**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.***

1. 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.

***N/A***

3) Jove reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

***We have included the available DOIs in the reference section.***

1. 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.***

1. 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.***

* 1. and 7.2 should be combined into 7.1.

***We have changed 7.2 into the sub steps of 7.1.***

* 1. 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).***

* 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.***

1. 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***

1. 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.***

1. 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.***

1. 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.***