\_sample$$

4. Normalization for HDL cholesterol amount (HDL-C): Normalize the HDLox lipid peroxidation value for each sample (including the pooled control) by the HDL-C) (mg/dl). Throughout the results section, HDL_{ox} is presented as $n_{(HDL\text{-}C)}$ HDLox measure to reflect the adjustment for HDL-C. Use the following equation:

$$Fluorescence\ in\ HDL\ sample\ (FU)/\ HDLC\ (mg/dl) = n(HDLC)\ HDLox\ (FU * \frac{dl}{mg})$$

5. Standardize the expression of HDLox lipid peroxidation value of each sample against the value of a pooled control. Normalize the $n_{(HDL\text{-}C)}$ HDLox lipid peroxidation value for each sample (adjusted for HDL-C) by the $n_{(HDL\text{-}C)}$ HDLox lipid peroxidation value of the pooled control. Throughout the results section, HDL_{ox} is presented as $nHDL_{ox}$ measure to reflect the adjustment for experimental variability and HDL-C. Use the following equation:

$$\frac{n(HDLC)HDLox(sample)\left(FU * \frac{dl}{mg}\right)}{n(HDLC)HDLox(pooled\ control)\left(FU * \frac{dl}{mg}\right)} = nHDLox\ (sample)\ (no\ units)$$

NOTE: This approach is similar to other established experimental approaches to reduce experimental variability of measurements such as international normalized ratio (INR)[44,45]. This approach has been validated in clinical studies[32,33,34,35,36,37,38,39].

6. Perform quality control of experimental results using standard quality control samples. Each laboratory should establish their own quality controls (QCs). Ideally there is a QC for nHDLox from a sample with known dysfunctional HDL and a QC for nHDLox from a sample from healthy donors [e.g. use pooled sample from young adults who are established blood bank donors and have no known comorbidity and risk factors for cardiovascular disease including smoking]. The latter control standardizes results among different laboratories. The average values of these QCs are established based on at least 5 replicates (typically we use 10 replicates for this important step).
    1. Check whether measured QCs in each assay have values within the expected range of values. Assuming an acceptable maximum experimental variability of 15%, all the measured experimental values should fall within 15% of the known average values of established QC. Use the following equations:

$$Expected\ range\ of\ values\ for\ QC\text{:}\ (Minimum\ acceptable\ value\ of\ QC) < experimental\ values < (Maximum\ acceptable\ values\ of\ QC)\ OR$$

$$[(Known\ average\ of\ QC - 0.15 * (known\ average\ of\ QC)] < experimental\ values < [(Known\ average\ of\ QC + 0.15 * (known\ average\ of\ QC)]$$

NOTE: If either of the included quality control samples (normal versus dysfunctional HDL) gives HDLox values outside expected range (established in each laboratory) then repeat the experiment.

7. Perform quality control of experimental results using the coefficient of variation (CV) to quantify experimental variability: Repeat all samples with CV% > 15%. Use the following equation:

$$\frac{Standard\ deviation\ of\ values\ of\ replicates}{Average\ of\ values\ of\ replicates} * 100 = CV\%$$

NOTE: A typical intra-assay (within the same plate) and inter-assay (among different plates) experimental variability is < 15%. 2) A typical intra-assay CV is between 1-7% and a typical interassay CV is between 3-10%.

Representative Results

50 µL of each HDL sample are added into each well as in step 7.3. 50 µL of HRP solution 5 U/mL (0.25 U) are then added into each well as in step 7.5. Samples are incubated for 30 min at 37 °C as in step 7.6. 50 µL of fluorochrome reagent are then added into each well as in step 7.7 (final concentration of 300 µM). The fluorescent readout (in dark) is then assessed every minute over 120 minutes at 37 °C with a fluorescent plate reader (530/590 nm filters). Representative fluorescence data for blank, pooled control, sample with known dysfunctional HDL and sample with normal HDL are shown in **Figure 4**. Raw representative data and step by step analysis of results using the equations described in section 8 of the protocol are shown in **Table 1.** In this example, fresh HDL samples were used and higher HDLox values are generally obtained with fresh HDL. For example, HDL known to be dysfunctional based on independent assays of HDL function and from HIV-infected person had approximately 2-fold relatively higher amount of lipid peroxide compared to pooled HDL from healthy participants. **Figure 5** shows representative results from a study with subjects known to have impaired HDL function[28,32]. The HIV-infected persons had approximately 60% higher mean HDL-lipid peroxide content (per mg of HDL-C) compared to the uninfected persons. In our prior published studies with cryopreserved HDL, this method was able to demonstrate at least 10% relative differences in HDLox compared to HDL from control groups (without disease e.g. chronic HIV infection)[36,37,38].

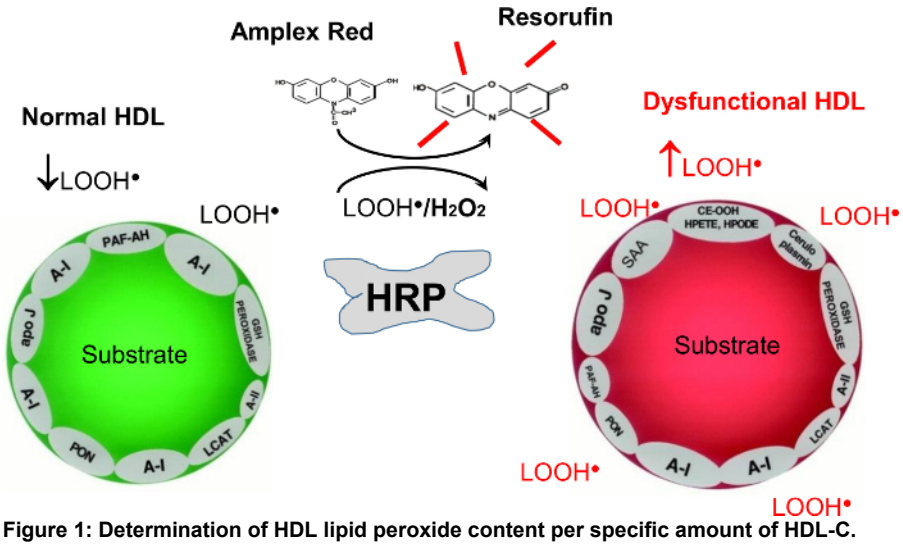


Figure 1: Determination of HDL lipid peroxide content per specific amount of HDL-C.
In states of systemic inflammation and oxidative stress HDL has increased lipid peroxides (LOOH) (HDLox) that are associated with impaired HDL function. HRP catalyzes the oxidation of non-fluorescent fluorochrome to fluorescent resorufin red. This oxidation can be driven by both endogenous peroxides present in the reaction (OH-) and HDL-lipid peroxides (LOOH-). Without cholesterol oxidase, resorufin (with HRP) can quantify the intrinsic HDL lipid peroxide content of a specific amount of HDL cholesterol. The background production of OH- as a result of air oxidation of the buffer is subtracted from the fluorescent readout of each well. Resorufin has minimal autofluorescence in most samples.This figure has been modified [32]. Please click here to view a larger version of this figure.

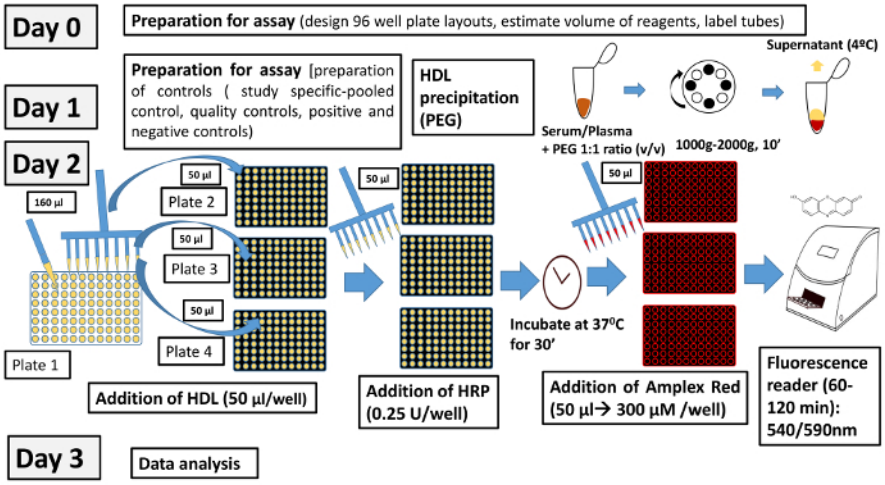
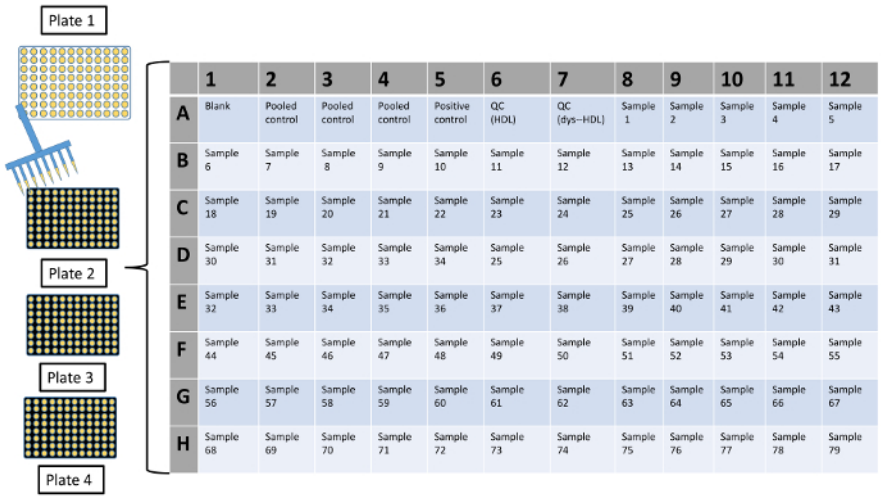


Figure 2: Overview of the workflow for the fluorochromeassay of HDL lipid peroxidation.
Day 0: Preparation for the assay: A) design 96 well plate layouts, estimate volume of needed samples (plasma/serum) and reagents (HRP enzyme, fluorochrome Reagent, buffers, PEG reagent), label tubes
Day 1: Preparation for the assay and HDL isolation: A) preparation of controls (study specific-pooled control, quality controls, positive and negative controls) B) HDL precipitation using PEG reagent.
Day 2: Preparation for the assay (if not done in Day 1) and fluorochrome assay: A) preparation of controls (study specific-pooled control, quality controls, positive and negative controls) A) addition of HDL samples: To minimize experimental variability and ensure that addition of reagents and samples will be done consistently and in a timely manner it is recommended that all additions of samples (e.g. 160 µL) are first done in a separate 96 well round-bottom, clear, polystyrene or polypropylene plate (Plate 1). Then a multichannel pipette can be used to transfer specific volumes (e.g. 50 µL) into 3 96-well plates (Plates 2-4: polypropylene, flat bottom, black plates) (that have identical layout). B) addition of HRP: Add 50 µL of 5 U/mL (0.25 U) to each well using a multichannel pipette C) Incubate at 37 °C for 30 min D) Add 50 µL of 300 µM /well of fluorochrome Reagent to each well using a multichannel pipette E) Assess the fluorescent readout (in dark) every minute over 120 minutes at 37 °C with a fluorescent plate reader (530/590 nm filters).
Day 3: Data analysis. Please click here to view a larger version of this figure.



	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Pooled control	Pooled control	Pooled control	Positive control	QC (HDL)	QC (dys-HDL)	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
B	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17
C	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29
D	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 25	Sample 26	Sample 27	Sample 28	Sample 29	Sample 30	Sample 31
E	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43
F	Sample 44	Sample 45	Sample 46	Sample 47	Sample 48	Sample 49	Sample 50	Sample 51	Sample 52	Sample 53	Sample 54	Sample 55
G	Sample 56	Sample 57	Sample 58	Sample 59	Sample 60	Sample 61	Sample 62	Sample 63	Sample 64	Sample 65	Sample 66	Sample 67
H	Sample 68	Sample 69	Sample 70	Sample 71	Sample 72	Sample 73	Sample 74	Sample 75	Sample 76	Sample 77	Sample 78	Sample 79

Figure 3: Representative layout of 96 well plates that are typically used in the fluorochrome HDL function Assay.
Differences in timing of addition of HDL samples into wells of a plate can lead to differences in spontaneous oxidation between different wells. To minimize experimental variability, avoid variable spontaneous oxidation between different wells and ensure that addition of reagents and samples will be done consistently and in a timely manner, it is recommended that all additions of samples (e.g. 160 µL) are first done in a separate 96 well round-bottom, clear, polystyrene or polypropylene plate (Plate 1). Then a multichannel pipette can be used to transfer specific volumes (e.g. 50 µL) into 3 96-well plates (Plates 2-4: polypropylene, flat bottom, black plates) (that have identical layout). Please click here to view a larger version of this figure.

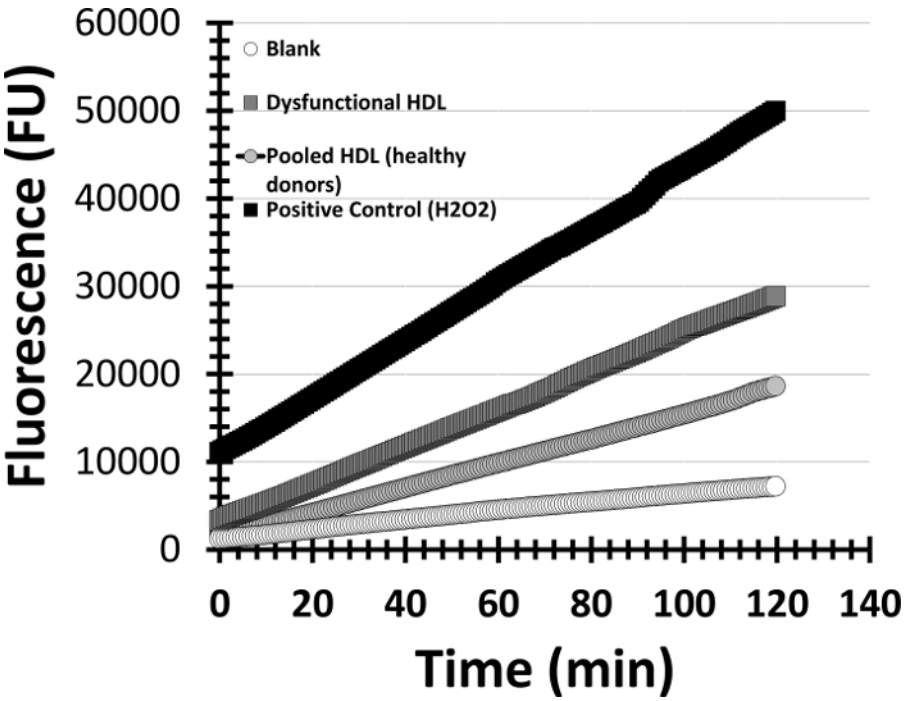


Figure 4: Representative data of HDL lipid peroxidation assay.
The fluorescent readout (in the dark) is then assessed every minute over 120 minutes at 37 °C with a fluorescent plate reader (530/590 nm filters). Representative data (arbitrary units) are shown for blank (no HDL; negative controls), positive control (H_2O_2), pooled HDL control and HDL from patient known to have dysfunctional HDL (based on two independent HDL function assays-cholesterol efflux and monocyte chemotaxis assay). Please click here to view a larger version of this figure.

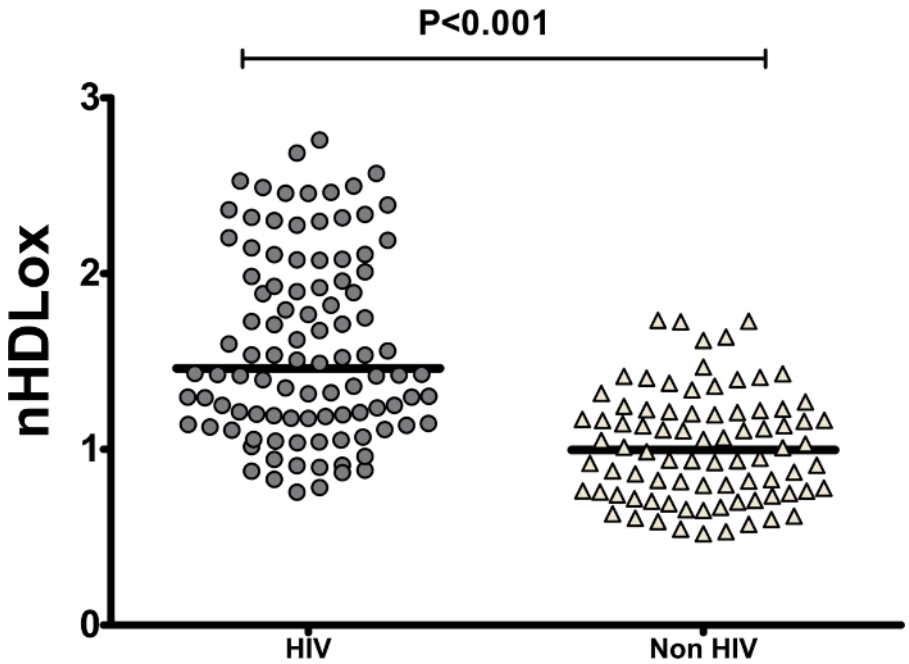


Figure 5: The lipid peroxide assay of HDL function can detect high lipid peroxide content per specific amount of HDL-C *in vivo*. HDL was isolated and HDLox was determined as described in the Protocol in 50 healthy subjects and 100 patients with HIV infection. The HIV-infected persons had higher HDLox (1.59±0.53) compared to the controls (1.01±0.31) (p<0.001). This figure has been modified [32]. Please click here to view a larger version of this figure.

Discussion

The protocol described here offers a robust tool to answer important research questions regarding the role of HDL function in atherosclerosis and human disease. The assay quantifies the HDL lipid peroxide content per mg of HDL-C using enzymatic amplification (HRP). This approach avoids known limitations of prior HDL function assays (e.g. the cholesterol efflux assay) including significant heterogeneity with regards to types of cells used, type of readout reported, lack of standardization and confounding effects of triglycerides[7,15,32]. Determination of biochemical rather than biological properties (e.g. cholesterol efflux) of HDL may be more reproducible. The inter-assay experimental variability of <10% compares favorably with the cell-based assays of HDL function, where interassay experimental variability is often >15% (or not reported). In addition, this approach may limit biochemical interactions between lipids and fluorescent probes[32]. Determination of HDL oxidation is relevant in the context of cardiovascular diseases given the key role of oxidative stress within the arterial wall in pathogenesis of atherogenesis [46]. We have used the fluorochrome HDL function assay to detect impaired HDL function in states of chronic oxidative stress such as atherosclerosis and Human Immunodeficiency Virus infection (HIV)[32]. Using biochemical assay of HDL function, several studies have confirmed the association of HDLox with obesity and cardiovascular disease [19,21,27,34,35,36,47]. Significantly, in human pilot studies we demonstrated associations of HDLox with validated cell-based assays, surrogate measures of cardiovascular disease, systemic inflammation, immune dysfunction and associated cardiovascular and metabolic risk phenotypes[32,36]. Although this assay has been used in several studies to address the role of HDL function in different diseases such as chronic HIV infection[32,38], atherosclerosis [32] and obesity[36], it remains to be validated in large scale studies with available clinical endpoints of CVD (e.g. heart attacks).

The reaction of the fluorochrome with peroxides in the presence of HRP to produce highly fluorescent resorufin is well established [48,49]. HRP catalyzes the oxidation of non-fluorescent fluorochrome to fluorescent resorufin red[50,51]. This oxidation can be driven by both endogenous peroxides present in the reaction (OH-) and HDL-lipid peroxides (LOOH-). Without cholesterol oxidase, resorufin (with HRP) can quantify the intrinsic HDL lipid peroxide content of a specific amount of HDL cholesterol. The background production of OH- as a result of air oxidation of the buffer is subtracted from the fluorescent readout of each well. Resorufin has minimal autofluorescence in most samples. Addition of catalase (1-4 U/mL) in the reaction buffer can quickly attenuate endogenous peroxides so that the increase in the fluorescent readout over time is driven by HDL lipid peroxides. The assay is versatile and can assess lipid peroxide content in HDL cholesteryl esters versus free HDL cholesterol[32,48,49] .

There are many variations of the fluorochrome HDL function Assay[32]. Briefly there are at least 3 major approaches: a) add a specific volume of HDL (e.g. 50 µL) per well and later normalize the HDL lipid peroxidation value by the level of HDL-C as determined in the clinical laboratory; b) determine the concentration of HDL-C in each sample based on standard colorimetric cholesterol assays and then add a specific amount of HDL-C (e.g. 1 µg) per well; and c) normalize the HDL function readout by HDL or apoA-I protein content[32]. There are also at least 3 major approaches on how to isolate HDL for HDL lipid peroxidation assays: a) HDL Cholesterol Precipitation e.g. with polyethylene glycol; b) Immunoaffinity capture; and c) other standard not high-throughput methods for HDL isolation such as µLtracentrifugation[32]. In addition, the assay is versatile and can assess lipid peroxide content in HDL cholesteryl esters versus free HDL cholesterol [32]. There are several ways to report the results e.g. arbitrary fluorescence units, standardized resorufin fluorescence units, normalized ratio to a pooled control[32]. Herein we present the most reproducible approach.

If plasma is used it is important to prepare plasma in tubes that have sodium citrate as the anticoagulant [27,28]. EDTA[40] and heparin sulfate can interfere with oxidation reactions [41,42]. Heparin sulfate can interfere with biochemical assays of HDL function [27,28]. Although cryopreserved serum and plasma are suboptimal for determination of lipids, HDL function and HDL lipid peroxidation, cryopreserved samples can quantify

relative differences in HDL lipid peroxidation per mg of HDL. It is important to process all samples within a specific study in the exact same way (e.g. same freeze-thaw cycles) and include a pooled control in each plate to account for potential confounders related to differences in sample processing among different studies. Long-term cryopreservation can compromise the results of HDL function assays[43] but relative differences in HDL lipid peroxidation among samples within one study may still be assessed if an appropriate pooled control is used [27,28].

Importantly, prior HDL function assays are not standardized. Here we describe an approach where use of appropriate controls ensures standardization of the readout. More specifically there are several known parameters that may affect the readout of biochemical assays of HDL function such as matrix effects (serum vs method of plasma preparation, presence of albumin and other proteins), cryopreservation, freeze-thawing[32]. The HDL function fluorochrome assay can be standardized with the use of commercially available standards and experimental reagents[32]. However, there are also several unknown (or poorly characterized) confounders in each study and among different persons that may affect determination of HDL function results. Thus, in each plate we use a pooled HDL control prepared from the specific specimens of interest within a given study (that have been processed identically) that minimizes artifacts and confounders[32]. Use of plasma blood bank samples and HDL-C values from the clinical laboratory can further standardize the assay[32].

We have previously shown that different methods of HDL isolation can significantly affect the readout of biochemical assays of HDL function [27,28,32] but the fluorochrome can reliably measure HDLox irrespective of the HDL isolation [32]. HDL isolation methods are a major limiting factor for high-throughput studies of HDL function. Ultracentrifugation is considered the reference method today, but remains a time consuming method[52]. Electrophoresis is imprecise and not standardized [53]. HDL precipitation methods involve the use of polyanions such as polyethylene glycol (PEG) to precipitate low density lipoproteins leaving the HDL in the supernatant [54]. Although these methods displayed certain drawbacks such as variable results with lipemic serums and interferences with enzymatic cholesterol procedures [55] prior modifications on the original procedure using standardized commercially available reagents yield a simple, reliable and accurate procedure[56]. Although PEG precipitation is commonly used method to isolate HDL[21,57] from patient serum, a major issue is the presence of non-HDL proteins such as albumin[58]. Immunoaffinity isolation can be used to minimize the effect of albumin on HDL function [32]. Although relative differences in HDLox for a specific capture method can be assessed, specific antibodies against HDL may not fully capture complex HDL structures. Using appropriate controls as described in this protocol, relative differences in HDLox among HDL specimens (isolated by PEG precipitation) can be reliably determined.

This assay, like all other assays of HDL function, has key limitations. *In vivo* oxidation of HDL in the arterial wall is quite complex and heterogeneous and lipid peroxide content may only partially reflect HDLox[46]. HDL structure and function continuously changes *in vivo* and measurement of HDL function at one timepoint may not reflect the impact of HDL dysfunction in end organ disease over time[59]. It is not known whether the HDLox readout should be normalized by HDL-C, or HDL protein [22]. Future clinical studies should validate the importance of the readout of the assay (HDL lipid peroxide content per mg of HDL-C).

In conclusion, the fluorochrome HDL function assay is a reliable method for determination of the HDL lipid peroxide content per specific amount of HDL (HDLox). Higher HDLox values are associated with reduced HDL antioxidant function. The readout of this assay is associated with validated cell-based assays, surrogate measures of cardiovascular disease, systemic inflammation, immune dysfunction and associated cardiovascular and metabolic risk phenotypes [19,21,27,32,34,35,36,37,38,39,47]. This method offers a convenient, yet robust tool for examining the role of HDL function in human disease.

Disclosures

This protocol and assay are relevant to the patent PCT/US2015/018147.

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