Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations upon first use.

The text was revised as suggested.

2. Your Abstract is over the 150-300 word limit. Please rephrase it to be within 300 words.

It was shortened as suggested. Thank you

3. JoVE cannot publish manuscripts containing commercial language. 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.
For example: Akkermansia mucinophila ATCC BAA-835, Bifidobacterium dentium ATCC 27678, nCS1 Viewer, Tecnai G2 SpiritBT, Nano-Glo Luciferase etc.

The commercial products were deleted from the main manuscript as suggested

4. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The text was revised as suggested.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.

The protocol was revised as suggested.

6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

The protocol was revised as suggested.

7. 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.

It was corrected

8. Please use the symbol µ instead of u throughout the manuscript.

It was corrected

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Step 2: How was plasmid linearized and how was the promoter and gene sequence deleted? How were the promoter and reported inserted? How was the gfp replaced and the linker inserted? How were the cloning, verification of cloning, extraction, and transformation done? If all this has been described in a publication, please cite that publication.

More details were added to this paragraph, and the whole paragraph was moved to the supplementary information to avoid distracting from the main protocol as it describes only cloning steps which are not relevant to EV isolation technique.

Step 3.1.1: Please specify the cell cultures used here, are they from Step 1 or 2 or both.

We clarified that the bacterial culture was generated by inoculation of bacteria from Step 1.

Step 3.1.2: How was the filtration done?

We added the sentence, "Filter by connecting the filtration device to a vacuum wall supply set to the open position".

Step 3.2.1.2.3: Please specify volume of ethanol, NaOH, water, and PBS used.

Volumes were added to protocol

Step 4.2: How were the fluorimeter-based measurements done: wavelengths etc

Information was added, "The kit works with a matching proprietary fluorimeter, at excitation/emission wavelengths of 485/590 nm."

Step 4.3: How was the value corrected? What was the particle concentration obtained here and how was it diluted to obtain the desired concentration?

Information has been clarified:

"Dilute samples in PBS supplemented with 1 % Tween-20 that has been filtered through 0.02 µm syringe filter to a protein concentration of approximately 0.1 µg per mL.

Note. The goal of dilution is to reach an expected particle concentration in the range of 10^{10} particles/mL. The optimal dilution may need to be determined empirically. For later fractions (beyond Fraction 6) few EVs are expected. Thus, despite analyzing at low dilutions, particle concentration will likely be $<10^{10}$ particles/mL."

Step 4.3, 6.1.2, 6.2.4: Please include button clicks for software usage, parameters for image acquisitions, etc.

Information has been added:

"3.3.2 Load 3 µL of each sample into the disposable microfluidics cartridge with a micropipette, insert the cartridge into the MRPS instrument, and push the metal button that has a blue light rim.

3.3.3 Click "Go!" on the acquisition software and wait for the sample to be analyzed by the instrument. Acquire 1,000 to 10,000 particle events to minimize the technical statistical error of analysis. At this point, click "Stop," and "End Run" to complete sample acquisition.

3.3.4 Together with the raw data files, the instrument outputs a summary spreadsheet listing the particle concentration in the sample. Correct this value according to the sample dilution made"

AND

"**On the microscope acquisition software, adjust exposure to obtain optimal quality of image. This may need to be determined empirically, for example 0.80851 seconds was used in this particular setup, by typing this value into the "exposure time" option box. Select the 80 kV option, then click "Start Acquisition" to capture the image. "**

Step 5: What was the aliquot volume? Please mention.

Information was added.

"**Aliquot individual or pooled fractions to 25-50% of individual fraction size (depending on the size of column used) in low protein binding tubes and store at -80 °C to avoid freeze-thaw cycles. Different applications may require smaller or larger aliquots depending on the expected amount to be utilized in each experiment. This will need to be determined empirically. Also, you may choose to discard non-EV containing fractions if not applicable to your research. "**

10. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.
Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader. Please do not highlight any steps describing euthanasia.

The layout was corrected as suggested.

11. As we are a methods journal, please add any limitations and the significance of your technique with respect to existing methods to the Discussion.

Updated wording to clearly reflect significance and limitations:

"**The significance of this methodology compared to widely used ultracentrifugation-based methods are that it is comprised of steps that can be easily reproduced by different users, and is highly scalable. This is especially important to facilitate the generation of sufficient material for *in vivo* studies. It was used to isolate EVs from cultures of 100 mL to 2 L. Given the wide range of available TFF devices, it is possible that this protocol could be adapted to larger-scale purifications with some modification. The isolation protocol described is primarily based on the physical properties of EVs, namely their size, and is likely applicable to bacterial species beyond those described in this study. Regarding limitations, there are other protocols that can likely attain higher EV purity (for example density gradient ultracentrifugation or immuno-isolation). However, these methods lack the throughput and scalability of the methods described in this study. Modification of this protocol with additional purification steps in the future may further increase the yield and purity of preparations, which could be important for experimental and therapeutic applications**."

12. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

A) We included a sentence in the legends of figures 3, and 4 to indicate that all images were taken with the same scale.

13. Figure 5A: Please provide y-axis values and y-axis titles for B. breve and B. dentium.

Figure has been updated

14. Figure 5B: Please provide y-axis values.

Figure has been updated

15. Supplementary Figure 3 and 4: Please include a short description of each figure. All figures and/or tables showing data must include measurement definitions, scale bars, and error bars (if applicable).

A) The descriptions were added as suggested to supplementary figures 3 and 4.

16. Please move information about sources of plasmids (e.g., lines 129-130) to the acknowledgment section.

A) It was moved as suggested

17. Please do not abbreviate journal names in references.

Journal names have been updated

18. Please ensure the inclusion of all items (tubes, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please sort the Materials Table alphabetically by the name of the material.

A) The table was sorted alphabetically

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript the authors propose a combination of ultrafiltration and SEC separation to isolate bacterial EVs from conditioned medium.

Major Concerns:

This method seems to enrich for very small EVs less than 100 nm. However, many bacteria produce EVs that are more heterogeneous in size 60-300 nm and an average of ~100-150.

Many factors can affect the size distribution, including the producing species, growth conditions, and enrichment protocol. In our protocol, most of the isolated EVs are <100 nm. As the reviewer suggests, it is possible that there exist other sub-populations of EVs that are larger. We added the sentence below to acknowledge this limitation:

"One limitation of the protocol described is that it favors isolation of small EVs, particularly <100 nm, as seen in the representative results. Prior reports also describe the presence of larger bacterial EVs[15,16]. Isolation of larger bacterial EVs may require modifications of the protocol above, for example by using SEC columns optimized for larger EVs. Such SEC columns are also commercially available."

Minor Concerns:
Temperature for large volume concentration (TFF) is not mentioned. Was this step done at 4 degrees or Room temperature?
TFF was done at room temperature. The manuscript text has been updated:

"2.2.2.2 At room temperature, begin circulating the filtered conditioned medium at approximately 200 mL/min (minimum 100 mL/min)."

Luciferase assay (How long is the incubation). Positive control for luciferase activity should be used in the assay.

information on incubation time was added to the manunscript:

"Similarly, for luminescence measurement, 20 µL aliquot of each fraction was mixed with equal volume of the Luciferase assay solution in 384-well plate, incubated for 15 minutes, and visible light luminescence was measured."

We also added the information regarding positive/negative controls to the text:

"Background signal from equal amount of negative control EVs (isolated from matched bacterial strain lacking nanoluciferase expression) was >1,000-fold lower in EV fractions; signal from positive control (nanoluciferase-expressing bacterial cell pellet) was approximately 1,000-fold higher than the EV fractions (not shown). The latter is normalized to the initial volume of cell culture material yielding the analyzed cells or EVs, respectively."

Is MCherry secreted into the medium where it can "contaminate EVs" if it is not secreted I do not see the rational of this control.

Thank you for this comment. Our data suggest that some mCherry was indeed released into the culture medium (Figure 3B). Excretion of cytoplasmic proteins through cell lysis or other mechanisms, has been previously reported (reviewed for example in PMID 25596889).

Fractionation of the culture medium by SEC led to elution of mCherry in fractions that lacked EVs (as assessed by both MRPS and transmission electron microscopy), which indicates that the detected mCherry fluorescence was independent of EVs. Taken together, the data presented in Figure 3B provides evidence in favor of our bacterial EV isolation protocol's ability to separate EVs from non-EV-associated secreted proteins.

For animal experiment - A Cy5.5 (alone) in PBS control is more appropriate than PBS alone.

This is a great suggestion. Unfortunately, the quenching reagent with this particular kit turned out to be non-compatible with our filtration devices. This became evident when we repeated the staining reaction with Cy5.5 alone (no EVs). We thus decided to omit the biodistribution figure in the absence of the most appropriate control.

Reviewer #2:
Manuscript Summary: The manuscript describes two methods of purifying bacterial membrane vesicles adaptable to several bacterial species. Choice of protocol is largely determined by volume with the TFF method described being applicable to large-scale purification of bacterial vesicles. Protocol is well written and easy to follow. Would benefit from a better description of materials and equipment (though I may have missed these in the version I downloaded)

Thank you for this comment. Per the journal editor's instructions, detailed description of materials/equipment with commercial names is not allowed in the main manuscript text. This information is provided in the Materials Table spreadsheet.

Major Concerns:
- in section 4.1 the authors suggest checking for contamination but do not suggest a course of action if bacterial growth is observed. While most can infer that you should move back a step there should be some language here to explain or describe contingencies.

We added language clarifying that TFF should be performed in a biosafety cabinet:

Perform TFF within a Biosafety Cabinet to minimize risk of contaminating the EV preparation with environmental bacteria.

Furthermore, we added language describing contingency to bacterial contamination:

After the initial centrifugation/filtration:

"If bacteria are detected, it is recommended to further optimize the procedure above by performing additional centrifugations and/or filtrations."

And at the final quality control of the EV preparation:

“If bacterial contamination is detected, it not recommended to use the EV preparation for experimentation. Instead, it is recommended to repeat the isolation, taking care to minimize the risk of bacterial contamination by: (a) performing sufficient centrifugation/filtration of conditioned bacterial cell culture medium, (b) using clean bottles, tubing, filters, and chromatography columns, and (c) performing appropriate aseptic techniques.”

- in section 4.2 the authors indicate that EVs have low concentration of protein but this is not always the case. protein concentration can be affected by a number of conditions and often OMVs/MVs can be quite rich in proteins dependent on parental organisms, growth conditions, etc. Authors should provide references or data to support this statement.

The reviewer is correct. We have deleted this phrase, as it is not universally applicable.

- also in section 2 the authors talk about a highly sensitive protein quantification kit but give no details as to the manufacturer or product name.

As mentioned above, the journal does not allow providing commercial details in the main manuscript text. This information is provided in the Materials Table. In this instance, we are referring to the Qubit protein assay kit (ThermoFisher).

Minor Concerns:
- there is some discussion on whether or not bacterial vesicles should be defined as EVs. The EV nomenclature is more commonly used with vesicles from eukaryotic cells. OMVs for gram negative bacterial vesicles or MVs for gram positive vesicles are more commonly used.

Indeed, there is discussion on the appropriate nomenclature. The latest nomenclature recommendation from the International Society of Extracellular Vesicles (ISEV; PMID 30637094) recommends using the general term “extracellular vesicles” in their “nomenclature section”. The same consensus paper uses the term “EVs” to describe prokaryote-derived vesicles in Table 3. Thus, we decided to also uses the more general term “EVs” and “bacterial EVs” in this protocol, as it encompasses vesicles produced broadly by a variety of species.

- be consistent with the use of "mL" versus "ml" and "ul" versus 'uL" throughout there are sections in one format and others in the other format.

This has been corrected.

-section 6.1.1 the sentence describing the buffer is in an unusual format. suggest "5mM Tris (pH #.#)"

This has been corrected:

"**5 mM Tris (pH 7.1)**"

Reviewer #3:
Manuscript Summary:
In this paper, Watson et al describe a method to isolate bacterial membrane vesicles from monocultures using a combined approach with ultrafiltration and SEC. This method can be used for small volumes, but also larger volumes >1L. Several techniques have been used to validate the purity and the yield. Also recombinant bacteria have been used to validate the method. These recombinant vesicles have also been used to study the in vivo distribution in mice, showing that most vesicles end up in the liver.

Major Concerns:
Methods are described in great detail, with an extensive list of consumables/equipment needed. This will make is possible for most researches interested in this field to repeat the experiments. With respect to the results, however, I do have some concerns. In one of the first papers showing that SEC is very useful to separate EVs from (lipo-)proteins (Boing et al 2014) is was shown that the vesicles appear starting from fraction 5-6. This has been confirmed by many others in the meanwhile (depending a little on the samples size (0.5 vs 1.0 ml/fraction). In the current paper the authors claim that vesicles already appear in high numbers in fraction 2 (fig 2A) and they seem to have disappeared from fraction 5 eben though they also collect 0.5ml fractions. This is quite contradictory to the results by others. TEM pictures on the other hand suggest that the highest density of vesicles seems to be in fractions 5-8, which is more in accordance to findings of other groups.

Thank you for these comments. Boing et al 2014 (PMID: 25279113) used an in-house Sepharose CL-2B column to isolate EVs. We used a commercial SEC column from Izon, with undisclosed column bed composition. Thus, direct comparison may not be accurate. Nonetheless, we noted that there is a difference in the way fractions are numbered between our protocol and the work published by Boing et al. Specifically, we do not count the eluted column void volume in our fraction numbering. This was done to avoid confusion with fraction numbering of columns with different void volumes, and has also been adopted by Izon in their instruction manuals as they have expanded their column repertoire to many more sizes (ranging from 0.1 – 100 mL of sample loading capacity) in addition to their "original" column which accepts up to 0.5 mL sample.

Keeping in mind the potential for an imperfect comparison between columns with potentially different composition, our Fraction 1 of the smaller SEC column corresponds to the 3.0 – 3.5 mL elution, if we also consider the void volume. Using 0.5 mL fraction volumes, this would correspond to Fraction 7 (using the Boing numbering scheme). Our peak EV elution by resistive pulse sensing was observed at Fraction 3 (or Fraction 9, using the Boing numbering). This is very similar to the observed EV peak in the Boing paper (Fraction 10).

Regarding the differences observed in EV abundance between MRPS and TEM analyses, this can be explained by the significant secretion of EVs <50 nm in size by the bacteria we tested.

These are below the limit of detection of MRPS, and can be more accurately assessed with electron microscopy.

The text was updated to clarify this observation:
"**This is likely due to the presence of significant numbers of very small bacterial EVs (as visualized in TEM, Figure 2D) that are below the limit of accurate detection by MRPS and again lead to instrument noise.**"

Finally, we did find EVs in pooled Fractions 7-8, however these were less abundant than preceding fractions (Figure 2D). Together with the rising total protein concentration in Fractions 7/8 (as compared to the more EV-rich Fractions 5/6), this suggests that Fractions 7/8 are of lower purity, containing also non-EV-associated proteins. Thus, for our experiments, we do not use Fractions ≥7 for EV studies. Others may choose to also include these fractions in their studies.

The text was updated to discuss these nuances:

"**As described in this protocol, quantification of EV abundance, total protein concentration, and abundance of non-EV proteins in the eluted chromatography fractions can help users decide which fractions to use for downstream assays. For example, small EVs were detected in pooled Fractions 7-8 (Figure 2D); however, their abundance was lower than immediately preceding fractions, while the total protein concentration (Figure 2B) was higher. This may suggest that Fractions 7-8 contain higher amounts of non-EV-associated proteins and may thus not be desirable for certain downstream applications**."

Likewise, although the authors claim that there's only low amount of protein in the EV fractions, fig 2b tell a different story. It shows that the protein concentration in the fractions containing the vesicles is even higher than those fractions which are supposed to contain the highest concentration.

Thank you also for this comment. In our manuscript we specifically propose that TFF + size-exclusion chromatography is useful in separating EVs from non-EV associated proteins, resulting in a low amount (specifically) of non-EV proteins in the EV-enriched fractions. We used a combination of EV quantification by 2 different methods, total protein quantification, and a recombinant non-EV-associated protein (mCherry) to make this argument.

The reviewer correctly points out that the early EV-enriched Fractions 2-3 have a higher protein concentration than the later EV-enriched fractions 4-6. This likely reflects a different amount of protein loading in the larger vs. smaller EVs that elute in the early and later fractions, respectively. We speculate that this could be due to different biosynthetic pathways and/or biophysical constraints of the smaller EVs that might limit protein loading, but this falls beyond the scope of this manuscript.

I think it is really important to clarify these issues, which cannot simply be related to the difference between eukaryotic and bacterial vesicles. Kameli et al. (2021) recently demonstrated

by using a similar methodology that bacterial vesicles are predominantly found in fractions 7-11. Since it is of great importance to know in which fraction the vesicles end up after SEC, this issue needs to be addressed carefully.

Kameli et al. 2021 (PMID 34026664) references the Boing 2014 paper discussed above as the method of SEC. As discussed above, our Fractions 1-5 correspond to Fractions 7-11 using the Boing 2014 numbering scheme.

Minor Concerns:
none