**All the responses to the editors and reviewers are written in blue.**

**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.

Done to the best of our ability.

2. Please print and sign the attached Author License Agreement - UK. Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

Done.

3. Please obtain explicit copyright permission to reuse any figures (Figure 2) from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

This figure is adopted from another manuscript prepared by the authors, which is currently being revised to address reviewers’ comments from another journal. Since this figure is more relevant and critical to this manuscript, this figure will be removed from the other manuscript and put in this manuscript instead.

4. Keywords: Please provide at least 6 keywords or phrases.

Added.

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

Corrected.

6. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Corrected.

7. Please use centrifugal force (x g) for centrifuge speeds.

Corrected.

8. 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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: CELLine™, Sepharose®, GlutaMax™, Beckman Coulter, Micro BCA™, NanoSight LM10, Millipore, Philips CM 12, FEI Electron Optics, Nucleofector™, Lonza, Eppendorf, Costar®, CorningTM, BD FACSCaliburTM, etc.

Corrected.

9. 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. Some examples:  
1.1: Please specify the culture conditions.

Added

4.2, 4.3: Please describe how to measure size distribution and concentration.

Added

5.1: Please ensure that the protocol here can stand alone. As currently written, users must refer to another commercial protocol in order to complete this protocol.

Added

7.1: What concentration is considered to be proper?

Added.

10.2: Please provide more details here.

Corrected.

10.6: What volume of sterile PBS is used to wash?

Added.

10. 7.4: Please write this step in the imperative tense.

Modified.

11. Please include single-line spaces between all paragraphs, headings, steps, etc.

Included.

12. 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.

Highlighted in yellow.

13. 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.

Done.

14. 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.

Done.

15. Please reference Figure 1 and Figure 2 in the protocol.

Figure 1 is originally drawn by the author. For Figure 2, please see comment on (3) above.

16. Figure 1 and Table 1: Please change “ml” to “mL”.

Changed.

17. Figure 2: Please use “x g” instead of “g” for centrifugation force (i.e., 2000 x g, 500 x g). Please include a space between the number and temperature unit (i.e., 4 °C). In panel D, please only capitalize the first word of a phrase (i.e., Exosome characterization, Protein quantification, etc.).

Corrected

18. Figure 5: Please revise to refer to the treatment in a consistent manner. For instance, panel A uses w/ electro while panel c uses w electro.

Corrected.

19. Table 1: Please upload Table 1 to your Editorial Manager account as an .xls or .xlsx file.

Corrected.

20. Line 343: Please convert the reference to a superscripted numbered reference.

Corrected.

21. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available.

Symbols have been removed. Lot numbers and RRIDs of antibodies have been listed in table of materials.

22. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

Corrected.

23. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Corrected.

**Reviewers' comments:**

Reviewer #1:

Manuscript Summary:

This is a well-written manuscript detailing exosome isolation from tissue culture media, characterization of the exosomes, and loading of the exosomes with siRNAs. The methodology is clear and the figures supportive of the text.

Minor Concerns:

Some additional clarifications would aid the researcher attempting to follow this protocol in the laboratory.  
Is deutrium oxide required for the preparation of the sucrose? Many protocols use standard sucrose preparations thus comments on the advantages of this method are appropriate.

This is already discussed in the manuscript (lines 531-533).

Details on the nucleofection kit used as well as the program used for electroporation of exosomes.

The nucleofection kit is purchased. The detailed product information is provided in the table of materials.

The size of the sepharose columns used is not provided.

The column size is 2.9 cm (H) x 1.3 cm (W). This has been added to the manuscript.

Reviewer #2:

Manuscript Summary:

The manuscript #JoVE58814, entitled "Isolation and characterisation of exosomes for siRNA delivery to cancer cells" by Faruqu et al., demonstrated that basis protocols of isolated cell-secreted exosomes could be used as the gene delivery carrier. Procedures including cell culture, exosome isolation and purification, as well as exosome characterization and formulation have been presented in details.  
  
Major Concerns:

1. It is doubtful if the bioreactor flask is properly used for cell culture and media collection to produce high amounts of exosome. Please calcify if exosomes could pass across the semipermeable membrane of bioreactor. Do concentrated media with the high amount of exosomes have effect on the continuous release of exosomes in cells? Another concern is cell morphology and confluency in the bioreactor flask. As the authored discussed, "it can be assumed that the cells in the bioreactor flask are not growing in a monolayer like the regular cell culture," it is questionable if this culture would also impact on the exosome release. Have any studies reported that cells in the bioreactor is growing closer to physiological conditions compared to regulation culture flask?

* The semi-permeable membrane MWCO is 10 kDa, so exosomes would not be able to pass through (supplier’s manual and reference 40)
* The exosomes are harvested on a weekly basis, so this avoids the excessive accumulation of exosomes in the culture supernatant that can potentially harm/influence the exosome production. This has been demonstrated in a study (reference 40) that shows consistent high yield of exosomes from prolonged culture in the bioreactor flask for up to 1 year, suggesting there is no negative feedback mechanism that reduces exosome production by the cells in an exosome-rich milieu.
* Weekly harvesting of the culture supernatant also will remove dead/loosely attaching cells (i.e. less viable) so this prevents the build-up of materials on the-semi permeable that can adversely affect the exchange of gas, nutrients or waste products (reference 40)
* There hasn’t been a study that looked on how the cells in the bioreactor flask are growing closer to physiological conditions, this is the authors’ speculation. However, one study (reference 40) demonstrated that exosomes produced from the bioreactor flasks have similar morphology, phenotype and immunomodulatory functions as those sourced from regular culture flasks.

2. Is it useful for the yield rate per ml of exosomes from concentrated media? As more cells grow in the flask, more exosomes will be released. The later collected media are supposed to have more exosomes, right?

The p/mL unit used to express the exosome yield in Table 1 refers to p/mL of PBS the exosomes were resuspended in at the final stage of the isolation, and not the volume of conditioned-medium that was initially used for isolation. The exosomes are always harvested after 7 days, and are always resuspended in 400 µL of PBS at the final step of the isolation, so exosome yield between different harvests can be directly comparable.

3. High amount of exosomes could easily come from large volume of cell culture media using more and/or larger conventional flasks. While more times are needed to concentrate media in the exosome isolation, variability related to exosome quality and quantity could be minimized in the cell growth, cell morphology, exosome release, and exosome yield. The authors should have valuable discussion and insights on the related issues in the manuscript.

Added to Discussions – Significance to other method.

4. List detailed information about siRNA. What are the target and sequence of siRNA? Is the label step of siRNA missing?

The detailed information including sequence of siRNA and catalogue number are added in the table of materials. The labelling step is conducted by the company. The siRNA used in this protocol is a negative siRNA.

5. Provide more studies of exosome delivered siRNA in the cancer cells such as downstream knockdowns of target RNA and protein levels using PCR, ELISA and western blotting. As the labeled Atto665 may be dissociated, a fake positive result could be observed in the cellular uptake.  
This is a good question. The proposed protocol provides a general methodology of using exosome for siRNA delivery to cancer cells *in vitro*. We used negative control siRNA for an example. As for the knockdown efficacy of a specific siRNA, we strongly suggest readers to investigate thoroughly using techniques such as PCR, ELISA, and western blotting.

Minor Concerns:

The manuscript is poorly written. There are lot of unprofessional expression and writing inconsistently. For example,

Equipment resources: some are provided and some not.

The table of materials and equipments have been updated.

Delete steps 10.2 and 10.3 as they were presented in the steps 8 and 9.

The description of these two steps are simplified and detailed information are pointed out to refer to Steps 8 and 9.

Table 1: Yield (x 10exp12 p/ml), 6.99±0.22; P:P ratio (x10exp10 p/µg), 8.3±?

Added.

Figure 4, provide SD in the 4A and 4B

Provided.

Figure 5, treatments are named improperly

Corrected.

Provide statistical analysis method.

Added statistical analysis method in Figure legends.