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March 25th, 2019

Dear Dr. Dsouza,
We would like to take this opportunity to thank you for considering publication of our manuscript titled "Purification of High Yield Extracellular Vesicle Preparations Away from Virus" in the **Journal of Visualized Experiments**.

Both reviewers were favorable and considered the manuscript and described protocol very significant for the field of extracellular vesicles research. They were excited about potential for the separation of extracellular vesicles from virus, particularly RNA viruses such as HIV-1 and HTLV-1 which are similar in size to extracellular vesicles. This method opens new avenues of research to investigate the roles of extracellular vesicles in the pathogenesis of numerous viral infections, including HIV-1 which affects millions of individuals worldwide. Below, we have responded to every comment from these reviewers. The editorial and reviewer comments are in italics and our associated responses appear in bold.

Editorial Comments:

1. Protocol Detail: Please add a step to describe how cells were cultured and maintained; ensure that you mention incubation conditions and culture media.

We thank the editor for bringing this to our attention. To clarify the culture conditions, we have added the following to the Protocol section of the manuscript on lines 130-133 "To prepare culture supernatant (i.e. cell lines and/or primary cell), culture approximately 10 mL of cells for 5 days at 37°C and 5% CO_2 in appropriate culture medium. All culture medium reagents should be free of EVs, and can be either purchased or prepared in-house by pre-ultracentrifugation of serum at 100,000 x *g* for 90 min."

2. Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We are grateful to the editor for this comment and we feel that review of our discussion section has made our paper stronger and more complete. We have reviewed the discussion to ensure it addresses all of the topics listed including modifications, limitations, significance, and future applications. This can be found on lines 376-451.

3. Figures: Please remove the text "DeMarino et al…Fig #" from all figures.

We had included these labels to aid the reviewers in referring to the appropriate figure during the review process. We have now removed the text for final publication purposes.

4. Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are ExoMAX, Nanotrap, ExoMAX Opti Enhancer, OptiPrep, ZetaView Nanotracking, ZetaView 8.04.02.

Thank you to the editor for bringing this to our attention. We have removed all commercial sounding language from the manuscript/figures and replaced it will more general terms to describe the product used. While we feel it is important to thoroughly describe the specific EV-enriching nanoparticles used in the introduction section, rather than describing them as "Nanotraps" we have referred readers to the "Table of Materials" in order for them to specifically reference the nanoparticles used in this study.

5. Please define all abbreviations at first use.

We appreciate this comment and have reviewed the manuscript to ensure that abbreviations are defined at the appropriate place in the manuscript.

6. Please use standard abbreviations and symbols for SI Units such as µL, mL, L, etc., and abbreviations for non-SI units such as h, min, s for time units. Please use a single space between the numerical value and unit.

We have reviewed all abbreviations and symbols in the manuscript to confirm that SI and non-SI units adhere to standard practices and contain a space between the value and unit.

7. If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

We have uploaded the re-print permission text from Scientific Reports as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. A copy of the

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Title: Antiretroviral Drugs Alter the Content of Extracellular Vesicles from HIV-1-Infected Cells
Author: Catherine DeMarino, Michelle L. Pleet, Maria Cowen, Robert A. Barclay, Yao Akpamagbo et al.
Publication: Scientific Reports
Publisher: Springer Nature
Date: May 16, 2018

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permissions is shown below. Additionally, the text in the figure legends for Figures 2, 3, and 4 have been changed to reflect the verbiage requested.

Reviewer #1:

We would like to thank this reviewer for all of the positive comments regarding our paper including: "This is an important accomplishment as EVs are now considered to play an important factor in cell-cell communication and affect viral pathogenesis… The authors innovatively use their original approach in combination with common protocols to isolate a virus-free fraction of EVs… In general, the protocol is well described with important small details that are necessary to reproduce the separation by the readers."

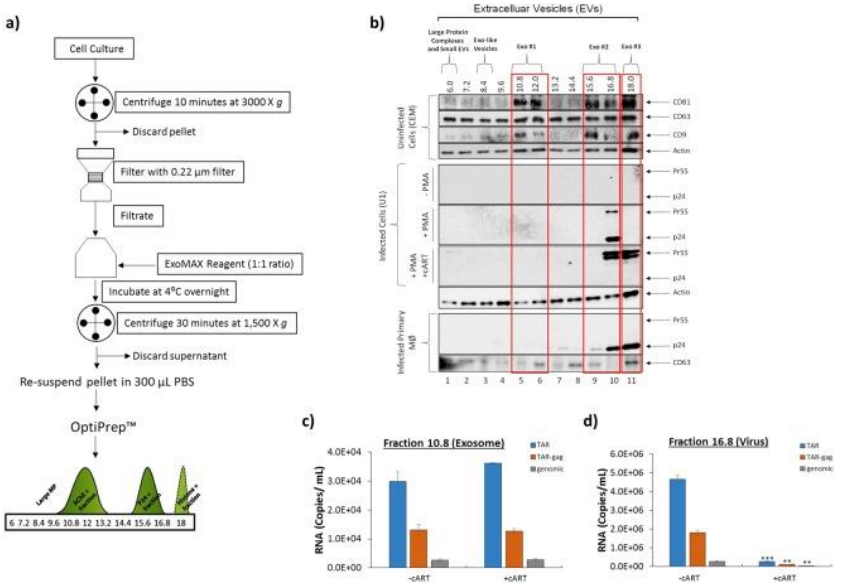
Minor Concerns:

1. The authors refer to vesicles that carry three tetraspanins (CD81, CD63, and CD9) as "exosomes". However, this is not a proven fact as some experts think that membrane vesicles can carry these proteins as well. Fig. 3 shows the presence of all three proteins, but since this represents a bulk analysis it cannot prove that all three tetraspanins are present on the same vesicles.

We are grateful to the reviewer to brining this to our attention. We agree with this type of bulk analysis that we cannot definitively prove that all three tetraspanins are present on the same vesicles. To bring this to the attention of the reader we have included the following within the results section on lines 249-258 which reads "The data in **Fig. 3A** show our previously published results which demonstrate the presence or absence of three exosomal tetraspanins (CD81, CD63, and CD9) in each fraction (CEM cells; top panel). The results indicate that exosomes, as defined by the presence of all three tetraspanins within the fraction, are found in three distinct populations: Exo #1 which includes fractions 10.8–12.0, Exo #2 which includes fractions 15.6–16.8, and Exo#3 which includes only the 18.0 fraction. Despite the presence of three distinct populations, our protocol cannot rule out the possibility of the presence of additional types of EVs within each fraction. Furthermore, these results do not definitively exclude the possibility that there are vesicles in these populations that are positive for a combination of the tested tetraspanin markers. These EVs could then be separated further by additional purification strategies."

2. As the authors mention, what they call pure EV fraction can contain elements of viruses. Some of these EVs may be not different from what was earlier called "defective viruses". It is worth mentioning this for the reader.

We thank this reviewer for asking these comments, as they are important, challenging, and require many more experiments from multiple labs to properly address the issue. However, we believe that the wild-type virus would have 2 copies of the genomic RNA (9.5 kb) with 4 copies of TARs and 2 *env* regions (as



detected by PCRs). This population would be affected by cART since the protease inhibitor in cART would affect Gag maturation, as the p24 is mostly missing and level of unprocessed Gag (Pr55) has increased. Our EV preps in the middle of the iodixanol gradient do not have such a ratio, as they contain mostly TAR and minimal *env* RNAs (DeMarino et al. Figure 1C-D).

Virus like particles (VLPs) in our system would not exist in the classical sense, since 100% of the cells are already infected and the genome is still wild-type after cART (a shift from wild-type genomes is an effect that would be seen from RT inhibitor effects at early stage of infection). VLPs by definition would be missing parts or most of the genome in the classical sense, as described in the past by transfection of multiple plasmids that make empty core shells (VLPs) without the genome.

An immature virus could be present in all of the fractions only if we started with a fresh infection and put cells under some kind of a selective pressure (i.e., cART), but in our case the genome is already integrated (latent cells) and the RT portion of the virus life cycle is not affected or regulated, therefore presence of an immature virus without a selection pressure in these cells would not make sense.

3. When separating other viruses, the authors mention that that (15.6-18.0) fraction contained EV-encapsulated Zika viruses. In my mind, density is not an ultimate proof of EV encapsulation of ZIKV.

We agree with the reviewers in that the presented data is not ultimate proof of EV encapsulation of ZIKV. However, we have additional data supporting this conclusion from infectivity assays of primary astrocytes and CCF-STTG1 astrocyte cell line. At this point, the manuscript containing these assays is still in preparation.

Reviewer #2:

We would like to thank this reviewer for his/her thorough review of the manuscript and all of their constructive comments. We feel that addressing these concerns and recommendations has helped to make the manuscript stronger and clearer to the reader.

Major Concerns:

1. Neither differential ultracentrifugation nor velocity density gradients are separating particles in base of buoyant densities (lines 70 and 94). This type of separation requires to be carried out for sufficient time to allow particles to reach the part of the gradient with equivalent density. Instead, authors should state that separation is based on different densities, sizes...

We thank the review for this clarification. We agree that these types of centrifugations separate the particles based on density and separate particles by allowing the particles to localize to the fraction with equivalent density. To clarify this distinction in the text we have edit the two phrases identified by the reviewer in the introduction section. They now read as the following:

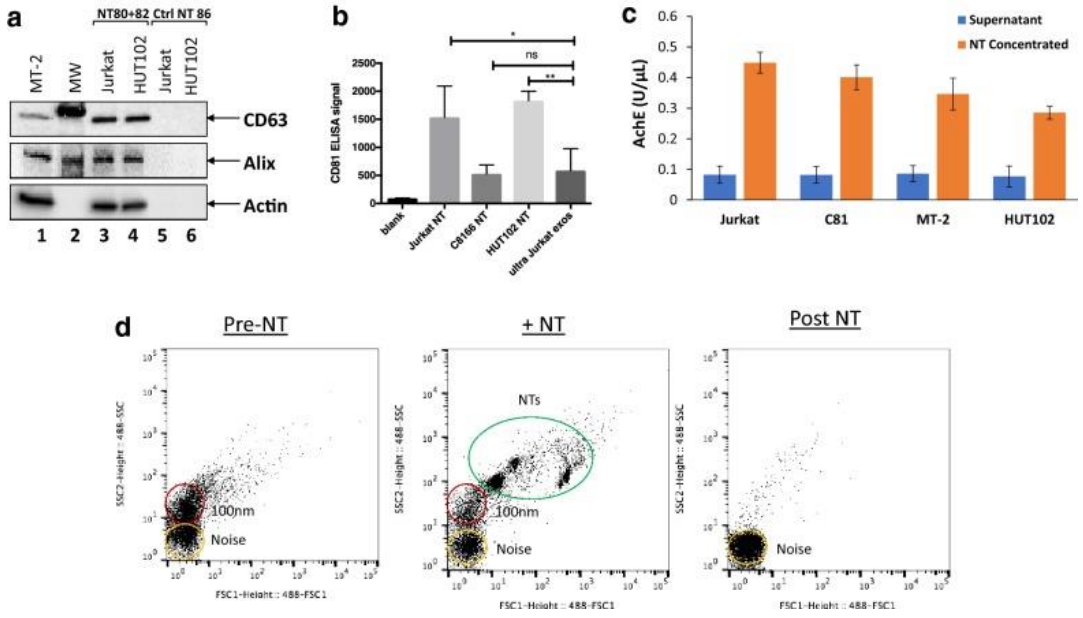
- **Lines 71-74:** "The current gold standard of EV isolation is ultracentrifugation. This technique makes use of the various vesicle densities, which allows the vesicles to be separated by centrifugation with differential sedimentation of higher density particles versus lower density particles at each stage [1, 2].."

- **Lines 94-98:** "To overcome the heterogeneity of EVs obtained from various precipitation methods, density gradient ultracentrifugation (DG) is utilized to better separate particles based upon their density. This method is carried out using a stepwise gradient using a density gradient medium, such as iodixanol

or sucrose, which allows for the separation of EVs from proteins, protein complexes, and virus or virus-like particles (VLPs).”

2. Authors compared precipitation of extracellular vesicles with ExoMax with a protocol using ultracentrifugation and demonstrated that yield recovered is higher with the precipitation protocol. Only comparison of first step with ultracentrifugation is done in the methodology described. However, authors state over the manuscript that their 3-step protocol is advantageous over ultracentrifugation. As second step requires UC, and no comparison between nanotrap capture and ultracentrifugation have been done, authors should limit comparison to UC to the first step. In fact, necessity for nanotrap technology in the last step is not clear. Can the authors explain better in the text why Nanotrap technology is better than other methods for concentration/precipitation?

While it is difficult to directly quantitate the number of particles captured by Nanotraps, previous studies from our lab (Anderson et al. 2018) have shown that Nanotrap particles can be used to concentrate EVs at equal to or higher levels than those isolated from 10 times the amount of starting material obtained from ultracentrifugation as measured by a CD81 ELISA assay (Panel B shown below). Specifically, in this data, CD81 levels from EVs isolated from 1 mL of supernatant (Jurkat, CD8166, and HUT 102) was equal to or greater than the CD81 signals obtained from EVs isolated from 10 mL of Jurkat supernatant using ultracentrifugation. Furthermore, in comparison to unenriched culture supernatant, NT enriched samples showed a 4-fold increase in AchE activity (Panel C) and removal of approximately 3.5x10^{9} vesicles post-trapping as measured by nanoFACS analysis of 300 uL of culture supernatant.



To describe the advantage of these nanoparticles to the readers we have included a statement within the discussion that illustrates the efficiency of these nanoparticles over ultracentrifugation and therefore the overall increased efficiency of the outlined protocol on line 375-378 which reads “Overall, the described protocol includes the combination of several well-known techniques and therefore should present limited difficulties. However, the incorporation of EV-enriching nanoparticles introduces a new technique which can require troubleshooting and/or modifications to achieve desirable results depending on the downstream assay or biological target of interest. In comparison to traditional ultracentrifugation, these EV-enriching nanoparticles have been shown to capture vesicles more efficiently, yielding a greater than or equal to amount of vesicles from 1 mL of culture supernatant compared to 10 mL of ultracentrifuged culture supernatant [15].”

3. Figure 3A shows analysis by Western blot of fractions obtained after density gradient separation of extracellular vesicles from different types of cells. CD9, CD81 and CD63 are only shown in fractions coming from non-infected T-lymphoblastic cell line. In contrast, viral proteins are shown only in fraction coming from infected promonocytic cells or infected primary macrophages. Definition of different types of vesicles based on tetraspanin expression is done in a non-infected T-cell line and viral proteins are shown only in infected monocyte/macrophages. Authors cannot assume that behavior of different vesicles and virus would be the same independent of infectivity status or cell type. Specially, because CD63 is primary macrophages behaves different that in non-infected CEM cells. Authors should add information for tetraspanins in infected monocyte/macrophages cells. Or at least, comment this fact clearly in the text.

We thank the reviewer for this important distinction. Our EV isolation procedure was developed and optimized using EVs and exosomes from CEM cells and for this reason the top panel of this figure shows CEM cell EVs. We have validated these results in myeloid cell lines in an independent experiment. To make this distinction more clear to the readers we have added text to clearly state the cell type used in the top panel of the figure within the representative results section. Lines 246-254 now read "In order to characterize the vesicles present in each fraction following nanoparticle enrichment, EVs were isolated from uninfected CEM or HIV-1 infected U1 cell culture supernatant using the outlined protocol and characterized using Western blot of each nanoparticle enriched iodixanol fraction. The data in **Fig. 3A** show our previously published results which demonstrate the presence or absence of three exosomal tetraspanins (CD81, CD63, and CD9) in each fraction (CEM cells; top panel). The results indicate that exosomes, as defined by the presence of all three tetraspanins within the fraction, are found in three distinct populations: Exo #1 which includes fractions 10.8–12.0, Exo #2 which includes fractions 15.6–16.8, and Exo#3 which includes only the 18.0 fraction."

Minor Concerns:

1. In general for all the text and, particular, referring to figure 2, I will change the term exosomes for extracellular vesicles because the presence of 3 tetraspanins in the preparation is not a guarantee for having all the vesicles with the 3 proteins together (bona fide markers of exosomes). Particles shedding from plasma membrane can contain also cd81, cd9....

We thank the review for their suggestion regarding the nomenclature used throughout the manuscript. However, we believe these three populations (Exo#1, Exo#2, and Exo#3) contain exosomes as indicated by the presence of CD81, CD63, and CD9. We do note within the results section that the presence of other extracellular vesicles with similar densities, perhaps those that do not contain CD81, CD63, and CD9, may be present in the populations due to the heterogenous nature of EVs and the fact that the presented assay is a bulk analysis of the EV preparation using our described protocol. Additionally, nanotracking analysis of each fraction suggests that that all fractions contain vesicles of approximately 100 nm in diameter. These data imply that exosomes released from cells may vary in their densities and therefore can localize to different fractions (i.e. 10.8-12.0 and 16.8).

We agree with the reviewer that the presence of 3 tetraspanins in the preparation does not guarantee that vesicles in the Exo#1 may not possess all three EV markers on the same vesicle. In fact, we believe that the EV preps are relatively heterogenous in that they contain vesicles which are only CD63+, vesicles that are only CD9+, and vesicles that are only CD81+. Furthermore, the prep may contain vesicles which may contain a number of combinations of these three markers. To clarify this to the reader we have modified the results section, lines 246-258 to now read: "The data in **Fig. 3A** show our previously published results which demonstrate the presence or absence of three exosomal tetraspanins (CD81, CD63, and CD9) in each

fraction (CEM cells; top panel). The results indicate that exosomes, as defined by the presence of all three tetraspanins within the fraction, are found in three distinct populations: Exo #1 which includes fractions 10.8–12.0, Exo #2 which includes fractions 15.6–16.8, and Exo#3 which includes only the 18.0 fraction. Despite the presence of three distinct populations, our protocol cannot rule out the possibility of the presence of additional types of EVs within each fraction. Furthermore, these results do not definitively exclude the possibility that there are vesicles in these populations that are positive for a combination of the tested tetraspanin markers. These EVs could then be separated further by additional purification strategies."

2. In the discussion, authors point SEC as an alternative to density gradients to purify EVs away from virus. This methodology is based on size and probably can be only used when viral particles are very different in size to the EVs which are the object of the study. The authors should explain this better or removed from discussion.

We are grateful to the review for bringing this to our attention. To better delineate the appropriate usage of SEC columns for the isolation of EVs from viruses, we have added additional details which stipulate that this method is appropriate for use in the context of large viruses who size varies greatly from the EV population of interest. This is included in the discussion section on lines 412-418 and now reads "For the isolation of EVs away from virus in patient material we have incorporated the use of size exclusion chromatography (SEC) columns, which are utilized in place of a density gradient. These columns are beneficial in that they are disposable and therefore are ideal in high containment laboratories. However, SEC columns separate particles according to size, therefore, it follows that this type of separation is only applicable for the separation of large or very small viruses, such as EBOV (1 µm) or ZIKV (40 nm), respectively, from EVs or separation away from free protein."

We thank the reviewers for all of their constructive comments. We hope that you will now find this manuscript suitable for publication in the **Journal of Visualized Experiments**. We look forward to moving on to video production.

We thank you in advance for your time and efforts.

Sincerely,

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