**Editorial comments:**  
Changes to be made by the Author(s) regarding the manuscript:  
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.  
2. Please provide figures with higher resolution if possible.   
3. Figure 2C: Please define error bars in the figure legend.   
4. Figure 3: Please change ml to mL.   
5. Figure 6B and Figure 8C: Please include it as a table and upload it separately in the form of an .xls or .xlsx file.   
6. Figures 8: Please line up panels A and B better.  
7. 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: Agilent RNA 6000 Pico Kit, Agilent 2100 Bioanalyzer, Illumina TruSeq, Pippin Prep, KAPA, Bowtie, etc.   
8. 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.

Thank you, for points 1- 7 we have reworked the manuscripts, figures and tables as you have suggested.

9. Please revise the protocol (107-122, 125-127, 135-142, 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.

These sections have also now been rewritten accordingly.

10. 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.

A more detailed detailed library construction protocol (which is the crux of this protocol) has now been added. (Section 5 on page 5)  
11. Please provide composition of MSC media, ExRNA collecting media, etc. If purchased, please cite the Materials Table.

The Materials table has been cited. It now reads (line 87-88): “Preparation: Mesenchymal Stem Cell Growth Medium (MSC media) is prepared beforehand as indicated in the Materials table.”

12. Line 95: What is the concentration of trypsin?

This has now been added: 0.05% Trypsin-EDTA (line 100 of the new draft)

13. Line 102: What container is used?

T175 flasks were used and this is now added(line 106-109) .

14. Line 123: How to dry the inside of the tube?

How the inside of the tube was dried is now revised (line 130-133): “Remove the supernatant, dry the inside of the tube by inverting the tube on absorbent paper and use small pieces of absorbent paper to remove the liquid inside the tube without touching the bottom of the tube, and resuspend the pellet in 200 μl of PBS by vortexing for 30 s and pipetting up and down 20 times.”

15. Line 162: Please specify what samples (i.e., from which this step) are used.

Now amended (line 169): “Thaw samples from step 2.9 on ice.”

16. Line 164: It is unclear. From where is the RNA eluted?

Now amended (line 171): “Elute the RNA from the column provided in the RNA isolation kit in 100 μl of RNase free water.”

17. For steps that are using a commercial kit (lines 172-174, 178-180, 191-195, 202-203, etc.), please ensure that the protocol steps have sufficient details to replicate.

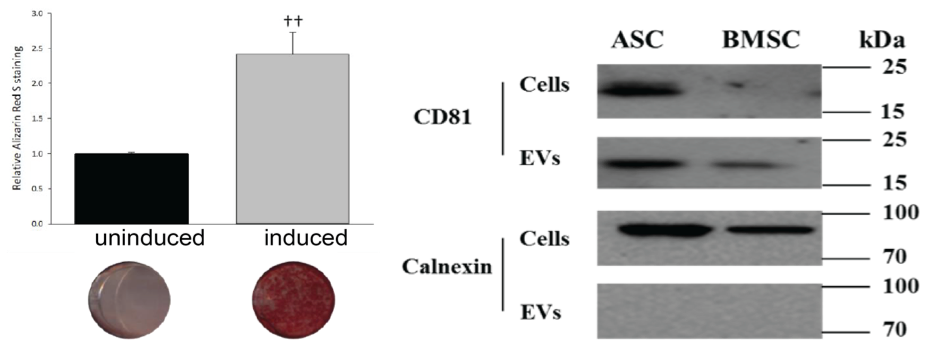
These sections have been revised accordingly. The Library Construction sections (Section 5) have been fully revised in detail.

18. Please include single-line spaces between all paragraphs, headings, steps, etc.  
19. 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.   
20. 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.   
21. 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.   
22. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: done  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique  
23. References: Please do not abbreviate journal titles.

Points 18-23 have been revised accordingly.  
  
**Reviewers' comments:**  
  
Reviewer #1:  
  
Manuscript Summary:  
Dear author,  
The manuscript reported the protocol details a complete guide to harvesting exRNAs from culture media, optimizing small RNA raw library data. This protocol provides a specific exosomal extraction process that can reduce the difficulty of exosomal acquisition. Although the authors presented some confidential results about the records for this study, there are several serious questions raised as followed:  
  
Major Concerns:  
  
1.Line 108-109："EV collecting media is normal MSC media, but prepared with EV-depleted FBS. "Whether there is FBS in EV collecting media? How"EV - depleted FBS"did not show in this article. Whether the medium treated by this method has any effect on cells growth.

Thank you for your comment. The EV-depleted FBS was bought from a company (refer to the Materials list). Since cells were grown in normal media (with normal FBS) we did not assess whether EV-free media affected proliferation or growth. The main reason is because the EV-free media mainly served as a medium for EV collection, for EV collection, meanwhile the normal media was used for both proliferation and differentiation.

2.Line 148: "Replenish the osteoblastic differentiation media once every 2-3 days ." This concept is too vague, please give the specific shape of the cells when you need to change the media,or quote the relevant literature to explain the state of the cell at this time.



Previous studies by our lab demonstrated that replacing the osteogenic differentiation medium every 2-3 days promotes osteoblastic differentiation (see references below) and the figure above.

<https://doi.org/10.1002/stem.1615>

<https://doi.org/10.1016/j.ymthe.2017.11.018>

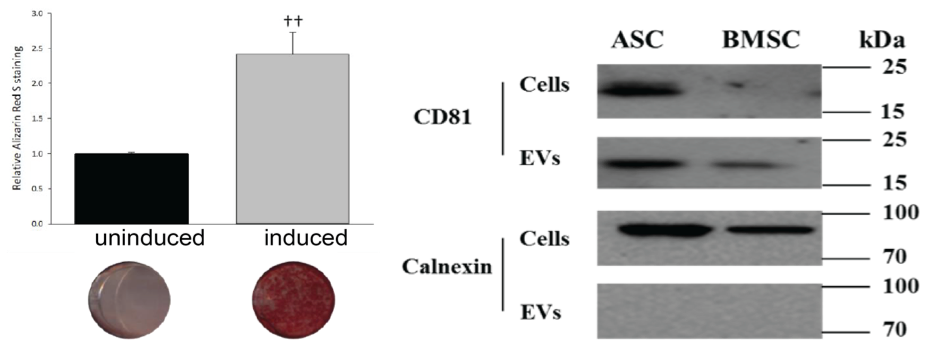
3.Line162："Remove the samples from -80°C and thaw on ice. "Is the freshly obtained exosomes treated in the article? Is there a comparison the efficiency of the two methods? Have other literatures tested for two different environmental efficiencies?

The freshly obtained EVs were not treated in the article. The main reason they are stored at -80°C is for optimal storage of the RNA for downstream usage. Storage conditions that evaluated the temperature effects on exosomes have been previously described (links to the references are supplied below).

<https://link.springer.com/article/10.1007%2Fs12257-015-0781-x>

<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4275651/>

4.Figure 2: The morphology of undifferentiated BMSCs (figure 2A) and BMSCs differentiated for 7 days (figure 2B) look similar. Pictures must be taken at higher magnification. A count of undifferentiated, mixed and differentiated colonies as it is usually done could help in understanding the level of differentiation observed in the cell populations.  
The level of differentiation was previously shown by means of Alizarin Red S staining, which is commonly used for the detection of *in vitro* differentiated BMSCs. The results were not included in this paper, however, the extent of differentiation (amount of red staining signifying the amount of calcium deposition from osteogenesis) is visible in the figure below.



Minor Concerns:  
  
5.Please provide the manufacturer and item number of the kit in the article.

All manufacturer items and numbers are in the Materials list that was submitted along with the manuscript. Jove specifically asked for us to leave out company names and trademarks.  
  
  
Reviewer #2:  
  
Manuscript Summary:  
The manuscript describes a method of measuring the RNA profile of extracellular vesicles from cultured MSC. The authors describe the culture of cells, the collection of media, the isolation of EVs from the medium, the isolation of RNA from the EVs, cDNA preparation, and a bioinformatic pipeline for measuring the library, all with quality checkpoints described. The protocol is concise but seemingly relatively complete. A few minor concerns exist.  
  
Major Concerns:  
Figures are unreadable.

My apologies, the system only allows for very small file sizes. We have reworked everything now.  
  
Please discuss why so many bacterial reads are appearing in the preps. Sine these are ostensibly clean cultured cell samples, this is highly unexpected.

Thank you for your comment. We too have been perturbed by this puzzling finding. We have discussed these findings in the discussion section: “One of the reasons for these contaminants is that bacteria overlap in size with extracellular complexes or EVs, and hence co-purify during the ultracentrifugation step. Previous publications have identified widespread contamination of certain bacteria in culture media which remain undetected under normal cell lab procedures. To date, this problem has largely been ignored but should be taken into account when purifying and analyzing exRNAs.”

Minor Concerns:  
Step 2.5: The authors recommend four centrifugation steps. Many protocols call for only three steps. Often time, EV isolation is some variation of low speed, medium speed, high speed. Authors may want to add a note as to why they recommend two medium speed steps.

The protocol we used was previously published in <https://doi.org/10.3402/jev.v3.25011>. We opted for this method because two medium steps of centrifugation ensured that cells and cellular debris was fully eliminated.

2.7: Authors tell the reader to "dry" the centrifuge tubes. The reviewer agrees that this is important, but they may want to add some detail about how they recommend drying. E.g., vacuum, air drying, etc. This is a nontrivial step and could help to avoid contaminants. Also resuspending is important. Tell the reader how many time they may expect to have to triturate their sample, as often the pellet is too small to see with the naked eye.

Thank you for your comment. We have revised this now and it now reads, “Remove the supernatant, dry the inside of the tube by inverting the tube on absorbent paper and use small pieces of absorbent paper to remove the liquid inside the tube without touching the bottom of the tube, and resuspend the pellet in 200 μl of PBS by vortexing for 30 s and pipetting up and down 20 times.”  
  
4.2: Please recommend to readers which small RNA isolation kits the authors have experience with, along with pros/cons if available.

Thank you for your comment. Unfortunately, we haven’t performed systematic experiments to demonstrate the efficiency of different small RNA isolation kits. The main takeaway from this paper is to know that when some results differ from recommended protocols, and that the reason is because the samples are not conventional samples (they are EVs).  
  
6.3: Some explanation of this formula would be helpful.

“Normalize miRNA expression using the following formula: (miRNA counts / the total counts of all mapped miRNAs)\*106 ”

This formula is a method to quantify the relative expression of miRNAs in a sample by representing it as the number of reads per million.

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