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TITLE:

Purification of High Yield Extracellular Vesicle Preparations Away from Virus

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KEYWORDS:

extracellular vesicles, exosome, virus, purification, density gradient ultracentrifugation, precipitation, EV-enriching nanoparticles

SUMMARY:

This protocol isolates extracellular vesicles (EVs) away from virions with high efficiency and yield by incorporating EV precipitation, density gradient ultracentrifugation, and particle capture to allow for a streamlined workflow and a reduction of starting volume requirements, resulting in reproducible preparations for use in all EV research.

ABSTRACT:

One of the major hurdles in the field of extracellular vesicle (EV) research today is the ability to achieve purified EV preparations in a viral infection setting. The presented method is meant to isolate EVs away from virions (i.e., HIV-1), allowing for a higher efficiency and yield compared to

conventional ultracentrifugation methods. Our protocol contains three steps: EV precipitation, density gradient separation, and particle capture. Downstream assays (i.e., Western blot, and PCR) can be run directly following particle capture. This method is advantageous over other isolation methods (i.e., ultracentrifugation) as it allows for the use of minimal starting volumes. Furthermore, it is more user friendly than alternative EV isolation methods requiring multiple ultracentrifugation steps. However, the presented method is limited in its scope of functional EV assays as it is difficult to elute intact EVs from our particles. Furthermore, this method is tailored towards a strictly research-based setting and would not be commercially viable.

INTRODUCTION:

Research centered around extracellular vesicles (EVs), specifically exosomes, a type of EV ranging 30–120 nm and characterized by the presence of three tetraspanin markers CD81, CD9, and CD63, has largely been shaped by the development of methods to isolate and purify the vesicles of interest. The ability to dissect multifaceted mechanisms has been hindered due to complex and time-consuming techniques which generate samples composed of a heterogeneous population of vesicles generated via different pathways with a wide range of contents, sizes, and densities. While this is an issue for nearly all EV research, it is of particular importance when studying EVs in the context of viral infection, as virions and virus-like particles (VLPs) can be similar in diameter to the vesicles of interest. For example, the Human Immunodeficiency Virus Type 1 (HIV-1) is approximately 100 nm in diameter, which is roughly the same size as many types of EVs. For this reason, we have designed a novel EV isolation workflow to address these issues.

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]. Several low-speed centrifugation steps are required to remove intact cells (300–400 x *g* for 10 min), cell debris (~2,000 x *g* for 10 min), and apoptotic bodies/large vesicles (~10,000 x *g* for 10 min). These initial purifications are followed by high speed ultracentrifugation (100,000–200,000 x *g* for 1.5–2 h) to sediment EVs. Wash steps are performed to further ensure EV purity, however, this results in the reduction of the number of isolated EVs, thereby lowering total yield [3, 4]. This method's utility is further limited by the requirement of a large number of cells (approximately 1 x 10^8) and a large sample volume (> 100 mL) to achieve adequate results.

To address the growing concerns, precipitation of vesicles with hydrophilic polymers has become a useful technique in recent years. Polyethylene glycol (PEG), or other related precipitation reagents, allows the user to pull down the vesicles, viruses, and protein or protein-RNA aggregates within a sample by simply incubating the sample with the reagent of choice, followed by a single low-speed centrifugation[1, 2, 5]. We have previously reported that use of PEG or related methods to precipitate EVs in comparison to traditional ultracentrifugation results in a significantly higher yield[6]. This strategy is fast, easy, does not require additional expensive equipment, is readily scalable, and retains EV structure. However, due to the promiscuous nature of this method, the resulting samples contain a variety of products including free proteins, protein complexes, a range of EVs, and virions thus requiring further purification to obtain the desired population[1, 2, 7, 8].

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). It is important to note that, while it was once thought that DG allowed for more precise separation of EV subpopulations, it is now known that sizes and densities of various vesicles can overlap. For example, exosomes are known to have flotation densities of 1.08–1.22 g/mL[9], while vesicles isolated from the Golgi ($COPI^+$ or $clathrin^+$) have densities of 1.05–1.12 g/mL and those from the endoplasmic reticulum ($COPII^+$) sediment at 1.18–1.25 g/mL[1–4, 9]. Additionally, if one desires to compare exosomal fractions against fractions containing viral particles, this may become more difficult depending upon the density of the virus of interest—there are viruses other than HIV-1 that likely equilibrate at the same densities as exosomal positive fractions[2].

Finally, enrichment of EV preps for downstream visualization and functional assays is vital to EV research. The use of EV-enriching nanoparticles, specifically, multi-functional hydrogel particles that range 700–800 nm in diameter, are a critical step in achieving concentrated EV preps. They possess a high affinity aromatic bait which encapsulated by a porous outer sieving shell to promote selectivity. The nanoparticles utilized in this study include two distinct preparations with different core baits (Reactive Red 120 NT80; and Cibacron Blue F3GA NT82) which have shown to increase capture of EVs from various reagents and biofluids (see the **Table of Materials**)[6, 10–15]. The particles offer easy enrichment of EVs from numerous starting materials including iodixanol fractions, cell culture supernatant, as well as patient biofluids such as plasma, serum, cerebral spinal fluids (CSF), and urine[6, 13].

The method presented here improves the efficiency of current EV purification techniques by combining several technologies; EV precipitation, density gradient ultracentrifugation, and particle capture, to streamline the workflow, reduce sample requirements, and increase yield to obtain a more homogenous EV sample for use in all EV research. This method is particularly useful in the investigation of EVs and their contents during viral infection as it includes a 0.22 µm filtration step to exclude large, unwanted vesicles and VLPs and separation of the total EV population based on density to effectively isolate EVs from virions.

PROTOCOL:

1. Filtration and precipitation of extracellular vesicles (EVs)

1.1. To prepare the culture supernatant from infected or transfected cells (i.e., cell lines and/or primary cells), culture approximately 10 mL of late-log cells for 5 days at 37 °C and 5% CO_2 in appropriate culture medium (i.e., RPMI or DMEM with 10% fetal bovine serum [FBS]).

NOTE: All culture medium reagents should be free of EVs, and can be either purchased (see **Table of Materials**) or prepared in-house by pre-ultracentrifugation of serum at 100,000 x *g* for 90 min.

This protocol has been successful for several commonly-used cell lines including: CEM, Jurkat, 293T, U937 (uninfected lines), U1, J1.1, ACH2, HUT102, MT-2 (HIV-1 and HTLV-1 infected lines), multiple transfected cells, and primary myeloid and T-cells (both infected and uninfected); however, this protocol can be used for any cell type, including those that require specialized media or culture conditions. Density of cells may need to be optimized for different cell types. It is recommended that the highest density be used with minimal cell death after 5 days.

1.2. Centrifuge the culture at 3,000 x *g* for 5 min to pellet cells and discard the pellet.

1.3. Filter the culture supernatant using a sterile 0.22 µm filter and collect filtrate in a clean tube.

1.4. Add equal volume of PEG precipitation reagent (1:1 ratio) to filtered supernatant. Invert tube several times to ensure a homogenous mixture.

NOTE: Do **not** vortex.

1.5. Incubate mixture at 4 °C overnight (O/N).

1.6. Centrifuge mixture at 1,500 x *g* for 30 min at room temperature (RT) to yield a heterogeneous EV pellet.

NOTE: EV pellet should appear white or off-white in color.

1.7. Discard the EV-depleted culture supernatant.

1.8. Resuspend the EV pellet in 150–300 µL of 1x phosphate-buffered saline without calcium and magnesium (PBS) and keep on ice.

2. Construction of a density gradient

2.1. Mix iodixanol density gradient medium with 1x PBS to create 11 different 1 mL density fractions from 6 to 18% iodixanol in 1.2% increments in separate microcentrifuge tubes as shown in **Figure 1A**.

2.2. Vortex each tube to mix.

2.3. Layer density fractions into a pre-cleaned and dry swinging bucket ultracentrifuge tube starting with fraction #18 and ending with fraction #6 as indicated in **Figure 1B**.

NOTE: All tubes should be sanitized using a 10% bleach spray followed by followed by rinsing 3x with deionized water and a final wash of sterile deionized water prior to loading of the gradient fractions.

2.4. Add resuspended EV pellet (300 µL) to the top of the layered gradient in the ultracentrifuge

tube.

2.5. Ultracentrifuge at 100,00 x *g* at 4 °C for 90 min.

2.6. Carefully remove the 1 mL fractions from the ultracentrifuge tube and transfer each fraction into new microcentrifuge tubes.

3. Enrichment of EV fractions using nanoparticles

3.1. Create a 30% slurry of nanoparticles using equal volumes of NT80, NT82, and 1x PBS.

NOTE: The mixture should be vortexed prior to use to ensure homogeneity.

3.2. Add 30 µL of the slurry to each microcentrifuge tube containing the density fractions and pipette/invert them several times to mix.

3.3. Rotate EV-enriching nanoparticle-containing density fraction microcentrifuge tubes O/N at 4 °C at approximately 20 rpm.

3.4. Centrifuge density fraction microcentrifuge tubes at 20,000 x *g* for 5 min at RT.

3.5. Discard the liquid and wash EV pellet twice with 1x PBS.

NOTE: Nanoparticle pellets can be frozen at -20 °C or immediately used for various downstream assays (i.e., PCR, Western blot, mass spectrometry, and other assays).

4. Recommended preparation of nanoparticle pellet for downstream assays

4.1. For RNA isolation

4.1.1. Resuspend the pellet in 50 µL of autoclaved deionized water treated with 0.001% diethyl pyrocarbonate (DEPC) filtered through a 0.2 µm filter and isolate RNA according to the kit manufacturer's protocol.

4.2. For gel electrophoresis

4.2.1. Resuspend the pellet directly in 15 µL of Laemmli buffer.

4.2.2. Heat sample 3x at 95 °C for 3 min. Vortex gently and spin down between each heat cycle.

4.2.3. Centrifuge sample for 15 s at 20,000 x *g* and load all eluted material directly onto the gel.

NOTE: For best results, limit the amount of particles loaded onto the gel and run the gel at 100 V to ensure any remaining particles are contained to the wells.

4.3. For trypsin digestion

4.3.1. Resuspend the pellet in 20 µL of urea prior to alkylation and trypsinization of sample. Nanoparticles can be pelleted by a 14,000 x *g* centrifugation at RT for 10 min. Sample containing the trypsinized peptide can be transferred into a clean collection tube.

REPRESENTATIVE RESULTS:

PEG precipitation increases EV yield.

Our combination approach to EV isolation is significantly more efficient in terms of EV recovery as compared to traditional ultracentrifugation, as evident by the 90% reduction in the volume of starting material required. Ultracentrifugation, the current gold standard in EV isolation, requires approximately 100 mL of culture supernatant to produce an adequate EV prep for downstream assays, whereas our novel protocol requires only 10 mL. This reduction is made possible through the use of the PEG EV precipitation reagent which provides a significant increase in EV yield as measured by nanotracking analysis (NTA). The results in **Figure 2A**, which have been previously published[6], indicate that when using 10 mL of culture supernatant PEG precipitation resulted in the recovery of 7.27×10^{10} EVs, which was approximately 500-fold more than the number of EVs recovered from the same starting material using ultracentrifugation (1.45×10^{8}). Increased efficiency of the isolation of exosomes, a specific EV subtype, was also observed as evident by increased levels of well-characterized exosome marker proteins, CD81, CD63, and CD9. Western blot analysis comparing EV preps produced from either ultracentrifugation or EV precipitation using a PEG precipitation reagent is shown in **Figure 2B**. These results show a 3,000-fold increase in CD81, a 4-fold increase in CD63, and a 40-fold increase in CD9 as measured by densitometry analysis. Taken together, these results suggest that the outlined protocol not only increases the total EV yield but also enhances recovery of exosomes when compared to ultracentrifugation.

Characterization of EVs and separation of EVs away from HIV-1 virus

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 **Figure 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, the 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.

The rapidly developing field of EV/exosome research has exploded since their discovery and has

resulted in many newfound functions and applications for these vesicles. In particular, their role as intracellular messengers in not only normal physiology but also in disease has led to the use of significant resources to dissect the effects and utility of extracellular vesicles, specifically exosomes, on recipient cells[16–18]. Numerous studies have implicated EVs in the pathogenesis of a variety of infections, including HIV-1[6, 10, 12, 19–22]. However, the size similarity between EVs and virions presents a potential obstacle in defining these EV-mediated mechanisms. To address this concern, we have designed our protocol to implement a density gradient to effectively separate EV populations from virions. To this end, cell culture supernatant from HIV-1 infected cells were subjected to the presented protocol and analyzed by western blot for the presence of HIV-1 Gag p24 to determine which fraction or fractions contained HIV-1 virions. The results shown in **Figure 3A** (middle panel) shows the localization of p24 in three independent conditions: in the absence of an inducer, in the presence of an inducer (Phorbol 12-myristate 13-acetate; PMA), and in the presence of PMA and combination antiretroviral therapy (cART), the standard treatment for HIV-1. These data indicate that the HIV-1 virus is localized to fraction 16.8 as measured by the presence of p24 and its uncleaved polyprotein Pr55. Furthermore, when cells are subjected to cART, which includes Indinavir, a protease inhibitor which prevents the cleavage of Pr55 to p24, no p24 was detected in any fraction. Instead, only Pr55 was detected and was present in the 16.8–18.0 fractions, indicating a shift of virus into more dense fractions. Similar results were obtained using primary macrophages (lower panel). Although the described protocol results in the co-sedimentation of Exo #2 and Exo #3 populations with virus, the Exo #1 population (fractions 10.8–12.0) remained virus-free, allowing for downstream assays free of virus contamination.

This type of separation of virus away from EVs allows us to address the possibility of pieces of virus entering EVs or being secreted from infected cells as free protein. Results in **Figure 3B** indicate that HIV-1 Nef protein can be found in the Exo #1 population in addition to the Exo #2 and Exo #3 populations. Nef also appears in the 6.0 fraction, indicating that Nef can be potentially secreted from infected cells as a free protein and within an EV. Additionally, HIV-1 Vpr protein is found in the 6.0 fraction while HIV-1 Tat protein is predominantly found in the 6.0 fraction, but also appears in fractions 7.2-9.6. We are currently testing all fractions for the presence of Tat protein by ELISA. These results indicate that both Tat and Vpr can be secreted from infected cells, likely as free proteins, while Tat could also be incorporated into EVs. Collectively, these data indicate that our method of EV isolation can successfully isolate exosomes (Exo #1) away from HIV-1 virions (Exo #2 and #3) and that EVs from HIV-1-infected cells contain some HIV-1 proteins, which may affect HIV-1 pathogenesis.

Characterization of EVs and separation of EVs away from other viruses.

To apply this protocol to other viral infection models, we have characterized vesicles from cells infected with several different viruses. **Figure 3C** shows an illustration of previously obtained vesicle and virus distributions following precipitation, separation, and enrichment according to the described protocol. As previously shown in **Figure 3A**, exosomes ($CD9^+CD63^+CD81^+$) are present in three different populations (Exo #1, fractions 10.8–12.0; Exo #2, fractions 15.6 –16.8, and Exo#3, 18.0 fraction) and HIV-1 virus localizes to the higher density 16.8 and 18.0 fractions (lanes 10-11). Not surprisingly, when applying this protocol to characterize EVs released from

Human T-lymphotropic virus Type 1 (HTLV-1), a retrovirus that is known to cause cancer in adults, we observed results similar to that of HIV-1-related EVs. The third panel in **Fig. 3C** shows that the HTLV-1 virus was primarily localized to the highest density fraction (18.0) and, to a lesser extent, fractions 13.2-16.8 as evidenced by the presence of HTLV-1 matrix protein (p19). Furthermore, we have previously found that the HTLV-1 transactivating protein, Tax, which is absent from the HTLV-1 virion, is present within exosomes released from HTLV-1 infected cells[15, 23]. The data in **Figure 3C** shows that Tax was present in the lower density fractions (6.0–12.0), two of which we have shown to contain exosomes (10.8 and 12.0).

Next, we tested the isolation protocol in the context of larger and smaller viruses such as Ebola virus (EBOV) and Zika virus (ZIKV), respectively, as HIV-1 and HTLV-1 virions are both retroviruses and very similar in diameter. Using VLP as a surrogate biosafety level 2 (BSL-2) model of EBOV assembly and exit, we found that VLP, which is approximately 1 µm in length, localizes to the highest density fraction (18.0) in the absence of 0.22 µm filtration (middle panel, **Figure 3C**). Moreover, when VP40, the EBOV matrix protein, is expressed at high levels, it is secreted from the cell through several different mechanisms resulting in the presence of VP40 in every density fraction, including those to which exosomes are localized[13, 14]. In contrast, ZIKV virions are significantly smaller than HIV-1 virions, measuring approximately 40 nm in diameter. The representative diagram in the bottom panel of **Figure 3C** shows the presence of ZIKV virions in both low density (6.0–8.4) and high density (15.6–18.0) fractions, suggesting the presence of both free virus and EV-encapsulated virus, respectively.

FIGURE AND TABLE LEGENDS:

Figure 1: Construction of an iodixanol density gradient. (**A**) Relative amounts of iodixanol and PBS utilized to create each density fraction (Fraction #). The Fraction # denotes the percentage of iodixanol included in each fraction. Their relative densities and placement in the gradient are depicted from heaviest to lightest. (**B**) Placement of the density fractions (6.0–18) in ultracentrifuge tube is shown (left side) as well as the location of the three EV populations (Exo #1, Exo #2, Exo #3), Exo-like vesicles, and protein complexes.

Figure 2: PEG precipitation reagent increases EV yield. (**A**) To compare EV recovery, EVs were isolated from 5 d HIV-1 infected monocyte (U1) culture supernatant (10 mL) using traditional ultracentrifugation (100,000 x *g*) or PEG precipitation reagent (incubation at a 1:1 ratio). EVs from both enrichment procedures were analyzed using nanotracking analysis to assess resulting EV concentration as described in DeMarino, et al.[6]. NTA was measured at 11 independent positions in technical triplicate. The blue bars represent an average of the 33 measurements ± S.D. Statistical significance was determined using a two-tailed Student's *t*-test; ***$p < 0.001$. **B.** EVs from 5-day CEM culture supernatant using either ultracentrifugation (UC) or PEG precipitation followed by iodixanol density separation. Ultracentrifugation was performed using 100 mL of culture supernatant (Lane 1) while PEG precipitation and subsequent iodixanol fractionation was performed using 10 mL of culture supernatant (lane 2). The total EV pellet obtained from ultracentrifugation and the 10.8 fraction from PEG/iodixanol separation were analyzed by western blot for the presence of exosomal marker proteins (CD81, CD63, and CD9) with Actin as

a control. For clarity, selected lanes from the same blot with identical exposures are shown in panel B. This figure has been modified from DeMarino, et al.[6].

Figure 3: Isolation of EVs away from viruses. (**A**) Five-day culture supernatants from uninfected CEM cells (top panel), HIV-1 (Ba-L; MOI: 0.01) infected U1 cells (±PMA and ±cART treatment; middle panel) or HIV-1 infected primary macrophages (bottom panel) were incubated with PEG precipitation reagent at 4 °C overnight. EVs were separated into fractions using an iodixanol density gradient. EVs from all fractions were enriched using NT80/82 particles overnight at 4 °C. CEM nanopellets were analyzed using western blot for the presence of exosomal marker proteins CD81, CD63, and CD9. U1 (±PMA and ±cART treatment) and HIV-1 infected primary macrophages were analyzed for the presence of HIV-1 Gag protein (p24 and Pr55; cleaved and uncleaved HIV-1 Gag polyprotein, respectively), as well as CD63. Blots were probed for actin as a control. True exosome populations ($CD81^+CD63^+CD9^+$) are outlined in red. This figure has been modified from DeMarino, et al.[6] (**B**) Five-day culture supernatants from infected U1 cells were incubated with PEG precipitation reagent at 4 °C overnight. EVs were separated into fractions using an iodixanol density gradient. EVs from all fractions were enriched using NT80/82 particles overnight at 4 °C. Fractions were run on a Western blot for the presence of HIV-1 Nef, Vpr, and Tat. Actin was used as a control. True exosome populations ($CD81^+CD63^+CD9^+$) are outlined in red as previously defined in DeMarino, et al.[6] (**C**) Representative illustration of previously characterized EV separation profiles from four different viruses: HIV-1, HTLV-1, EBOV, and ZIKV.

Figure 4: Protocol workflow. The novel workflow combines several techniques including filtration, EV precipitation using PEG precipitation reagent, iodixanol density gradient separation, and nanoparticle enrichment of EVs. Utilization of this protocol separates EVs from various viruses and provides a small volume sample for downstream assays including qPCR, Western blot, mass spectrometry, RNA sequencing, cytokine analysis, ELISA, and cell-based assays. This figure has been modified from DeMarino, et al.[6].

DISCUSSION:

The outlined method allows for enhanced EV yield and the separation of virus from EVs using a combination approach to isolation. Relatively large quantities of starting material (i.e., cell supernatant) can be filtered prior to EV isolation by precipitation, DG separation, and nanoparticle enrichment, resulting in a final volume of ~30 µL, allowing for immediate usage in a variety of downstream assays. The use of nanoparticle enrichment is essential as, compared 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]. 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. We recommend that nanoparticles be incubated with filtered cell culture supernatants overnight. If the target protein or RNA of interest is of low abundance in the model system, the protocol can be adapted to increase the incubation period to ensure capture of the target. The utilization of

nanoparticles in downstream assays can typically be accomplished by resuspending the pellet in the desired sample buffer for each assay. For example, nanoparticles can be resuspended directly in Laemmli buffer for western blot analysis. The captured material should then be more efficiently eluted from the nanoparticles in SDS via heating and vortexing cycles. To achieve adequate separation of protein, care should be taken to limit the amount of nanoparticles loaded into the gel, and the gel should be run at approximately 100 V to ensure any remaining particles are contained to the wells. Furthermore, for the visualization of low molecular weight proteins, an overnight wet transfer achieves optimal results. Although the use of nanoparticles results in significant enrichment of vesicle populations, additional steps must be taken to elute captured material from the particles. Furthermore, elution of the material can potentially limit the utility of EVs in downstream functional assays as many elution buffers can damage the integrity of the EV membrane. Additional research is needed to engineer a strategy to allow for the removal of intact vesicles from nanoparticles post incubation. Another disadvantage of these particles is the current lack of characterization. The outer shell of the particle has been designed to exclude high molecular weight molecules. However, specific targeting of the particles to EVs does not take place, and as a result many confounding factors and background signals can potentially be present in downstream assays.

The described method is primarily to be used for laboratory purposes. We have recently developed alternate methods to expand the capabilities of our combination approach to additional techniques for isolating EVs from small-scale, patient biofluids such as plasma and CSF and large-scale, commercial EV production. 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. In terms of large-scale EV production, recent advancements in filtration-based methods, namely tangential flow filtration (TFF), have allowed for the efficient isolation of EVs from large sample volumes. TFF has historically been used for the purification of nanosized biomolecules, including viruses, and through the optimization of protocols this system has successfully been adapted for the isolation of EVs[24, 25]. Briefly, during the TFF process, sample fluid flows tangentially across the surface of a semi-permeable membrane which selectively retains vesicles based on size and molecular weight, thus allowing for the separation of EVs away from free proteins and other contaminating biomolecules[26]. When compared to ultracentrifugation, it has been reported that TFF results in higher yields, less aggregation, and reduces the lot-to-lot variability of EVs[27]. Additionally, the use of TFF for the good manufacturing practice (GMP)-grade isolation of EVs for therapeutic application has also been reported[28]. Due to its scalability and reproducibility, this technology offers great potential for future EV research.

Viruses come in different sizes and densities, therefore depending upon the virus in question, optimization of this protocol may be required. For very large viruses such as Ebola (~ 1 μm or larger in length), simple filtration can be utilized to remove the vast majority of virions and large

contaminating bodies such as VLPs and apoptotic bodies[14]. This allows for most of the separation of virus away from EVs to be executable on the front end of purification. However, in the case of smaller viruses such as enterovirus (~30 nm), Zika virus (~40 nm), hepatitis B virus (~42 nm), hepatitis C virus (~55 nm), and others, the fraction in which virions sediment will need to be determined before further downstream functional analyses. One cannot always approximate the likely fraction of sedimentation based on particle diameter alone. For example, poliovirus, which is 30 nm in diameter, is also known to be encapsulated in secretory autophagosomes[29–33], and therefore is likely to be found on the right side of the gradient (denser fractions) rather than the left (less dense fractions). While we have optimized this protocol in the case of HIV-1, HTLV-1, and EBOV VLPs, additional viruses will need characterization to fine-tune the protocol for their specific purification away from EVs.

The protocol outlined here (**Figure 4**) allows, for the first time, the user to separate EVs from virus with enhanced efficiency and smaller volumes of starting material as compared to the gold standard of EV isolation, ultracentrifugation. This method can be easily adapted to the conditions at hand including varying sample sizes, required yields, starting material type (i.e., culture supernatant versus patient material), and various different viruses of different sizes.

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DISCLOSURES:

B.L. is affiliated with Ceres Nanosciences inc. that produces reagents and/or instruments used in this article. All other authors declare no potential conflicts of interest.

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Figure 1

A)

Gradient Fractions			
	Fraction #	Iodixanol (µL)	PBS (µL)
lightest	6	60	940
	7.2	72	928
	8.4	84	916
	9.6	96	904
	10.8	108	892
	12	120	880
	13.2	132	868
	14.4	144	856
	15.6	156	844
	16.8	168	832
heaviest	18	180	820

B)

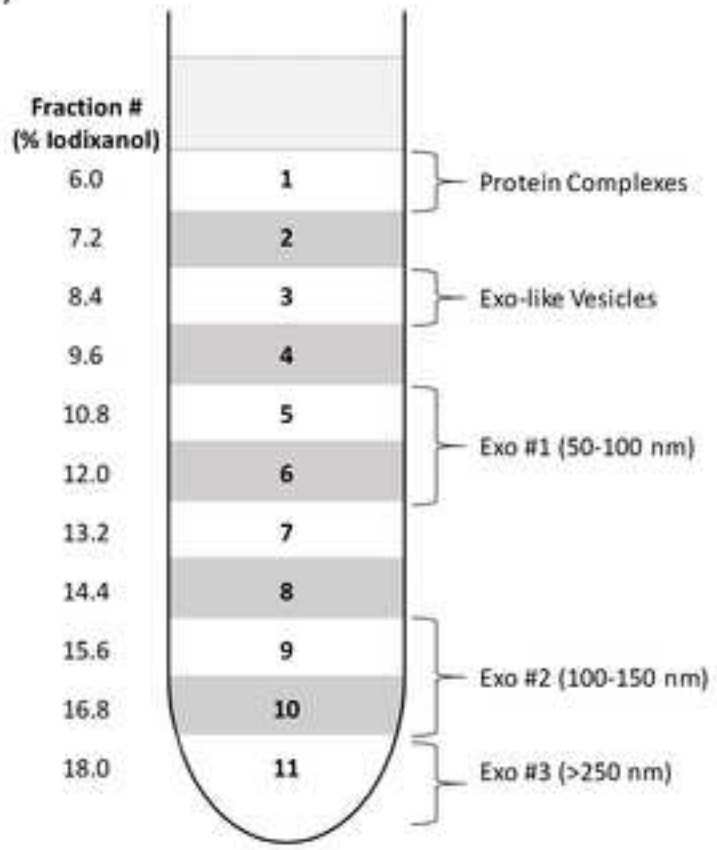


Figure 2

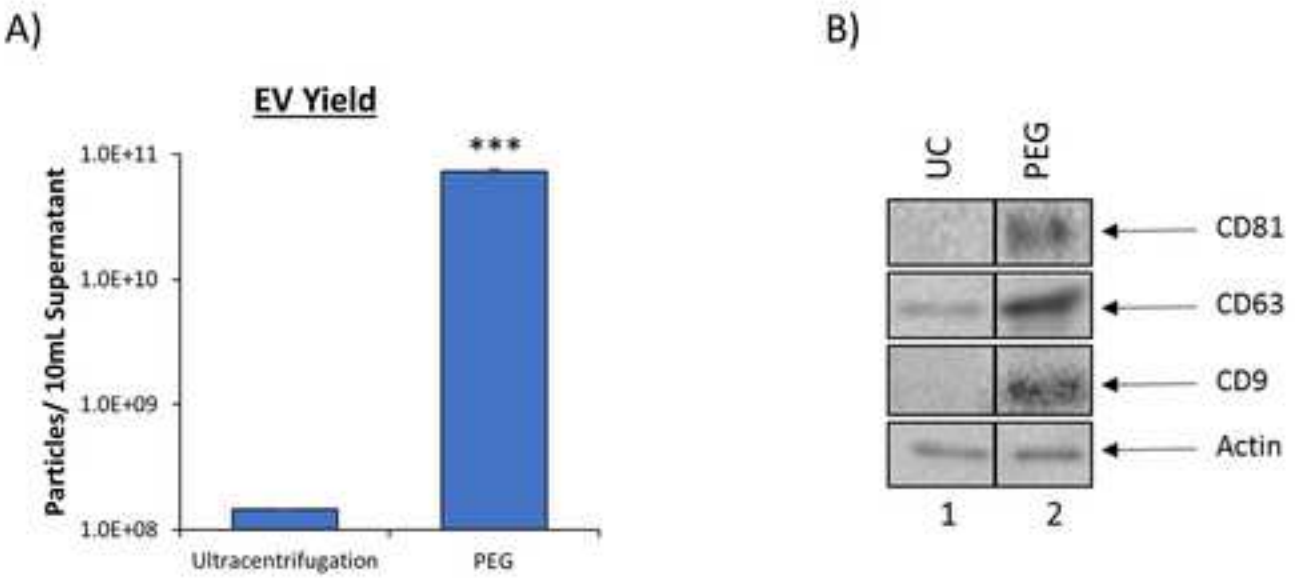


Figure 3

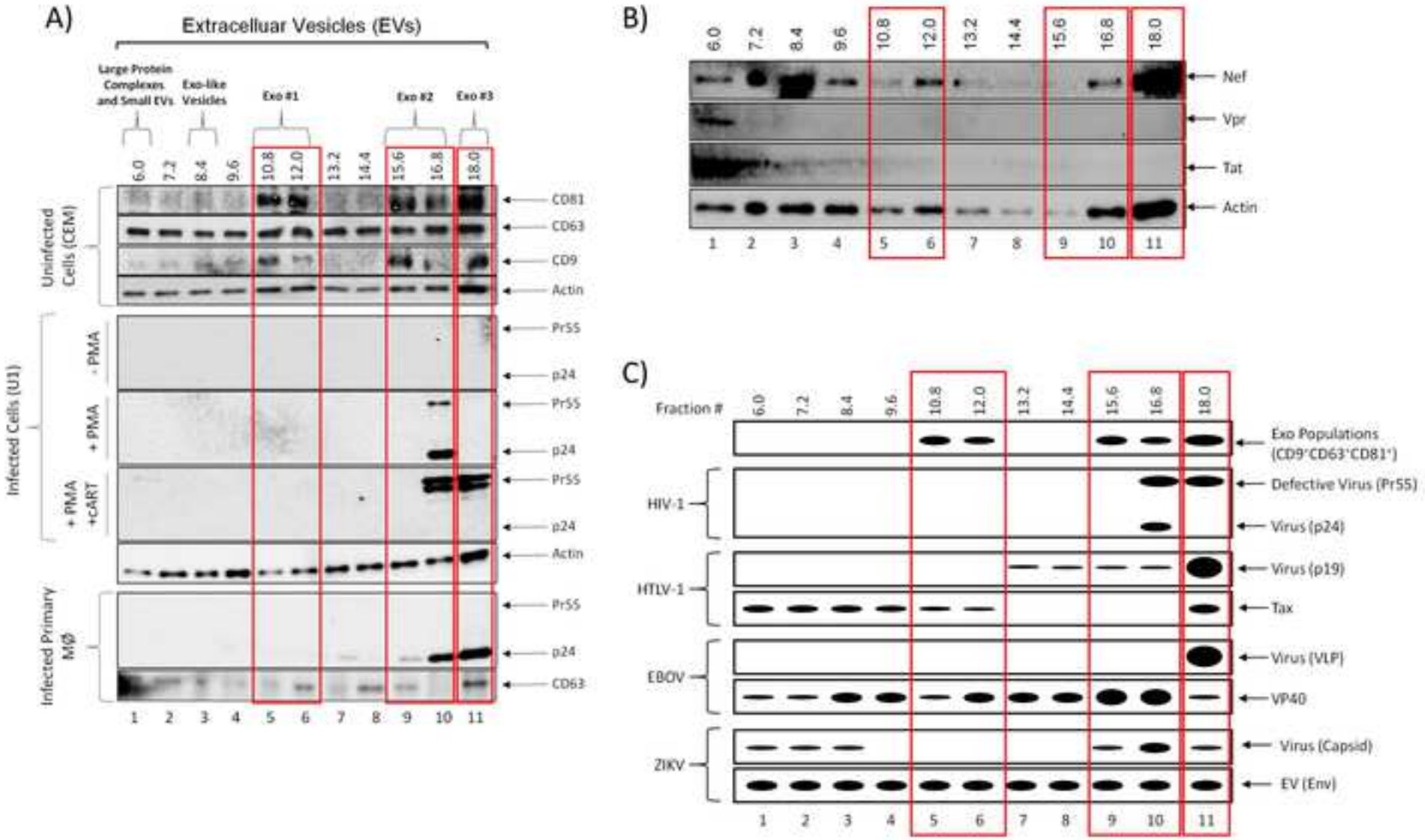
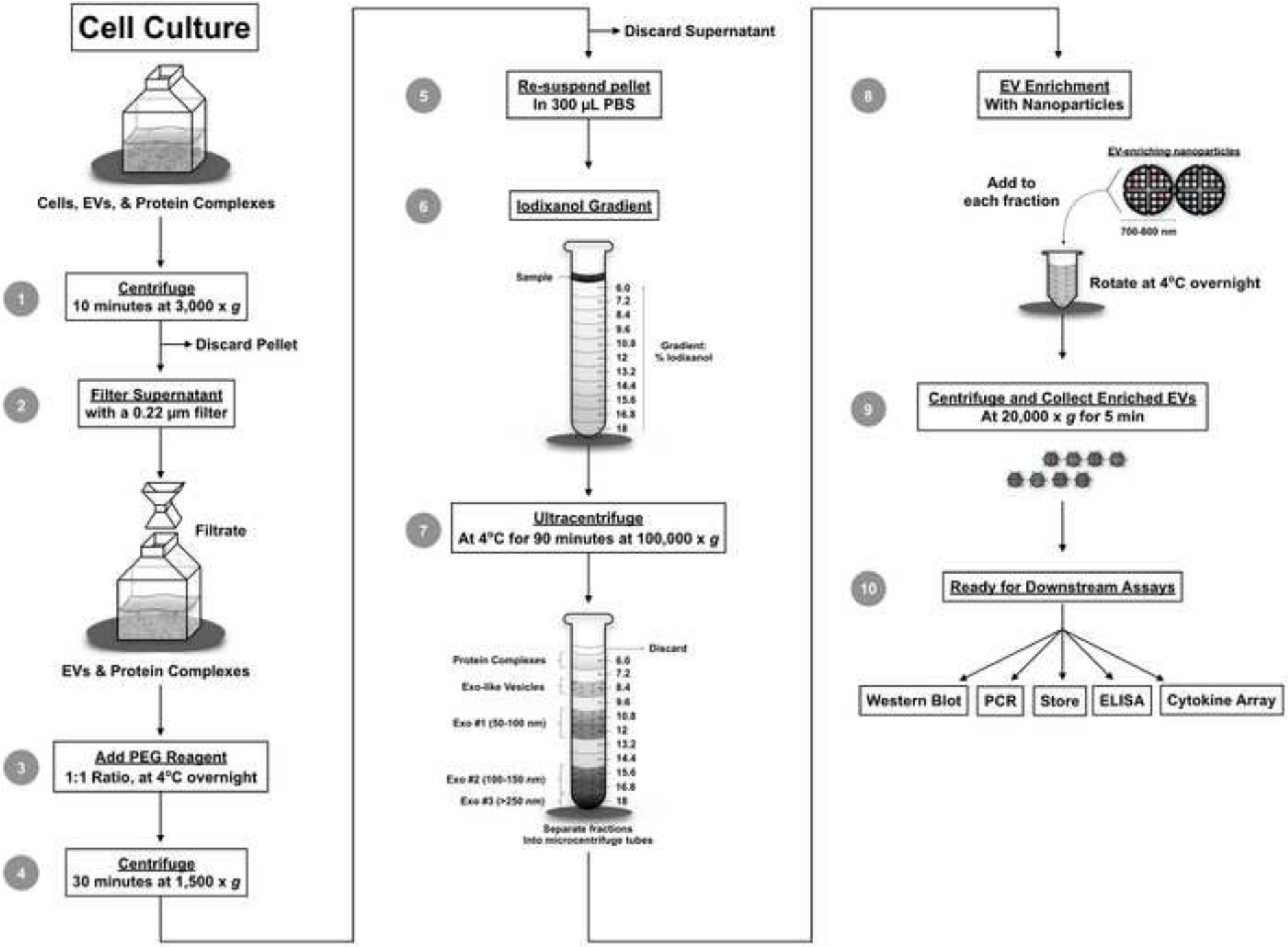


Figure 4



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
CEM CD4+ Cells	NIH AIDS Reagent Program	117	CEM
DPBS without Ca and Mg (1X)	Quality Biological	114-057-101	
ExoMAX Opti-Enhancer	Systems Biosciences	EXOMAX24A-1	PEG precipitation reagent
Exosome-Depleted FBS	Thermo Fisher Scientific	A2720801	
Fetal Bovine Serum	Peak Serum	PS-FB3	Serum
HIV-1 infected U937 Cells	NIH AIDS Reagent Program	165	U1
Nalgene Syringe Filter 0.2 µm SFCA	Thermo Scientific	723-2520	
Nanotrap (NT80)	Ceres Nanosciences	CN1030	Reactive Red 120 core
Nanotrap (NT82)	Ceres Nanosciences	CN2010	Cibacron Blue F3GA core
Optima XE-980 Ultracentrifuge	Beckman Coulter	A94471	
OptiPrep Density Gradient Medium	Sigma-Aldrich	D1556-250mL	Iodixanol
SW 41 Ti Swinging-Bucket Rotor	Beckman Coulter	331362	
Ultra-Clear Tube, 14x89mm	Beckman Coulter	344059	

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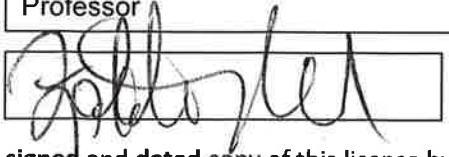
Signature:	
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March 25th, 2019

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."

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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.

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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."

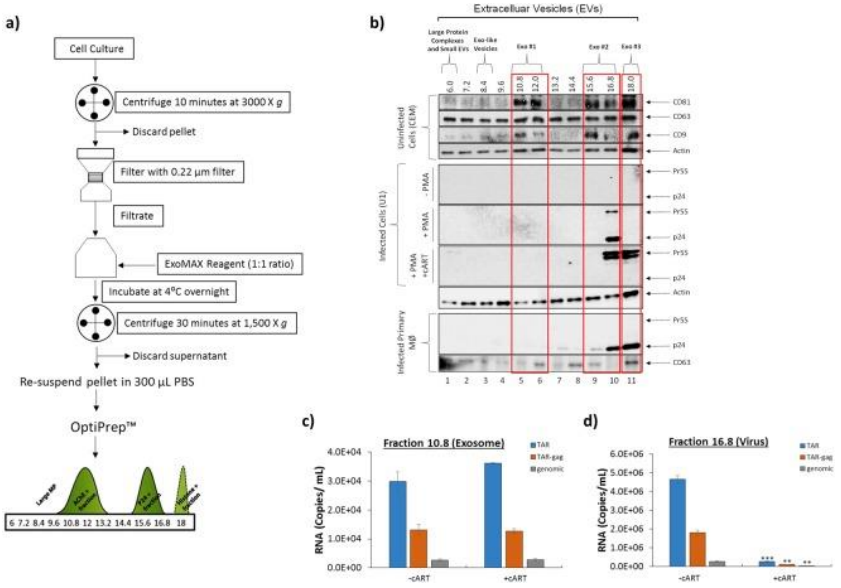
Minor Concerns:

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:

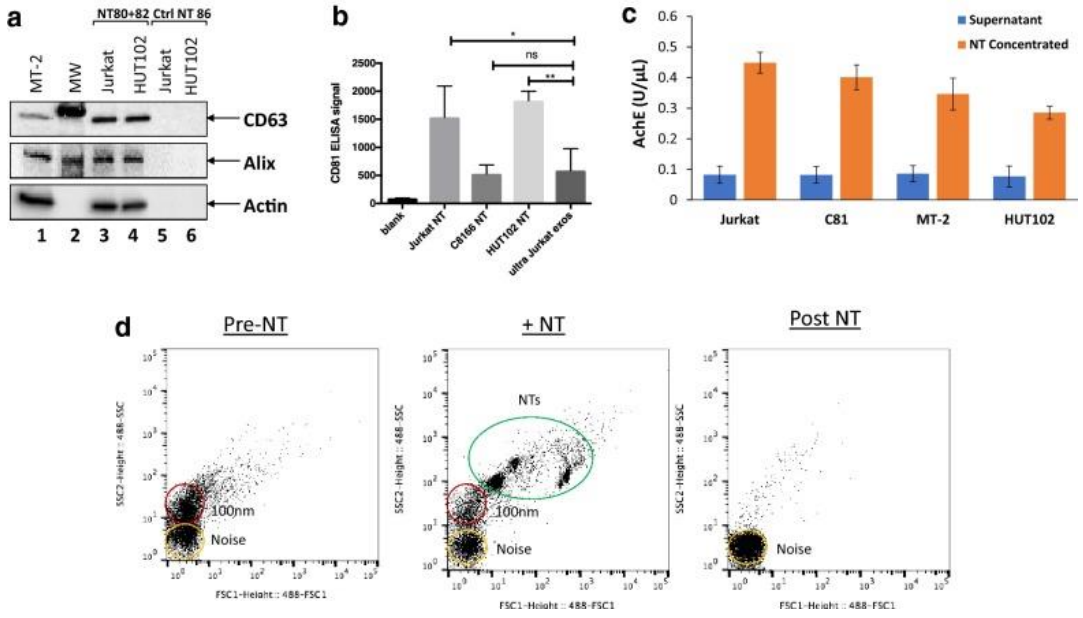
- **Lines 71-74:** "The current gold standard of EV isolation is ultracentrifugation. This technique makes use of the various vesicle densities, which allows the vesicles to be separated by centrifugation with differential sedimentation of higher density particles versus lower density particles at each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