The authors are grateful for the opportunity to revise the manuscript. In particular, we are thankful for the comprehensive review and the valuable suggestions of the reviewers. In the following, we would like to respond to the comments and questions of the reviewers in a point-by-point manner.

**Editorial comments:**

*Changes to be made by the Author(s):*

***Comment 1****: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*

Answer: We thank the editor for this hint and carefully proofread the manuscript.

***Comment 2****: Please revise lines 57-59, 63-67, 84-87, 89-90, and 238-240 to avoid previously published text.*

Answer: We are thankful to the editor for this important annotation and revised the stated lines.

***Comment 3****: Please revise the title to be more concise.*

Answer: We revised the title.

***Comment 4****: Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.*

Answer: We carefully reviewed the complete manuscript and revised the units, in particularly all units including the unit micro. Please state, if used abbreviations continued to be not conform to the International system of Units.

***Comment 5****: JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Exoquick, Snap-On, Fluoresbrite®, SuperSignal, etc.*

Answer: We thank the editor for this remark. We revised the complete manuscript and removed all commercial language.

***Comment 6****: Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.*

Answer: We adjusted the numbering of the protocol and removed the bullets.

***Comment 7****: Please revise the protocol (lines 206-214, etc.) to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.*

Answer: We thank the editor for this note. In the revised version now only imperative tense is used. The required safety procedures are included in the manuscript.

***Comment 8****: Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.*

Answer: Following your recommendations we have added more details to the protocol steps in the revised version. Because the processing of the sample is already published extensively in Mehdiani et al., we will not go into detail, but focus on the differences and specific steps. This fact was already stated in protocol step 3. For further alterations, please state more explicitly, which steps should be described in more detail.

***Comment 9****: Line 107: Please specify the blood source. What volume of blood is collected?*

Answer: Blood source and obtained volume are indicated now in the revised manuscript.

***Comment 10****: Line 109: Transfer 1 mL of serum or plasma? Please specify.*

Answer: We adapted the whole protocol for the use of serum to avoid ambiguities.

***Comment 11****: Line 224: Please specify incubation conditions.*

Answer: We specified the incubation conditions in the revised manuscript.

***Comment 12****: Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.*

Answer: As already stated in our response to comment 8, detailed information about the complete measurement process are already published in our previous JoVE publication. Please specify more explicitly, which steps should be more explicitly explained.

***Comment 13****: Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.*

Answer: We combined the shorter protocol steps as far as possible. Please note that some steps are critical and combining those steps could compromise the conduction of this protocol.

***Comment 14****: Please include single-line spaces between all paragraphs, headings, steps, etc.*

Answer: We included single line spaces between all paragraph, headings and steps.

***Comment 15****: After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*

Answer: We highlighted approximately 2.75 pages of manuscript.

***Comment 16****: Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.*

Answer: We thank the editor for these recommendations and highlighted complete sentences in imperative tenses only. Please state if there are any ambiguities left.

***Comment 17****: Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.*

Answer: We considered these recommendations at highlighting. The highlighted steps should include all relevant details to perform the step. Please state if there are any ambiguities left.

***Comment 18****: Figure 4: Please explain the right panels (4) in the figure legend.*

Answer: We thank the editor for this note. In the revised version of the manuscript, panel 4 is explained in the figure legend.

***Comment 19****: References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.*

Answer: We revised the references and wrote out the journal titles. Volume and issue numbers are included as far as they are provided by the National Center for Biotechnology Information.

**Reviewer #1:**

**Manuscript Summary:**

*Overall a good description of EV analysis by combination of nanoparticle analysis and labelling of EV. Simple to follow protocols that would be accessible to most laboratories starting work on EVs.*

Answer: We thank the reviewer for acknowledging the original character and the topic of our work. We also appreciate for bringing these points to our attention, all of which have been now addressed in the revised version of the manuscript:

**Major Concerns:**

*No major concerns*

**Minor Concerns:**

***Comment 1****: Lines 69 onwards in text - the authors use FACS as a term when I think they would be better to use flow cytometry. Reason - imaging flow cytometry instruments are now available for EV, and sorting is also an option, but analysis without sorting is probably used by most groups, therefore I think it would be better to go through the text and use flow cytometry as an overall term. They can expand on the various types of flow cytomerty - imaging, sorting, analysis etc in an additional sentence if they wish.*

Answer: We thank the reviewer for this suggestion. We revised the complete manuscript and replaced the term FACS by flow cytometry.

***Comment 2****: Line 122 - description of ultracentrifugation verges on dangerous! Many standard 1.5 ml tubes would not survive 110,000 x g and most rotors would not support these types of tubes anyway. Authors are using a bench top ultra - many other labs would use bigger floor standing ultras, so they need to be more specific to prevent accidental mis-use by individuals not well versed in use of ultra. I think some more explanation of tube types and machine compatibility would help here, and a recommendation that supervision should be sought in the use of ultracentrifuges.*

Answer: We thank the reviewer for this hint. We revised the complete paragraph and added further detailed information about the used materials and handling of the ultracentrifuge.

We revised protocol step 1.2 as follows (lines 137 – 154):

1. Take the pre-cooled rotor (TLA-55 fixed-angled) from the fridge and cool-down the ultracentrifuge before use.
2. Transfer 1.25 mL PPP (prepared in section 1.1) to a suitable 1.5 mL ultracentrifugation tube with cap and centrifuge the sample at 110,000 x g for 90 min at 4°C. Make sure the rotor load is balanced before starting.
3. Decant the supernatant and place tube turned upside-down on a paper towel for 2 min. Re-suspend the pellet in 500 μL PBS and centrifuge the sample at 110,000 x g for 90 min at 4°C.
4. Aspirate the supernatant and re-suspend the pellet in 50 μL PBS.

Note: Use only rotors and accessories designed for the ultracentrifuge you are operating. Pretest the tubes in the rotor by using water because the strength of the tubes can vary between lots.

***Comment 3****: line 139. Justify the use of water as a dilution reagent in this section and later sections. Does the change is osmolality between an EV being in serum versus water alter the results? I see no fixation step, so this is a concern. Insert reference to published data on serum free media or PBS versus water as evidence base for use in this protocol. Is there a limit on storage of EV preps in water?*

Answer: We thank the reviewer for this important question. Unfortunately, when we use PBS as dilution reagent, the detection of fluorescence labeled particles is quite difficult. Based on the high concentrations of ions in the diluent we suppose that the high osmolality affects the detection method. When analyzing an unstained suspension of particles, we generally used carefully selected water, which contains near to zero particles. The use of water as diluent did not affect the number of particles in the samples, but after a couple of hours, the particles begin to swell, however, we did not observe any particle burst. When diluting the samples for measurement, we recommend processing the sample as soon as possible to avoid variation in the detected size. We added an appropriate note in the protocol section as well as in the discussion.

We have added to protocol (lines 169 – 170):

“Note: It is crucial to use water as diluent for the EV suspension, because other diluents (e.g. PBS) can impair the measurement. “

We have also added to the discussion (lines 430 – 433):

“Normally, many researchers use PBS as diluent for EVs. For this method, it is crucial to use distilled water as diluent for the EV suspensions. When EVs are labeled with fluorescing dyes, the high osmolality and ion concentration of other diluents, such as PBS can interfere with the measurement and lead to altered results.”

***Comment 4****: Figure 4 a-c - why two lanes in the blots? Are they different loading or the same loaded twice? Please indicate in figure legend. Also CD63 blots are notorious for smearing from 20- 60 kDa range. It would be helpful to indicate that this might occurs in some samples.*

Answer: We thank the reviewer for this comment. All conducted experiments were performed with blood samples from two different donors, and therefore we used both samples for Western blot analysis. We added an appropriate annotation in the figure legend. We also added a note in the protocol that CD63 can be extensively and variably glycosylated, and that the molecular weight can vary and bands can appear between 40 - 65 kDa.

We added to the legend of figure 4 (lines 383 – 384):

“…representative Western blots of two different EV suspensions (4) for…”

We have also added to protocol (lines 309 – 310):

“Note: Because CD63 antigen is extensively and variably glycosylated, the molecular weight can vary and bands can appear between 40 - 65 kDa.”

**Reviewer #2:**

**Manuscript Summary:**

*Extracellular vesicles (EVs), including exosomes are nano-sized vesicles found in bodily fluids, which are released, from many cell types to play roles in regulating cell-cell communication in diverse range of biological processes. EVs studies are highlighted as potential biomarkers for early diagnosis of several diseases and/or prediction of disease progression. Exosomes are often described by the presence of molecules that they are specifically associated with regardless of the cell types they are driven from. Many methods are used for EVs purification methods to describe the characterization, however most of methods have disadvantages, such as time-consuming, difficult to analyze specific markers of interest due to the lack of discrete populations. There is still no standardized method for characterization of single EVs. Authors developed a semi-automated method for characterization of single EVs by fluorescence-based nanoparticle-tracking analysis. Their new advanced methods are based on their former method of nanoparticle-tracking analysis. Their protocol presents rapid isolation of EVs and characterization with both PKH67, a general cell membrane linker, as well as with specific surface markers such as CD63, CD9, vimentin and LAMP-1. They used human serum samples for EVs isolation, but their method is suitable for EDTA and citrated plasma and from cell culture supernatants. Their method resulted a high level of reproducibility, which is confirmed by Western blot. This manuscript has an interesting topic, innovated results, and showing potential significance. Reviewer suggests addressing few issues and data to revise their manuscript.*

Answer: We would like to thank the reviewer for careful and thorough reading of this manuscript and for the thoughtful comments and constructive suggestions, which help to improve the quality of this manuscript. In the following we would like to respond to the comments and questions of the reviewers.

**Major Concerns:**

***Comment 1****: In 1.1, please describe more detail about the methods used. For example, after whole blood collection, did you incubate at RT for how long before the centrifugation? The description is not user friendly.*

Answer: We thank the reviewer for this suggestion. We revised the complete paragraph and added more details about the isolation process.

The paragraph was revised as follows (lines 110 – 132):

1. Collect 2 mL human whole blood in serum-separating tubes (SST) via venipuncture and incubate the tube for 15 min at room temperature (RT) until coagulation is finished.
2. Centrifuge the SST at 1,700 x g for 10 min at RT to separate cells from serum and transfer 1 mL of the serum to a 1.5 mL reaction tube. Centrifuge the platelet-rich plasma (PRP) at 3,000 x g for 15 min at 4°C to remove platelets and transfer 100 μL of platelet-poor plasma (PPP) to a new 1.5 mL reaction tube.
3. Add 25 µL exosome precipitation solution (4 parts PPP, 1 part exosome precipitation solution) and vortex thoroughly. Incubate the sample for 30 min on ice. Keep the tube upright and do not rotate or mix the tube during the incubation period.
4. Centrifuge the sample at 1,500 x g for 30 min at 4°C to pellet the EVs. After centrifugation, the EVs appear as a beige or white pellet at the bottom of the vessel. Aspirate the supernatant and centrifuge the sample at 1,500 x g for 5 min at 4°C.
5. Remove all traces of fluid and re-suspend the pellet in 100 µL phosphate-buffered saline (PBS) by frequently pipetting up and down. Store the EV suspension at -80 °C when analysis is not be performed immediately.

Note: When isolating EVs from plasma, fibrinogen and fibrin can impede efficient recovery and re-suspension is heavier and takes more time.

***Comment 2:*** *Spell PBS in full, at least for the first time such as phosphate-buffered saline (PBS).*

Answer: We thank the reviewer for this hint. We reviewed and revised all the used abbreviations in this manuscript.

***Comment 3****: Authors used PKH67 to staining the EVs. This dye is non-specific membrane dye and now exosomes specific dyes are available in commercially. Reviewer suggests using such dyes to in their method.*

Answer: We thank the reviewer for this suggestion. We also tested an Exo-Glow staining kit but unfortunately, these fluorescing dyes are not stable enough for the applied method. Of course, we will consider the use of novel dyes for refining our method for future applications

***Comment 4****: Lowry protein assay needs to be described.*

Answer: We added a more precise explanation how to handle and prepare the samples for Lowry protein assay.

We have added to protocol (lines 291 – 297):

1. Measure the total protein by Lowry protein assay kit. Dilute 1 μL of the isolated EV suspension with 49 μL RIPA buffer and use 5 μL in triplicates for analysis. Dilute the EV suspension with 2x Laemmli loading buffer to a final concentration of 2 µg/ μL and heat for 10 min at 95°C.
2. Load 20 μg of protein per well. Separate and transfer the proteins by polyacrylamide gel electrophoresis and tank blotting according to standard protocols.

***Comment 5****: How did you store the purified exosomes?*

Answer: Normally, we store the purified exosomes at -80°C for long-term storage. But all analyses for this manuscript were performed immediately after isolation. We added a respective comment in 1.1.

We have added to protocol (lines 131– 132):

“Store the EV suspension at -80 °C when analysis is not performed immediately.”

**Minor Concerns:*****Comment 1****: Add detailed information for the reagents you used, such as Exoquick, PKH67, etc.*

Answer: We thank the reviewer for this hint. We added more details for the used reagents in the results and in the discussion as well as in the materials table.

We have added to representative results (lines 328– 330 and 354 - 355):

“PKH67 is often used for proliferation monitoring but has also proven useful for monitoring exosome or liposome uptake as well as for in vivo cell trafficking. Due to the non-specific labeling of PKH67, a wide variety of EVs can be labeled and detected”.

“EVs were isolated by a polymer-based exosome precipitation solution containing polyethylene glycol”

We also have added to the discussion (lines 411 – 414)

“In this protocol, the EV suspension was generated from 100 μL serum using an exosome precipitation reagent, which contains a proprietary polymer that gently precipitates exosomes and EVs according to a corpuscular size ranging from 30 nm to 200 nm”

***Comment 2****: Please correct the reference for LOTAN etc. This format needs to be revised to match other references.*

Answer: Because there is no reference for “LOTAN” we edited all references according to the journal guidelines

***Comment 3****: The last paragraph of Introduction can be revised to strengthen the discovery.*

Answer: We revised the last paragraph of the introduction and added further useful information for practical application.

We revised in the introduction (lines 97– 102):

“In this protocol, we describe the complete workflow for rapid isolation of EVs from human whole blood and fast characterization of specific markers by fluorescence-based nanoparticles-tracking analysis. EVs can be detected by staining with PKH67, a general cell membrane linker, as well as with specific exosomal markers, e.g. CD63, CD9 and vimentin. Our protocol is also suitable for EDTA and citrated plasma as well as other body fluids and cell culture supernatants.”

***Comment 4****: Revising Abstract is suggested in the last few sentences to highlight the discovery.*

Answer: We revised the abstract in the last few sentences.

We revised in the abstract (lines 49– 55):

“In the conducted experiments, we exclusively used EVs isolated from human serum samples, but this method is also suitable for plasma or other body fluids and can be adjusted for characterization of EVs from cell culture supernatants. Irrespective of the future progress of research on EV biology, the protocol that is presented here provides a rapid and reliable method for rapid characterization of single EVs with specific markers.”

Again we would like to thank the reviewers for their critical review and suggestions. Following their recommendations we feel that the manuscript has gained in clearness and validity, and we hope that the revised form of our work will find the approval of the reviewers as well as the editors of JoVE.