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Fast and simplified method for high through-put isolation of miRNA from highly purified high density lipoprotein

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Abstract:	Small non-coding RNAs (miRNAs) have been implicated in a variety of human diseases including metabolic syndromes. They may be utilized as biomarkers for diagnosis and prognosis or may serve as targets for drug development, respectively. Recently it has been shown that miRNAs are carried in lipoproteins, particularly high density lipoproteins (HDL) and are delivered to recipient cells for uptake. This raises the possibility that miRNAs play a critical and pivotal role in cellular and organ function via regulation of gene expression as well as messenger for cell-cell communications and crosstalk between organs. Current methods for miRNA isolation from purified HDL are impractical when utilizing small samples on a large scale. This is largely due to the time consuming and laborious methods used for lipoprotein isolation. We have

	developed a simplified approach to rapidly isolate purified HDL suitable for miRNA analysis from plasma samples. This method should facilitate investigations into the role of miRNAs in health and disease and in particular provide new insights into the variety of biological functions, outside of the reverse cholesterol transport, that have been ascribed to HDL. Also, the miRNA species which are present in HDL can provide valuable information of clinical biomarkers for diagnosis of various diseases.
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January 11th 2016

To,
Dr. Nandita Singh
Senior Science Editor
JoVE

Dear Dr. Nandita:

We are hereby submitting the revised version (R2) of invited manuscript entitled ***"Fast and simplified method for high through-put isolation of miRNA from highly purified high density lipoprotein"*** by Mulugeta Seneshaw, Faridoddin Mirshahi, Hae-Ki Min, Amon Asgharpour, Shervin Mirshahi, Kalyani Daita, Sherry Boyett, Prasanna K. Santhekadur, Michael Fuchs and Arun J. Sanyal for publication in ***JoVE***.

We thank the reviewers for their valuable time and effort and highly appreciate their inputs which definitely helped improve the quality of our manuscript. We have taken all their comments to heart and have worked to incorporate all of their suggestions into the revised manuscript. Our specific responses to individual comments are also provided.

We have thus revised the manuscript in accordance with the reviewer's suggestion and hopefully have addressed all their concerns. We once again thank the editor and reviewers in processing and reviewing the manuscript. We hope this revised manuscript will now be considered suitable for acceptance for publication. We look forward to hearing from you regarding our submission. We would be glad to respond to any further questions or comments that you may have.

Sincerely,

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TITE:
Fast and simplified method for high through-put isolation of miRNA from highly purified high density lipoprotein

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KEYWORDS:
High density lipoprotein, isolation, density gradient ultracentrifugation, plasma, microRNA, exosomes.

SHORT ABSTRACT:
MicroRNAs play an important regulatory role and are emerging as novel therapeutic targets for various human diseases. It has been shown that miRNAs are carried in high density lipoproteins. We have developed a simplified method to rapidly isolate purified HDL suitable for miRNA analysis from human plasma.

LONG ABSTRACT:
Small non-coding RNAs (miRNAs) have been implicated in a variety of human diseases including metabolic syndromes. They may be utilized as biomarkers for diagnosis and prognosis or may serve as targets for drug development, respectively. Recently it has been shown that miRNAs are carried in lipoproteins, particularly high density lipoproteins (HDL) and are delivered to recipient cells for uptake. This raises the possibility that miRNAs play a critical and pivotal role in cellular and organ function via regulation of gene expression as well as messenger for cell-cell communications and crosstalk between organs. Current methods for miRNA isolation from purified HDL are impractical when utilizing small samples on a large scale. This is largely due to the time consuming and laborious methods used for lipoprotein isolation. We have developed a simplified approach to rapidly isolate purified HDL suitable for miRNA analysis from plasma samples. This method should facilitate investigations into the role of miRNAs in health and disease and in particular provide new insights into the variety of biological functions, outside of the reverse cholesterol transport, that have been ascribed to HDL. Also, the miRNA species which are present in HDL can provide valuable information of clinical biomarkers for diagnosis of various diseases.

INTRODUCTION:
MicroRNAs are endogenous non-coding tiny RNA species that are highly conserved and are considered key players in the regulation of various biological processes by degrading or repressing specific target messenger RNAs[1]. Because miRNAs act intracellularly they have been explored as tissue-derived biomarkers which led to the discovery of tissue-specific functions of these miRNA. However, miRNAs are also found extracellularly either associated with proteins or in exosomes/micro vesicles that effectively can shield them from degradation by extracellular RNases[2]. More recent studies have shown that the protective effect of HDL may not be closely linked to its capability to promote cholesterol efflux but rather to its non-cholesterol cargo, in particularly as a circulating miRNAs carrier [3, 4]. These miRNAs may not only modulate lipid metabolism but are also associated with anti-inflammatory, antioxidant and antithrombotic effects of the HDL-miRNA complex [5, 6].

To further explore the role of miRNAs carried in HDL particles, a simple and easy protocol needs to be established for miRNA extraction from isolated highly purified HDL for use in clinical routine. Numerous methods have been described to isolate HDL. These methods are either very time consuming or require large volume of plasma that may require sample pooling, extensive dialysis for desalting isolated lipoproteins and they do not completely remove exosomes as a source of miRNAs[3], respectively. Here we describe a simple and rapid method that can isolate miRNA from highly purified HDL utilizing small volume of blood samples on a larger scale. We believe that this method may serve as good reference to promote research into the role of circulating miRNAs and in particular the role of HDL in facilitating communication between various cells and organs.

PROTOCOL:

1. Collection of Blood samples.

1.1. Collect fasting peripheral venous blood samples into 10 ml plastic tubes containing anticoagulant Ethylenediaminetetraacetic acid (EDTA) (which has several advantages over other anticoagulants) by standard venipuncture of a prominent vein in the antecubital fossa.

1.2. Centrifuge the blood samples at 1,600 x g for 20 min at 4 °C in a tabletop centrifuge to obtain plasma free of red blood cells and small amounts of RNA.

1.3. Sequentially centrifuge the supernatant at 3,000 g (4 °C) in a swinging bucket rotor for 10 min to remove WBC & Platelets and then additional 15 min to remove remaining cell debris respectively.

1.4. Measure the density of the plasma using a densitometer at room temperature as per manufacture instructions. NOTE: Adjustment of the density (d=1.023g/ml) with 0.9% saline solution may be required after removal of exosomes but prior to density gradient ultracentrifugation.

2. Exosome removal from plasma.

2.1. Remove the circulating exosomes that have a density similar to HDL and represent a quantitatively significant source of miRNA[3].

2.1.1 Do this by adding 252 µl exosome precipitation solution to 1 ml plasma followed by incubation for 30 min at 4 °C. To pellet out the exosomes, centrifuge the mixture for 30 min at 1,500 g at 4 °C.

2.1.2. To isolate HDL, transfer 1 ml of the resulting supernatant to a polycarbonate thick-walled ultracentrifuge tube for further processing with density gradient ultracentrifugation (see below).

3. Density gradient ultracentrifugation (Fig. 1).

3.1. To seperate HDL use a 3-step process employing a floor ultracentrifuge with a fixed-angle rotor operating at 448,811 x G and 8 °C, respectively.

3.2. Prepare three different density solutions sequentially and fresh for each isolation.

3.2.1 Prepare Solution A (isolation of VLDL, d=1.006 g/ml) by dissolving 11.4 g NaCl (NaCl: 0.195 mol), 0.1g EDTA2Na and 1 ml 1N NaOH in 1000ml of autoclaved-distilled water. Then add an additional 3ml of autoclaved-distilled water.

3.2.2 Prepare Solution B (isolation of LDL, d=1.182 g/ml) by adding 25.2 g NaBr to 100ml solution A (NaCl 0.195 mol, NaBr 2.44 mol).

3.2.3 Prepare Solution C (isolation of HDL, d=1.470g/ml) by mixing 78.8g NaBr with 100ml of solution A (NaCl 0.195 mol, NaBr 7.7 mol). Confirm the appropriate density at room temperature using a densitometer. Keep all solutions at 4 °C until further use.

4. Isolation of VLDL.

4.1. Mix 1 ml of plasma (average density = 1.023 g/ml) and nuclease free 200 µl of Fat Red 7B in a 6.5 ml polycarbonate thick-walled ultracentrifuge tube.

4.2. Then carefully layer 5 ml of solution A on top of the mixture. If needed, add additional Fat Red 7B on top of solution A to balance the weight of each tube. Centrifuge for 2 hr (acceleration - 5), (deceleration - 7). NOTE: During centrifugation, the lipoproteins are accumulated as a band at their equilibrium density regions.

4.3. At the end of the run observe 2 layers. Remove 1.5 ml of the VLDL fraction representing the top layer and store at 4 °C.

4.4. Finally, using a pipette transfer 4 ml from the bottom of the tube containing the LDL and HDL fraction to a new polycarbonate tube for LDL isolation.

5. Isolation of LDL.

5.1. Mix 2 ml of solution B and 100 µl nuclease free Fat Red 7B into the tube containing the LDL and HDL fraction (section 4), respectively.

5.2. Then centrifuge out for 3 hr (acceleration 9, deceleration 7). Thereafter, remove 1.5 ml of the LDL fraction representing the top layer and keep at 4 °C or store at -80 °C. Finally, transfer 4 ml from the bottom of the tube containing the HDL fraction to a new polycarbonate tube.

6. Isolation of HDL.

6.1. Mix 2 ml of solution C, 100 µl nuclease free Fat Red 7B and 15 µl of 98% β-mercaptoethanol into the tube containing the HDL fraction, respectively.

6.2. Centrifuge for 3 hr (acceleration 9, deceleration 7). Thereafter remove 2 ml of the HDL fraction representing the top layer and either keep at 4 °C or store at -80 °C.

7. Desalting and concentration of lipoprotein fractions.

7.1. To avoid interference with subsequent agarose gel electrophoresis and PCR, remove excessive salt added during density gradient ultracentrifugation using centrifugal filter devices with the appropriate molecular weight cutoff (3K tube for VLDL and 15K tube for LDL/HDL) as described by the manufacturer's instructions.

7.1.1 Briefly, after adding 2.5ml cold PBS (137 mM NaCl, 2.7 mM KCL, 8 mM Na2HPO4, 2 mM KH2PO4; pH 7.4) centrifuge the entire VLDL fraction collected-during density gradient ultracentrifugation at 4 °C for 60 min using a swinging bucket rotor.

7.2. Desalt the LDL fraction twice with 10ml PBS for 30 min each. Next, Use 13 ml PBS twice for desalting the HDL fraction. The higher PBS volume is necessary to improve mobility with agarose gel electrophoresis. After centrifugation, remove the lipoprotein containing solutes and keep at 4 °C or stored at -80 °C.

8. Agarose gel electrophoresis.

8.1. Perfrom lipoprotein agarose gel electrophoresis employing the kit with minor modifications of the manufacturer's instructions as follows NOTE: This step is just to assess the quality and purity of the concentrated lipoprotein samples.

8.1.1 Briefly, obtain 6 µl of the desalted lipoprotein fraction with density gradient ultracentrifugation and load onto a pre-cast lipoprotein gel. Use human lipoprotein standards for VLDL, LDL and HDL as size reference. Carry out electrophoresis at room temperature at 100 V for 60 min using Rep Prep buffer.

8.1.2 Dry the gel for 10 min and then stain for 10 min at room temperature with Fat Red 7B. Destain the gel in a mixture of methanol-water 75:25 (v/v) and dry again for 5 min.

9. RNA extraction and purification.

9.1. Perfrom isolation of miRNA by purified human HDL using the serum/plasma miRNA isolation and purification kit.

9.1.1 Briefly, add 1 mL of RNA lysis reagent to 200 µl of purified HDL, mix with a vortexer and then incubated for 5 min at room temperature to ensure complete dissociation of nucleoprotein complexes and inactivation of RNases.

9.1.2 Then spike 3.5 µl of synthetic Caenorhabditis elegans microRNA (cel-miR-39; 1.6 x 10^8 copies/µl) into the mixture. Then carry out RNA extraction according to the manufacturer's instructions.

9.2. Perform purification of extracted-miRNA with elute spin columns as per manufacturer's instructions. Measure the concentration of miRNA from purified HDL with a spectrophorometer. NOTE: Elution of miRNA from the spin columns employed 16 µL of RNase-free water.

10. Reverse transcription (RT-PCR).

10.1. Isolate 100 ng of the miRNA from HDL spiked with synthetic miRNA (cel-miR-39)

and reverse-transcribed in a 20µL reaction volume employing the reverse transcription kit and according to the manufacturer's instructions.

10.2. Perform appropriate controls without template miRNA (NTC) and without reverse transcriptase enzyme mix (NRT).

11. Real-time PCR (qRT-PCR).

NOTE: All primers used were purchased from commercial companies.

11.1. Perfrom Real-time PCR in a total volume of 20 µl with 2 µl of a 1:2 dilution of the cDNA, 10 µl PCR mix, 2 µl universal primer, 2 µl of miRNA primers and 4 µl RNase-free water.

11.2. Run the reaction in 96-well plates at 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 s and 55 °C for 30 s and an extension phase at 70 °C for 30 s. Perfrom all reactions in triplicates.

11.3. Next, Calcuate relative quantities of miRNA by using the $2^{-\Delta\Delta}$Ct method after normalization to the synthetic housekeeping gene as per manufacturer's instructions .

REPRESENTATIVE RESULTS:

Isolation of high density lipoprotein after removal of exosomes

To obtain miRNA from highly purified HDL it is necessary to remove exosomes that represent a source of miRNA contamination[7]. This was done prior to density gradient ultracentrifugation with a commercially available kit. For practical purposes a three step standard density gradient ultracentrifugation protocol developed by commercial company was modified (**Fig 1**). This protocol requires centrifugation with a fixed-angle rotor at a speed of 140,000 rpm which is substantially faster than commonly used protocols with centrifugation forces up to 54,000 rpm[3, 8, 9, and 10]. Employing a widely available T-1270 rotor with a maximum force of 70,000 rpm, centrifugation was initially carried out with polyallomer tubes which have failed to resist the centrifugation forces. To avoid collapse of tubes, polycarbonate tubes were successfully used. Centrifugation time is critical for lipoprotein oxidation and potentially miRNA degradation[11]. Therefore several different centrifugation times, ranging from a total of 8 to 96 hr were tested. Furthermore temperature at which centrifugation was carried out was adjusted based on centrifugation time and force, respectively.

Purity of the high density lipoprotein fractions

Purity of isolated high density lipoprotein fractions were checked with agarose gel electrophoresis. **Fig 2.** illustrates a typical electrophoresis result for the HDL subtraction isolated by gradient ultracentrifugation. It clearly showed that HDL was devoid of any contamination of VLDL and exhibits typical a-mobility. The absence of any a-migrating lipoproteins in the VLDL and LDL fractions demonstrates the complete recovery from HDL during the ultracentrifugation step. The HDL fraction, however, also showed some trace of b-mobility, a known electrophoretic banding pattern due to contamination with lipoproteins (Lp(a)) [12].

Elimination of Lp(a) from isolated HDL

Studying miRNAs carried in HDL requires isolation of HDL of highest purity. Interference of Lp(a) with HDL potentially contributes to cross-contamination of HDL with miRNAs carried in LDL. To minimize this contamination of HDL, 15 ul of β-mercaptoethanol [10] was added to solution C during the last centrifugation step (**Fig 1**). Addition of β-mercaptoethanol has been shown not to change HDL density properties[10]. As displayed in **Fig 3.**, addition of β-mercaptoethanol resulted in the absence of any b-migrating lipoproteins consistent with very effective removal of Lp (a).

Purification of miRNA from HDL

Isolation of miRNA was initially attempted employing lysis reagent which is commonly used for RNA extraction from blood. Although this method resulted in a very good RNA yield (91.45 ng/µl) but after spectrophotometric analysis showed unsatisfactory RNA purity. Additional purification of the isolated RNA by removing phenol improved the purity but the RNA yield was now significantly low. Next, another kit was tested which showed acceptable RNA purity (260/280 nm ratio of 1.7) but the RNA yield was 26-fold lower compared with the lysis reagent (3.45 vs. 91.45 ng/µl). The best results for acceptable RNA purity with a good yield were obtained with the miRNeasy serum/plasma kit (48.2 ng/µl; 260/280 nm ratio of 1.6) and therefore this extraction procedure was subsequently employed for the detection of miRNA from the isolated HDL lipoprotein fraction.

To demonstrate the feasibility to quantify miRNAs carried in human HDL purified with this method, we chose to amplify miR-223. This miRNA was chosen because miR-223 was identified in cargo of HDL3 and was shown to repress HDL cholesterol uptake5. The **Fig 4 A**. shows a typical log plot of amplification curves comparing baseline threshold and threshold cycle values after optimization of the PCR reaction for the miR-223 and the spiked-in Cel-mir-39. This synthetic gene was used as internal control as there are no reports of known normalization control for miRNA in plasma. In addition, several potential established endogenous housekeeping genes like RNU6-2, RNU-48, HY3 could not be detected and SNORD95 could be detected in purified HDL, but, the difference in Ct values of miR-223 and SNORD95 is less than five (data not shown). Melting curve analysis illustrated in **Fig 5.** clearly shows a distinct single peak consistent with amplification of a selective miRNA in the preceding PCR. As illustrated in Fig 4 B., both miR-223 and the reference Cel-mir-39 could consistently be detected in all six pro bands. Relatively small variations among all individuals were observed for Cel-mir-39 compared with miR-223. These findings support isolation of HDL at high purity to allow detection of its miRNA cargo.

Detection of miR-223 in purified HDL

Real time quantitative PCR method was used to quantify double hairpin-structure miRNA precursors to single strand miRNA. This method needs a forward/reverse gene-specific primers and a thermostable reverse transcriptase to convert the hairpin structure of the miRNA to cDNA. The cDNA was subsequently amplified and quantified using real-time qPCR with the help of SYBR green detection. MicroRNA from serum,

plasma and purified HDL plasma can be accurately profiled using the miRNA RT PCR system.

Our experimental product of mean miR-223 gene expression showed a Ct value of 30.9 in purified HDL and its corresponding negative controls were above cut-off 35 (NTC = 38.1 Ct value, NRT and NAC were not amplified). In this experiment, we saw in the purified HDL samples the formation of Primer-dimer with low melting temperature (73 °C in miR-223 and 75.5 °C in Cel-miR-39) and higher C_t value than the specific miRNA assays (**Table 1**). The Primer-dimers in **Table 1.** occur probably because of high concentration of primers in the solution. This occurs mostly when primer molecules attach to each other after 30 PCR cycles [13]. This allows them to anneal to other primer molecules and in effect, become linear mini-templates and it will cause higher background and may lead to a generation of C_t value < 40 for NTC (No template control) samples.

FIGURE LEGENDS:

Figure 1.Schematic representation of the HDL isolation procedure. HDL was prepared by density gradient ultracentrifugation in a series of three centrifugation steps. Distribution of lipoprotein bands and intermediate fractions in the density gradient are illustrated.

Figure 2. Agarose gel electrophoresis of lipoproteins isolated without β-mercaptoethanol. VLDL, LDL and HDL fractions isolated by density gradient ultracentrifugation without adding *β*-mercaptoethanol to solution C demonstrate the presence of Lp (a) in the HDL fraction. Lanes 1, 2, 9, and 10 each represent the size marker; Lanes 3/4, 5/6 and 7/8 represent VLDL, LDL and HDL respectively.

Figure 3. Agarose gel electrophoresis of HDL isolated with β-mercaptoethanol. Adding *β*-mercaptoethanol to solution C before the last centrifugation step resulted in the removal of Lp(a) from the HDL fraction. Lanes 1, 2, 9 and 10 each represent the size marker; Lanes 3-8 represent HDL.

Figure 4. Amplification plot of two different miRNAs and expression of Cel-miR-39 and miR-223. (A) Real-time quantitative PCR employing isolated HDL was carried out as described. The PCR was run for 45 cycles and the point at which the curve intersects the threshold is the C_T-Value. The data show the expression of Cel-miR-39 (left panel) and miR-223 (right panel), respectively. The horizontal line represents the detection threshold. A cycle number of 35 was set as cutoff for positive amplification. (B) Representative real-time PCR data from isolated HDL obtained from 6 samples are shown. Raw mean Ct values are shown for Cel-miR-39 and miR-223, whose expression levels vary less than 2 Ct value in purified HDL plasma fraction between 6 samples, each from a different donor. Expression profiling was performed using the PCR System and the Human Serum & Plasma miRNA qRT-PCR.

Figure 5.Melting curves for Cel-miR-39 and miR-223. As illustrated, the presence of a single peak indicates specific amplification of Cel-miR-39 (left panel) and miR-223

(right panel), respectively.

Table 1. Row C_t value of negative and positive control of synthetic Cel-miR-39 and miR-223 characterized in serum, plasma, cDNA, and purified HDL fraction. NTC (No template control), NRT (No reverse transcription enzyme), NAC (No amplification control, only water and reagents of qRT-PCR).

DISCUSSION:

Identification of novel biomarkers from blood will aid in the clinical diagnosis and prognosis of various diseases. MicroRNAs have known to possess all the qualities of biomarkers and have been shown in various studies [14-17]. In this study we have demonstrated rapid and simple easy method to isolate miRNA from plasma HDL. Conventional density gradient ultra-centrifugation method of isolation of VLDL, LDL and HDL depends on accurate sampling of plasma, precise preparation of the buffer solution, measurement of density and quantitative transfer of bottom lipoprotein fractions[8]. There are numerous methods that have been described to isolate HDL[8, 9,10 & 11]. These methods are often either very laborious or require large volume of plasma and extensive dialysis for desalting isolated lipoproteins and do not completely remove exosomes and lipoproteins as a source of miRNAs[3]. To address these shortcomings we have established this simple and fast method of isolation of miRNA from plasma HDL.

The key goal and primary purpose of this study was to separate and isolate HDL-miRNA complex by ultracentrifugation without RBCs, buffy coat, exosomes, secretory vesicles, VLDL, LDL and Lipoproteins (a) interference, then isolate miRNAs from purified HDL. Contamination and interference of any of these components with HDL will alter the expected experimental results and miRNA profiling data. Isolation of DNA, RNA and miRNAs from blood is a very difficult task due to its complex nature. It has always been posed with lot of technical challenges to nucleic acids (including miRNAs) extraction and purification compared to cells, tissues and organ samples[18]. Also there are relatively less miRNA genes present in circulating blood cells. Blood is a known carrier for exosomes, secretory vesicles and HDLs along with free circulating miRNAs, which are relatively less[18]. Therefore, larger amount of blood plasma sample volume is required for processing and isolation of HDL-miRNA complex. In addition, the short nature of the miRNAs and their target sequences, makes it even difficult to achieve sufficient specificity with standard PCR oligonucleotide technologies. The cellular components of blood are always know to secrete miRNA and mRNA in response to change in external environment including temperature, microorganisms and toxins or stress. Also presence of nucleases, RNases, circulating proteins and other enzymes in blood will affect these miRNA isolation.

The circulating miRNA amplification also lacks a well-established known housekeeping genes like β-actin or GAPDH for data normalization while using the $\Delta\Delta$Ct method of qRT-PCR. This again poses one of the major hurdle for miRNA profiling from circulating blood serum or plasma[19]. Commonly used short non coding snoRNAs and snRNAs are rarely expressed in blood and this makes it further difficult in utilizing them as internal control genes. To solve these demerits of snoRNAs and snRNAs and to avoid any

technical difficulties we used Serum/Plasma synthetic Spike-In control in the assays as internal control gene as followed according to the manufacturer protocol. It helps in normalization for any nonspecific amplification and changes occurred during miRNA purification and PCR reaction[18]. Even in the case of degradation of miRNA or absence of any specific miRNA amplification signals, the spike-in control should amplify in qRT-PCR reactions and give an expected signal with a reasonably constant and uniform Ct value. Based on the spike in control amplification along with three negative controls and positive controls we got good optimal data validation for miR-223 gene. This confirmed that we have got good yield of miR-223 from circulating plasma HDL from this simple method.

In conclusion, we have established a method of density gradient ultracentrifugation (DGUC) with the addition of β-mercaptoethanol for purification of miRNA from plasma HDL. The method has several advantages: (a) VLDL, LDL and HDL are separated within a short period of time by floor ultracentrifuge compare to other methods that needs 24-96 hr [8-11]; (b) LDL and HDL dialysis, desalting/concentrating, take place within one hour compare with other methods that took 24 or more hours[8,9]; (c) Contamination of Lipoproteins is removed by β-mercaptoethanol in HDL fraction, but is not intended to be used in the LDL fraction; (d) The sample volume required is only 1 ml for all stepwise VLDL, LDL and HDL isolations compare with the other methods that needs more specimen volume; (e) It minimized oxidative damage to HDL during isolation[11]; (f) A maximum of 12 samples can be analyzed in a single lipoprotein separation analytical run; (g) The 250 µL sample volume of extracted HDL is enough to analyse miRNA concentration/purity, agarose gel electrophoresis, protein concentration, microarray and RT-qPCR procedures. The limitations of our method is that small percentage of HDL-miRNA may lose during the exosome precipitation step and the required plasma sample volume should be more than 200 µl to isolate HDL-miRNA.

Finally, the plasma microRNAs are not only derived from damaged or renegade blood cells in the circulation but also from healthy normal blood cells and cells from other parts of the body which includes various healthy and diseased tissues and organs affected by ongoing health status of the body20. This present study has systematically purified mature miR-223 from isolated HDL of human plasma. This study clearly demonstrates that levels of mature miR-223 in the highly purified HDL plasma are detectable, stable, reproducible and consistent among individuals sample, thereby greatly facilitating clinical use of futurity tests for lipoproteins, liver, cardiovascular and other metabolic syndromes. Surprisingly, mature miRNAs, particularly miR-223 from HDL plasma are probably resistant to nuclease digestion and other harsh conditions which potentially explain the stability of miR-223 in purified HDL. The mechanism of resistance of miR-223 to RNases requires further study. Obviously, studying miRNA expression profiles in these purified HDL plasma would shed light on future miRNA markers in studying various maladies. These HDL associated microRNAs in the plasma can be used as a potential clinical diagnostic biomarkers and personalized medicine in the future.

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DISCLOSURES:
The authors have nothing to disclose.

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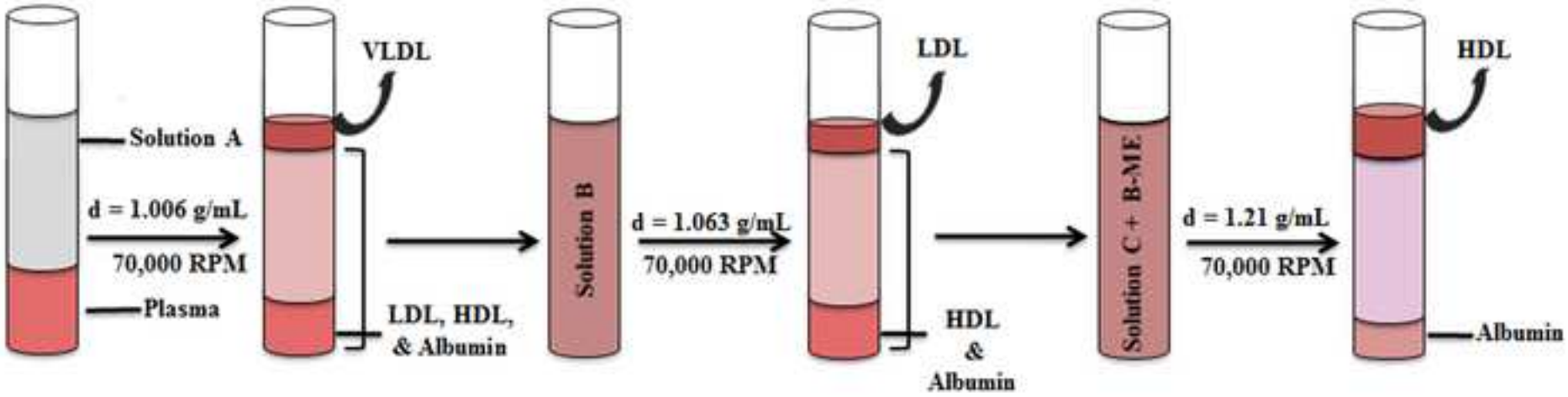

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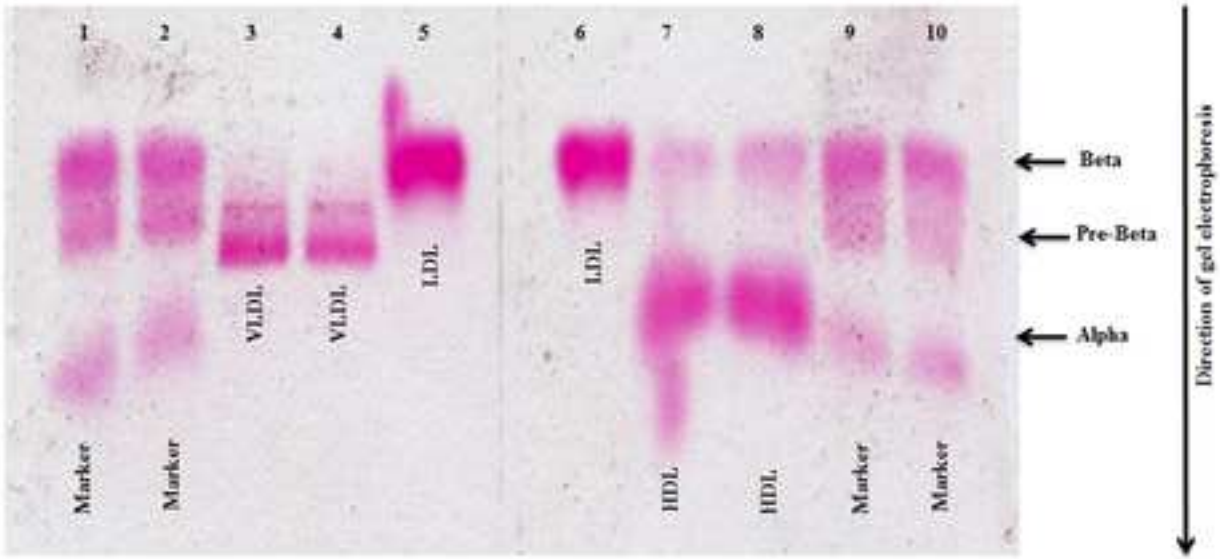

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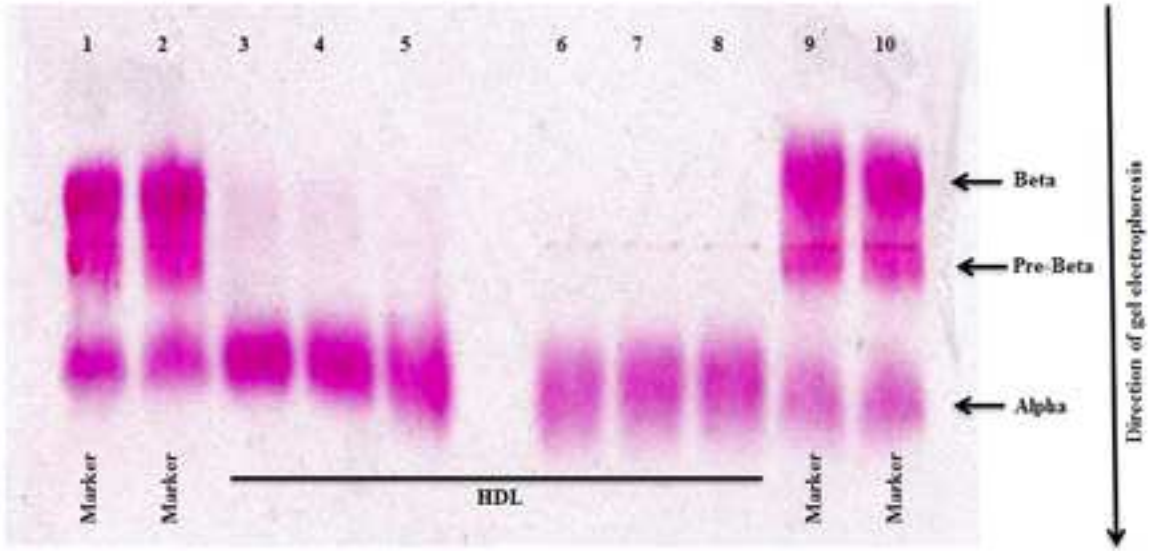

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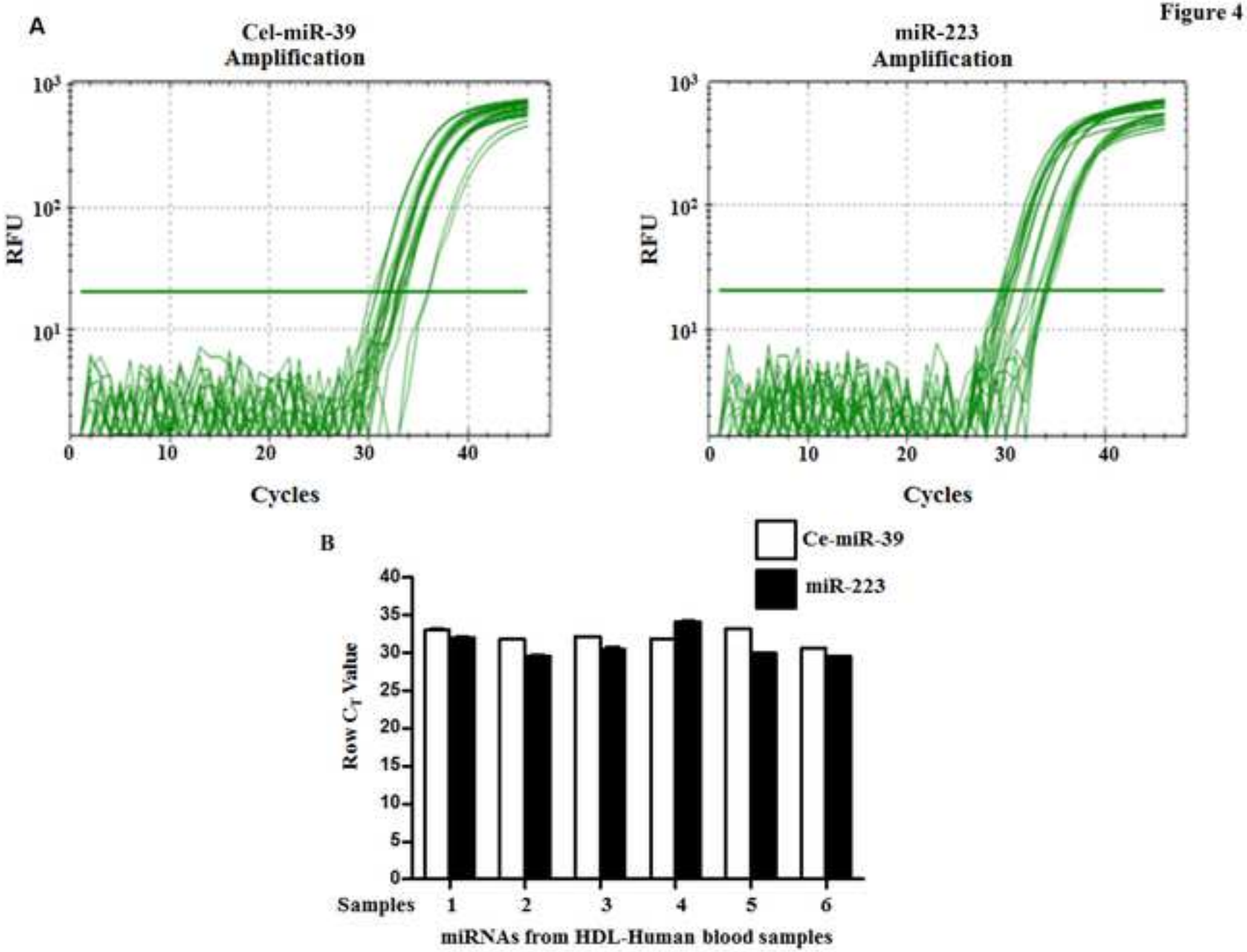

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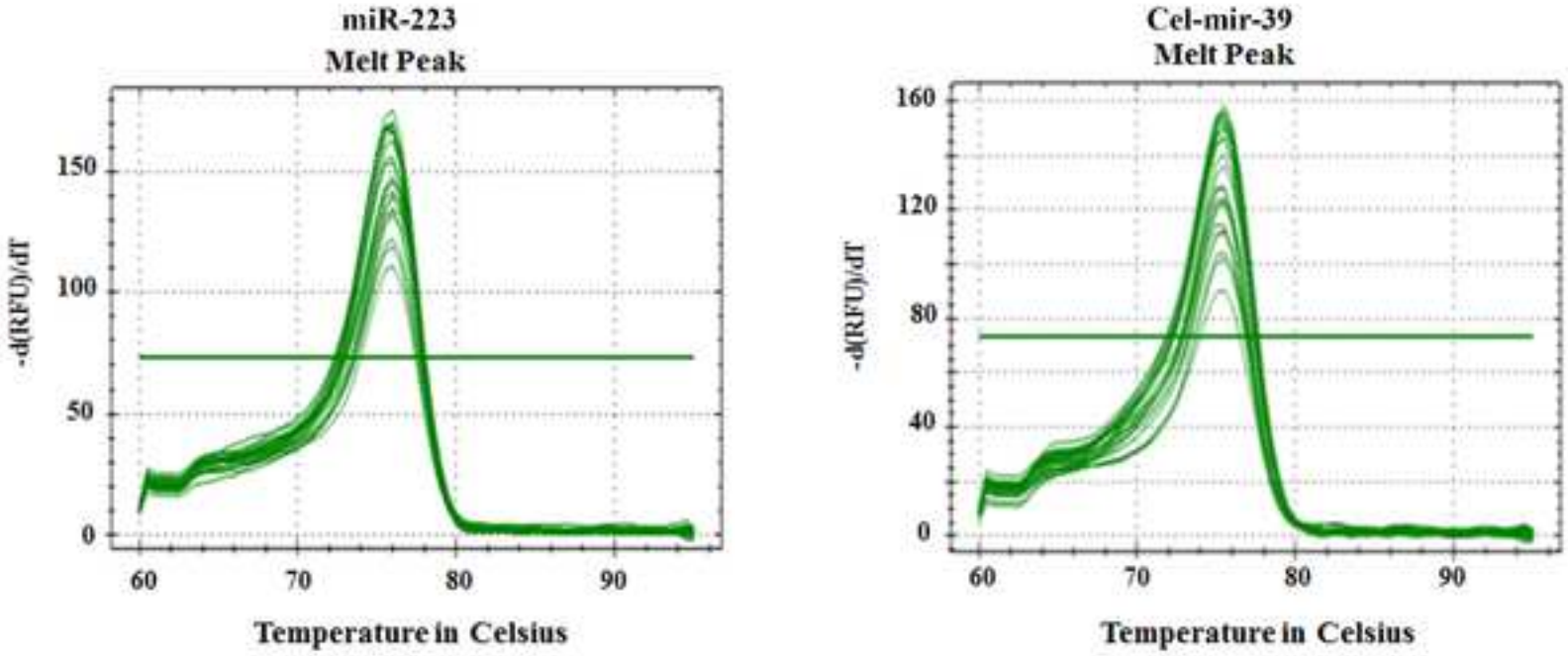

Table 1

Gene	microRNA	NTC	NRT	NAC	Sample Tm	NTC Tm
Cel-miR-39 added during miRNA extraction in the plasma	24	35	38.0	N/A	78.5 °C	None
Cel-miR-39 added after miRNA extraction in the plasma RT-PCR (cDNA)	21	35	38.5	N/A	78 °C	None
Cel-miR-39 added during miRNA extraction in the purified HDL plasma	32	37	39.1	N/A	76 °C	75.5 °C
Cel-miR-39 added after miRNA extraction in the purified HDL plasma RT-PCR (cDNA)	16	35	38.0	N/A	78.5 °C	78 °C
miR-223 in serum	22	33.5	N/A	N/A	75 °C	74 °C
miR-223 in plasma	20	33.5	N/A	N/A	75 °C	74.5 °C
miR-223 in purified HDL serum	31	33.5	N/A	N/A	74.5 °C	74 °C
miR-223 in purified HDL plasma	31	38.1	N/A	N/A	75.5 °C	73 °C

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog #
Plastic Vacutainer Lavender K2EDTA tubes	Becton, Dickinson and Company	366643
Centrifuge	Thermo Scientific, Sorvall Legend X1R	75004261
Densito 30PX densitometer	Mettler Toledo	MT51324450
ExoQuick solution	Invitrogen	4484451
Polycarbonate thick-walled ultracentrifuge tube	Thermo Scientific	O3237
Sorvall WX100 ultracentrifuge	Thermo Scientific	46902
Fat Red 7B	Sigma-Aldrich	201618
β-mercaptoethanol	Sigma-Aldrich	
Amicon Ultra-15 Centrifugal filter devices 10K	Millipore	UFC901008
Amicon Ultra-centrifugal filter devices 3K	Millipore	UFC800308
QuickGel Lipo kit	Helena Laboratories	3344,3544T
Human lipoprotein standards for VLDL, LDL and HDL	LipoTrol; Helena Laboratories	5069
Rep Prep buffer	Helena Laboratories	3100
RNeasy MinElute spin columns	Qiagen	
NanoDrop 1000 analyzer	Thermo Scientific	
miScript II RT Kit	Qiagen	218161
CFX96 Touch real-time PCR detection system	BioRad	
miRNeasy Serum/Plasma Kit	QIAGEN	217184
miScript Primer Assays	QIAGEN	141078139
miScript SYBR Green PCR Kit	QIAGEN	218073
miRNeasy Serum/Plasma Spike-In Control	QIAGEN	219610

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Response to Reviews

1) There are a number of formatting issues to be corrected:

-Steps 2.2-2.3 should be sub steps of 2.1

-3.3-3.5 should be sub steps of 3.2

-7.2-7.2 should be sub steps of 7.1

-8.2-8.3 should be sub steps of 8.1

-9.2-9.3 should be sub steps of 9.1

•Unnecessary branding should be removed:
-10.2 - miScript

-Figure 6 legend (miScript 2x)

-Trizol (Rep Results, Line 334/340)

-LipoTrol (Figure 2 legend (TM)).

We have corrected the formatting issues and removed all the commercial language as suggested by the editorial board.

2) If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from.” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

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We have included the available DOIs in the reference section.

4) NOTE: Please copyedit the entire manuscript for any grammatical errors you may find. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol. Please thoroughly review the language and grammar of your article text prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

We have copy-edited the manuscript for the grammatical errors and have thoroughly reviewed the language and grammar prior to resubmission.

5) NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

We have included a line-by-line response letter to the editorial and reviewer comments.

Reviewers' comments:

Reviewer #1:

Major Concerns:

Protocol:
1.1 Different anti-coagulants will likely alter the HDL miRNA profile, particularly heparin. Is EDTA preferred over sodium citrate? The authors should state the anti-coagulant in step 1.1.

We agree with the reviewer that different anti-coagulant will likely alter the HDL miRNA profile (Heparin). The advantage of EDTA over sodium citrate is to prevent autoxidation and also to chelate calcium, which is involved in various enzymatic reactions. EDTA has also been recommended as the anticoagulant of choice for hematological testing because it allows the best preservation of cellular components and morphology of blood cells and prevents miRNA from these cells in interfering with HDL miRNA. We have stated this in step 1.1.

1.3 What is the need to spin for 10 min and then 15 min? Why not just state 25 min here?

The reason behind centrifuging two times with 10 min and 15 mins is to remove WBCs and platelets first and then to remove remaining cell debris.

2.1 and 2.2 should be combined together into 2.1

We have combined 2.1 and 2.2 together into 2.1 as suggested by reviewer.

2.2 ExoQuick will likely break apart or affect all lipid structures, including lipoproteins. How do the authors know that lipoproteins, namely HDL, are not broken apart, and thus, lose miRNAs in this step? Moreover, ExoQucik may likely pellet lipoproteins or lipoprotein remnant proteins / lipids possibly containing miRNAs, thus depleting the HDL miRNA pool moving forward. A simple FPLC chromatogram showing the distribution of lipoprotein lipids, protein, and cholesterol in plasma before and after ExoQuick solution treatment and in the supernatant after pelleting is warranted.

ExoQuick will not break apart the HDL and other lipid structures. It will only pellet out exosomes and lipoprotein remnant proteins in a very small scale leaving behind high percentage of HDL, VLDL, LDL in supernatant. It is convinced from our studies that we have got good yield of HDL, VLDL, LDL in supernatant. This claim is also supported from commercial companies' technical note.

4.2 Most density gradient ultracentrifugation of lipoproteins (conventional method) requires 24 h at ~40,000 g. The method here only requires 2h at 7,000 g Can the authors describe what is different between the rapid method and the conventional method that allows such a big reduction in times and forces? Although Fig.1 states 70,000 g, which would make much more sense than the text for 3.1.

We have corrected the typo of density gradient ultracentrifugation timings from 7,000 to 70,000 for 2 hours in text.

7.1 How many times should the VLDL, LDL, or HDL be filtered down, resuspended, and re-filtered to remove salts from the lipoprotein concentrates - twice for LDL and once for VLDL and HDL?

We have filtered twice for HDL.

7.1 and 7.2 should be combined into 7.1.

We have changed 7.2 into the sub steps of 7.1.

7.2 How much volume and total protein of VLDL, LDL, and HDL are in the final concentrates? Are all the concentrates resuspended to the same volume or aliquoted for the same total

lipoprotein concentration for downstream RNA isolation steps? This would likely be important as if the samples are not normalized by volume or protein concentration across all samples the high variability in filtered concentrate volumes may add variability to miRNA

All the volumes of VLDL, LDL and HDL are in good yield and resuspended to the same volume and also we have measured HDL protein concentration and we have used equal amount of HDL for miRNA isolation (48.20ng/µl).

8.1 and 8.2 should be combined together into 8.1

We have changed 8.2 and 8.3 into sub steps of 8.1.

10.2 It may also be helpful to suggest a water control that underwent both the RT step and PCR.

We have used suitable negative control for that underwent both the RT step and PCR.

1. The authors claim the procedure is a method that alleviates some issues with conventional lipoprotein isolation methods that require large starting plasma volumes. This method starts with 1mL of plasma. Many stored lab plasma samples are either below 1 mL or are limited in supply and companies or labs may not want to distribute 1mL of plasma for this method. Moreover, mouse plasma samples almost always are below 1 mL in volume and would still need to be pooled for the method described here. How low in sample volume can this method go in terms of starting input and getting quality results at the end?

We can use minimum plasma volume of up to 250µl starting input to get quality result of miRNA at the end. Also, our method is fast and simple compared to the conventional method.

2. Can the authors show the data from the ultracentrifugation time optimization studies stated on line 308?

Data from the ultracentrifugation time optimization is out of the scope of this work for publication in JoVE.

3. Where are the data described in lines 363-365 comparing pre-miR-223 levels to mature miR-223 levels, etc.? If this was not done or shown, please remove text related to pre-miRNA analysis and comparison to mature miRNAs.

As suggested by reviewer, we have removed the text related to pre-miRNA analysis and comparison to mature miRNAs, which is not consistent with the data.

4. In lines 367-371… Are the samples from different human subjects? Were the serum, plasma, and HDL all matched for the subjects? How did the authors compare HDL values to serum and plasma values? For example, one is purified from the total pool. How was everything normalized to compare the concentrated HDL to the total plasma pool?

These samples are pooled from single human subject and plasma and serum HDL yield is similar. miRNA from serum and plasma has almost similar Ct values. Serum has 2 Ct values more compared to plasma.

5. Why did adding the spike-in (cel-miR-39) to the HDL prior to RNA isolation impact the Ct value so much more that spiking plasma prior to RNA isolation?

This is may be due to loss of small amount miRNA during processing of serum.

Minor Concerns:
1. (7.3) please change "Desalted the LDL" to "Desalt the LDL".

We have changed the text as suggested by reviewer.

2. (9.3) please change "1.6 x 108 copies/uL" to 1.6 x 10^8 copies/uL".

We have corrected as per the suggestion by reviewer.

3. Pease reference or state the commercial company that the density gradient ultracentrifuge protocol was modified from.

As per the Editorial board suggestion and instructions to the authors we have avoided all the commercial company names. We have modified our protocol from Thermo scientific company.

4. (8.1) I assume the agarose gel electrophoresis step is just to assess the quality and purity of the concentrated lipoprotein samples (e.g. LDL without HDL). Can the authors state this to not add confusion that the authors are suggesting that the gel electrophoresis be an additional purification step and that each lipoprotein class (e.g. VLDL) be cut from the gel and resuspended?

We have stated as per the suggestion of reviewer.

5. Please change "Next the another kit" to "Next, another kit" on line337. What was this other kit?

We have changed as per the suggestion of reviewer. We have mentioned the kit name previously. As per the suggestion by Editorial board and journal policy we have removed the kit name.

6. Please change "miRNA-223" to "miR-223" and "Ce-miRNA-39" to "cel-miR-39" throughout the article.

We have changed "miRNA-223" to "miR-223" and "Ce-miRNA-39" to "cel-miR-39" throughout the article as per the reviewer suggestions.

7. Please label the left and right panel for Figs.4 and 5.

We have labelled the left and right panel for Figs.4 and 5.

8. Line 362…Was this relative quantitative value based real-time PCR or true quantitative PCR with a standard curve using miR-223 and cel-miR-39 mimetics or single-stranded oligos?

This is relative quantitative value based on real-time PCR.

9. Due to the purity of the final product by electrophoresis and the exosome removal step, it is unlikely that contaminating exosomes and other lipoproteins account for variation between samples. I would remove text in lines 367- 371.

We have removed the text in lines 367-371 as per the suggestion by reviewer.

10. Another round of editing for grammar and spelling mistakes is needed.

We have edited for grammar and spelling mistakes.

Reviewer #2:

Minor Concerns:

1. The author mentioned removal of plasma exosome by using exosome precipitation solution (L 172). The author may need to detail the formula of the precipitation solution or whether it is commercially available and the company.

We have used ExoQuick solution to precipitate exosomes. This is a commercially available solution from Invitrogen. Due to journal policy we have avoided mentioning it in the text. We have provided all the detail of these chemical in chemicals list.

2. Descriptions for the contents in the bracket ln line 203, line 216 and line 225 (' acceleration X, deceleration X) should be given.

We have described the acceleration (increase of speed or velocity) and deceleration (slow down) in the text as suggested by the reviewer.

Reviewer #3:

Major Concerns:

1. According to the statement, the developed method in this manuscript was based on the known commercial methods, the author should give the compared results for the quality and quantity of miRNAs extracted from the commonly used methods and your improved methods.

Based on our experiments the quality of the miRNA isolated is very good. It is confirmed by our RT-PCR experiments, Quantity and Ct values shows good yield of miRNA. We also have microarray data for the result. Further, we have a plan to continue this work in clinical arena and this is out of the scope and for the publication in JoVE. Our method is very fast and simple in compare to commonly used HDL isolation and our HDL-miRNA isolation method is modified from previously published papers and also from commercially available ExoQuick kit.

2. It will be better to detect at least another known miRNA carried by HDL to demonstrate the robustness of the method.

We have detected some other miRNAs from this method as previously stated we have analyzed miRNA profile from microarray. This is out of the scope of this work for the

publication in JoVE. We have a plan to extend our study further by using this method in clinical and translational research.

3. The limitations of the present method should be discussed.

The limitations of this method is included in discussion section as suggested by the reviewer.

Minor Concerns:

1. It is described in representative results that "Therefore several different centrifugation times, ranging from a total of 8 to 96 hr were tested. Furthermore temperature at which centrifugation was carried out was adjusted based on centrifugation time and force, respectively." (line 307-310) How to decide which time point or temperature or force is the most proper one? What is the criteria? Please describe the results from experiments under different conditions.

Based on Thermo scientific technical note and previously published papers we have used S140-AT Thermo scientific rotor and 70,000rpm for total 480 min at 8 °C. We found that this is optimal temperature. Experiments under different condition is out of the scope of this work for the publication in JoVE.

2. Fig 4 and Fig 6 are shown for the same data and should be presented in one figure.

Figure 4 and 6 are presented in one figure as suggested by the reviewer

3. As mentioned in Table1, Ct value of microRNA-223 in purified HDL serum is close to NTC (31 vs 33.5). Please discuss the reason for the difference of NTC between microRNA-223 in purified HDL serum and plasma.

The small Ct value difference in serum and plasma may be due to different method of processing the blood. The difference is by only 2 Ct values.

4. In 3.3. "Prepare Solution A (isolation of VLDL, d=1.006 g/ml) by dissolving 11.4 g NaCl, 0.1g EDTA2Na and 1 ml 1N NaOH in 1000ml of autoclaved-distilled water. Then add an additional 3ml of autoclaved-distilled water (NaCl: 0.195 mol)." (NaCl: 0.195 mol) should be after "11.4 g NaCl"? Or the concentration should 0.195 mol/L? Please clarify.

We have clarified the confusion in the text 3.3. That should be after 11.4 g NaCl.

5. The authors need to have the MS proof read carefully to avoid the typos.

We have proof read carefully to avoid any spelling mistakes and typos.