Peer-Review

Mody, Veda, and Sandhya Bansal. 2025. "Characterization of Biomarkers in Small Extracellular Vesicles during SARS-CoV2 Infections in Lung Transplant Patients." *Journal of High School Science* 9 (2): 363–74.

1.Why would a patient subject themselves to a blood draw when a nose-swab PCR test can be performed instead? Is there a difference in sensitivity? Specificity? detection of active infection verus only antigens from SARS-CoV2 (I would think not, since the exosome test also quantitates antigen)? Does anything preclude lung-transplant patients from a nose-swab test? Please describe, explain and justify in the manuscript.
2.Please present centrifugation settings as RCF or 'times g', rather than as RPM and minutes. The former allows inter-operability between different makes and models of centrifuges.
3.Change all tense from present perfect "…we have collected…" to past perfect "…we collected…." Manuscript should be written in third person, past perfect tense.
4.Since samples from 5 patients were pooled, the magnitude of the amount of any molecules assayed would be 1/5 th that reported (since obtained from one patient, because you claim diagnosis of SARS-CoV2). Would this amount of biomarker be enough to be detected by the SDS-PAGE, Western blot, BCA assay….? or be able to be statistically differentiated from control? Please present the LOD for all these methods and justify that a blood sample from one patient would contain enough biomarker to be > LOD (5 times or whatever is standard for signal/noise) for all these methods and be able to be differentiated from control. As a crude calculation, from figure 7B, your spike protein translates to 0.4/5 = 0.08 relative density, while your nucleocapsid translates to 0.3/5=0.06 relative density, assuming linear response.
5.Are these antibodies: "…..SARS-CoV-2 spike protein S2 (Thermo Fisher Scientific), SARS-CoV-2 nucleoprotein antibody (Thermo Fisher Scientific)…." specific for SARS-CoV2 ? Are there references that provide evidence that the SARS-CoV2 nucleoprotein antibody will not bind nucleoproteins from other viruses such as (say) RSV, cytomegalovirus, rhinovirus…..? Similar for spike protein.
6.Please explain the "BCA assay for protein estimation" in greater detail. What were the standards? Was the BCA reagent (A+B+C)able to extract the proteins from the exosomes? References? Where was the BCA reagent sourced from? What was the reason for employing different BSA concentrations ? Provide evidence that extraction of proteins was complete using the BCA reagents (references?)? How did you convert concentration from ug/uL (volume) to ug/20 ug (weight)? Where are these numbers reported in the manuscript?
7.You state "….Samples were reconstituted at equal concentrations to run on SDS PAGE (Sample + Buffer + Sample loading Dye = 20 microliters)….." This needs more explanation. Were the samples those after exosome digestion and extraction with BCA reagents? How much sample?
8.Please create a subheading called "materials" and list any reagents and/or chemicals that were not part of vendors' kits including their sourcing and grade.
9.you state "…..Coomassie brilliant blue dye and the transfer on to the PVDF membrane is shown in Figure VI……" Provide more detail. Readers must be able to replicate your work.

Answer to Reviewer's Comments

We would like to thank the reviewer for their comments to help us improve the manuscript. We have highlighted all the edited and added sections in yellow in the revised manuscript.

Q1. Why would a patient subject themselves to a blood draw when a nose-swab PCR test can be performed instead? Is there a difference in sensitivity? Specificity? detection of active infection versus only antigens from SARS-CoV2 (I would think not, since the exosome test also quantitates antigen)? Does anything preclude lung-transplant patients from a nose-swab test? Please describe, explain and justify in the manuscript.

Answer to reviewer's comment; I added the following sentences with references to the revised manuscript: We analyzed exosomes from a blood draw rather than a nose-swab and PCR test because a PCR test can provide false negative results (10, 11). Infections can be tracked using exosomes until the infection is gone with greater accuracy and can even be tracked past the two week infection period (12, 13). Exosomes can be more sensitive and specific as diagnostics as well. Nothing precludes lung transplant patients from the nose swab test but since some patients can be PCR negative despite having an infection, alternate detection methods are always needed.

Q2. Please present centrifugation settings as RCF or 'times g', rather than as RPM and minutes. The former allows interoperability between different makes and models of centrifuges.

Answer to reviewer's comment; I have added centrifugation settings as RCF in the revised manuscript. The changed values in the revised manuscript are 12,000 RPM to 15,294 RCF and 5,000 RPM to 2,655 RCF.

Q3. Change all tenses from present perfect "…we have collected…" to past perfect "…we collected…." Manuscript should be written in third person, past perfect tense.

Answer to reviewer's comment; I changed the wording from we have collected to we collected, we have discussed to we discussed, and we have also discussed to we also discussed in the revised manuscript.

Q4. Since samples from 5 patients were pooled, the magnitude of the amount of any molecules assayed would be 1/5 that reported (since obtained from one patient, because you claim diagnosis of SARS-CoV2). Would this amount of biomarker be enough to be detected by the SDS-PAGE, Western blot, BCA assay….? or be able to be statistically differentiated from control? Please present the LOD for all these methods and justify that a blood sample from one patient would contain enough biomarker to be > LOD (5 times or whatever is standard for signal/noise) for all these methods and be able to be differentiated from control. As a crude calculation, from figure 7B, your spike protein translates to 0.4/5 = 0.08 relative density, while your nucleocapsid translates to 0.3/5=0.06 relative density, assuming linear response.

Answer to reviewer's comment; In the initial submission of the manuscript, we did not specify that The samples from the 5 adult lung transplant recipients diagnosed as SARS-CoV-2 positive were not pooled. We have addressed this in the revised manuscript. The only samples that were pooled together were the samples of the healthy individuals. We don't expect the band of desired protein in the samples of these healthy individuals and thus the values for the negative control are considered the baseline.

We have established in the past in the laboratory that this loaded level of protein is enough for detection of desired protein. We then subtracted the baseline from the samples to analyze the data.

We have added the following in their respective subsections in the method section: In general, the detection levels for a protein in a micro BCA assay is 20 to 2,000 µg/mL. The detection limit for an SDS-PAGE is about 2-5 ng/protein band. The western blot can detect down to 10 pg/lane. The analysis of western blot is not done using signal to noise ratios, it needs to be done using internal control which is the light chain IgG. Each band in each lane of the sample is normalized to internal control before analysis.

Q5. Are these antibodies: "…..SARS-CoV-2 spike protein S2 (Thermo Fisher Scientific), SARS-CoV-2 nucleoprotein antibody (Thermo Fisher Scientific)…." specific for SARS-CoV2 ? Are there references that provide evidence that the SARS-CoV2 nucleoprotein antibody will not bind nucleoproteins from other viruses such as (say) RSV, cytomegalovirus, rhinovirus…..? Similar for spike protein.

Answer to reviewer's comment; Yes, these antibodies are very specific. They were designed by Thermo Fisher Scientific as targeted monoclonal antibodies. To the best of our knowledge, they don't cross-react with other viruses i.e., RSV, CMV or Rhino. We optimize and check each and every antibody before using them for research. We do not have any specific references for this.

Q6. Please explain the "BCA assay for protein estimation" in greater detail. What were the standards? Was the BCA reagent (A+B+C)able to extract the proteins from the exosomes? References? Where was the BCA reagent sourced from? What was the reason for employing different BSA concentrations ? Provide evidence that extraction of proteins was complete using the BCA reagents (references?)? How did you convert concentration from ug/uL (volume) to ug/20 ug (weight)? Where are these numbers reported in the manuscript?

Answer to reviewer's comment; We have explained the BCA assay for protein estimation in the revised manuscript. The standards are mentioned in Table I.

Yes, the BCA reagent was able to extract proteins from the exosomes according to the manufacturer's protocol. The BCA reagents were sourced from the Thermo Scientific kits and we used 5 ml of Reagent A, 5 ml of Reagent B, and 500 ml of Reagent C. I added these numbers in the BCA assay section of the revised manuscript.

The estimation of the optical density data validates the evidence that the extraction of proteins was complete using the BCA reagents. We employed different BSA concentrations to create a standard curve.

We have added the concentrations of BSA + Water in duplicates from 0 microliters of water and 10 microliters of BSA to 10 microliters of water and 0 microliters of BSA to create the standard. We have

added Table I to make this clearer. The table is formatted to reflect the Water to BSA ratio for the standard. The samples are in D3 and D4 to H3 and H4 and the control is in A5 and A6.

The conversion from concentration per microliter to 20 ug/microliter is added in Table II in the revised manuscript.

Q7. You state "….Samples were reconstituted at equal concentrations to run on SDS PAGE (Sample + Buffer + Sample loading Dye = 20 microliters)….." This needs more explanation. Were the samples those after exosome digestion and extraction with BCA reagents? How much sample?

Answer to reviewer's comment; The additional explanation on the SDS-PAGE process has now been included in the revised manuscript.

Yes, the samples were taken after exosome digestion and extraction with BCA reagents. We loaded 20 ul in volume and the concentration of protein in each sample was 20 micrograms which was adjusted to the volume of sample as shown in Table II in the revised manuscript.

Q8. Please create a subheading called "materials" and list any reagents and/or chemicals that were not part of vendors' kits including their sourcing and grade.

Answer to reviewer's comment; All the materials procured were research grade. List containing materials with their catalog number has been added in the revised manuscript.

Q9. you state "....Coomassie brilliant blue dye and the transfer on to the PVDF membrane is shown in Figure VI……" Provide more detail. Readers must be able to replicate your work.

Answer to reviewer's comment; I added the following paragraph to the revised manuscript in the SDS-PAGE subsection of the methods section: The gel was stained with Coomassie Brilliant Blue for visibility of protein bands. A separate clean replica gel without Coomassie Brilliant Blue staining was used to transfer the proteins on PVDF membrane and probing with the desired antibodies. We prepared a PVFT membrane by soaking it in methanol for 30 seconds and then leaving it in the TBST Buffer. We then took another section of gel and made a layer to put the gel in between. We added 2 sheets of blotting paper each 0.75 mm thick, then added the PVFT membrane, and then the gel, and finally another 2 blotting papers. We put this stack in the Power Blotter machine and ran it for 11 minutes 2 times.

The write up for the BCA assay procedure now is even more confusing. I suggest you leave out the proceudure and substitute with "per manufacturer's or vendor's instructions". Present the Standard curve with regression coefficient and highest concentration. Then present the total protein in the 5 individual SARS-CoV positive samples with average and standard deviation and that from the 5 pooled

negative samples. I am assuming that all experiments were performed at least in duplicate. If not, provide reasons.

1.What is the use of the BCA assay if it quantifies total protein in the exosome? There are undoubtedly other proteins besides the spike and nucleocapsid protein in the exosome. How does knowing the total concentration of protein in the exosome advance your objective of ID and quantification of the two proteins of interest? Please clarify and describe in the manuscript.
2.Provide evidence that the exosome cell wall is lysed using the reagents in the BCA assay.
3.Do not present colored in histograms (columns) in Figure 7 for relative density. Present the 5 points instead. This is because your nucleocapsid band for patient 4 is almost negligible in the Western blot. What does one star and 3 stars in the Figure represent? Provide a p value key in the Figures.
4.For the nanosight particle size analysis; include a phrase to the effect that the procedure followed was per the manufacturer's or vendor's instructions. What do the 6 figures in Figure III represent? I am assuming one negative and 5 individual SARS-CoV2 postive patients? Please label.
5.Figure legends or Figure Titles should NOT appear in the submitted JPEG or PNG figures. Remove these and resubmit the Figures. Submit the figure legends separately in another doc file.
6.Was protein extracted from the exosomes for the SDS-PAGE analysis? If not, present evidence that the SDS in the buffer lyses the exosomes and that the bands are not the result of different sized exosome migration; rather; from the extracted proteins from the exosomes.
7.The references need to list all the 6 authors followed by an et al. if the reference has more than 6 authors. The references need to list all the authors if the number of authors is less than 6. References need to follow a consistent format.
8.The English in this manuscript is riddled with grammatical, sentence structure and syntactic errors, which are too numerous for me to list. Please write in past perfect tense, third person. Remove all hyperbole and subjective exaggerations. Please use this manuscript as a guide: https://doi.org/10.1002/ctm2.576 . Note that we cannot publish unless the minimum requirement for proper English syntax and scientific reporting guidelines is met. Failure to do so will result in repeated 'revise and resubmit' iterations until iteration 3; after which your manuscript may be rejected.

Answer to Reviewer's Comments

We would like to thank the reviewer for their comments to help us improve the manuscript. We have highlighted all the edited and added sections in yellow in the revised manuscript.

1. The write up for the BCA assay procedure now is even more confusing. I suggest you leave out the procedure and substitute with "per manufacturer's or vendor's instructions." Present the Standard curve with regression coefficient and highest concentration. Then present the total protein in the 5 individual SARS-CoV positive samples with average and standard deviation and that from the 5 pooled negative samples. I am assuming that all experiments were performed at least in duplicate. If not, provide reasons.

Answer to reviewer's comment; I have removed the BCA assay procedure in the revised manuscript. I have added that the micro-BCA assay was performed according to the manufacturer's instructions instead.

I have added Figure IV to the revised manuscript with the following caption: Figure IV displays the BCA standard curve, with protein concentration (µg/µL) plotted on the x-axis and optical density (OD at 562 nm) on the y-axis. The curve demonstrates a strong linear relationship between concentration and absorbance, with a regression coefficient (R^2) of 0.9996. The highest standard concentration used in the assay was 20 µg/µL.

I added the following sentence to the revised manuscript: The average total protein concentration measured in the five individual SARS-CoV-2 positive samples was 18.47 µg/µL, with a standard deviation of 7.30 µg/µL.

For the SARS-CoV-2 negative group, only a single pooled exosome sample of the 5 individuals was available to us, with a total protein concentration of 39.14 µg/µL. The individual protein concentration values for the five SARS-CoV-2 negative control samples are unfortunately not available to us. As a result, we are unable to calculate the average or standard deviation for that group.

Yes, all experiments were performed in duplicate.

2. What is the use of the BCA assay if it quantifies total protein in the exosome? There are undoubtedly other proteins besides the spike and nucleocapsid protein in the exosome. How does knowing the total concentration of protein in the exosome advance your objective of ID and quantification of the two proteins of interest? Please clarify and describe in the manuscript.

Answer to reviewer's comment; I added the following sentences to the revised manuscript: The use of the BCA assay when it quantifies total protein in the exosome is to allow the loading of equal amounts of protein from each sample onto the gel for SDS-PAGE and western blotting. This advances our objective of identification and quantification of the two proteins of interest because this ensures that differences in band intensity reflect true differences in the proteins of interest, rather than variations in sample loading. It helps confirm the consistency and quality of the exosome preparations before specific protein detection.

3. Provide evidence that the exosome cell wall is lysed using the reagents in the BCA assay.

Answer to reviewer's comment; Since the reagents in the BCA assay are provided by the Thermo Scientific micro-BCA assay kit, there is no definite evidence that the reagents we use as a part of the kit are the ones that lyse the exosome cell wall. However, since we are able to receive a value for protein quantification, there is a lysis of the exosome cell wall that occurs without using a separate lysing buffer. I attempted to explain this through the color change that occurs after the assay is completed. Please let me know if I should explain this further or in a different way.

I added the following sentences to the revised manuscript: The BCA assay produces a dark purple color when proteins reduce copper ions (Cu^{2+} to Cu^{+}), which then form a purple complex with bicinchoninic acid (14). The intensity of this color corresponds to the amount of protein present. In the case of exosomes, proteins enclosed within intact vesicles may not be accessible to the reagent. Therefore, without lysis, primarily surface-associated proteins are detected, resulting in limited color development. The optical density measurement at 562 nm confirms that a color change occurred, indicating the presence of accessible proteins and supporting the conclusion that lysis was necessary for total protein quantification.

4. Do not present colored in histograms (columns) in Figure 7 for relative density. Present the 5 points instead. This is because your nucleocapsid band for patient 4 is almost negligible in the Western blot. What does one star and 3 stars in the Figure represent? Provide a p value key in the Figures.

Answer to reviewer's comment; I presented the 5 points in Panel C of Figure 7. I added a p value key for Figure 7 that explains what the different stars mean. I just wanted to let the reviewer know that I have left Panel B in the Figure because I thought that the statistical analysis does not make as much sense without it and I wanted to explain myself better. If the reviewer would like me to remove Panel B of Figure 7 for more clarity, I am able to do that.

5. For the nanosight particle size analysis; include a phrase to the effect that the procedure followed was per the manufacturer's or vendor's instructions. What do the 6 figures in Figure III represent? I am assuming one negative and 5 individual SARS-CoV2 positive patients? Please label.

Answer to reviewer's comment; We have included a phrase that says the procedure followed was per the manufacturer's instructions in the nanosight particle size analysis.

Yes, the 6 figures in Figure III represent the negative control and 5 individual SARS-CoV-2 positive patients. I have labeled this in the legend for Figure III of the revised manuscript.

6. Figure legends or Figure Titles should NOT appear in the submitted JPEG or PNG figures. Remove these and resubmit the Figures. Submit the figure legends separately in another doc file.

Answer to reviewer's comment; I removed all Figure legends and Figure titles from the submitted PNG figures. I have submitted figure legends in a separate doc file.

7. Was protein extracted from the exosomes for the SDS-PAGE analysis? If not, present evidence that the SDS in the buffer lyses the exosomes and that the bands are not the result of different sized exosome migration; rather; from the extracted proteins from the exosomes.

Answer to reviewer's comment; No, protein was not separately extracted from the exosomes for the SDS-PAGE analysis. I added the following paragraph to the manuscript to explain further: SDS is a strong ionic detergent that disrupts lipid membranes by solubilizing membrane proteins and lipids, while the heat aids in protein denaturation and release of internal contents. This approach is commonly used to lyse extracellular vesicles, including exosomes, for protein extraction prior to electrophoresis (15). Given that intact exosomes (30–200 nm) are too large to migrate through the polyacrylamide gel, the observed bands, such as those for SARS-CoV-2 Spike and Nucleocapsid proteins, represent proteins released from lysed exosomes rather than from intact exosome migration.

8. The references need to list all the 6 authors followed by an et al. if the reference has more than 6 authors. The references need to list all the authors if the number of authors is less than 6. References need to follow a consistent format.

Answer to reviewer's comment; I have listed all 6 authors followed by et al. for the references with more than 6 authors and all the authors for those that were less than 6. The references are following this consistent format in the revised manuscript.

9. The English in this manuscript is riddled with grammatical, sentence structure and syntactic errors, which are too numerous for me to list. Please write in past perfect tense, third person. Remove all hyperbole and subjective exaggerations. Please use this manuscript as a guide: https://doi.org/10.1002/ctm2.576. Note that we cannot publish unless the minimum requirement for proper English syntax and scientific reporting guidelines is met. Failure to do so will result in repeated 'revise and resubmit' iterations until iteration 3; after which your manuscript may be rejected.

Answer to reviewer's comment; We have carefully edited all grammatical, sentence structure, and syntactic errors in the revised manuscript. All sections have been rewritten in past perfect tense and third person, with subjective language and hyperbole removed.

I appreciate the responses to my comments. However, many comments remain either totally or partially unanswered.

1.Your control concentration in the BCA assay is 39.1 ug/uL (i am assuming that this is the case, since your heading in table says "Conc/uL)". Your standard max concentration is 20 ug/ul ? (don't know because your X and Y axes have neither headings nor units??, axes headings and units go with the figure.) What is this "Control" sample and why is it needed? Its concentration is outside the range of standard concentrations.
2.you state "….SARS-CoV-2 positive samples was 18.47 µg/µL, with a standard deviation of 7.30 µg/µL." I input the same numbers into a SD calculator which returned an SD value of 6.44. Please double-check the figures.

3.Please replace the block black colored columns in figure 7B with only the 5 points and remove the additional columns that you have created.
4.The p-Key rectangle is not necessary, simply state that *:p<0.05 and **p<0.0001** in the legend. Similarly, the C, M, P1…. should go in the legend.
5.Remove Table 1, it is too confusing and the headings are cryptic. Instead, present all the concentrations from P1 to P5, then present average and SD.
6.An ideal write up on any instrument would follow "NTA measurement with Nanosight NS300 (Malvern, UK)" in https://doi.org/10.1080/20013078.2019.1596016 . I tried to find the manufacturer's directions on how to perform EV analysis using the machine, but could not. Please provide a link on where this information may be obtained. Also, if you did not calibrate this machine with polystyrene sphere standards with known size, please include this omission and/or limitation in the manuscript.
7.Is Figure 1 reproduced from a public domain source? Please obtain permission to use figures. Merely citing a source is not enough. Please do not violate copyright.
8.Need manufacturer, model, operational settings for "…..Power Blotter system….."
9."….secondary Abs conjugated with horseradish peroxidase (HRP), specific to the primary Abs, were employed….." need vendor, cat.#
10.In the absence of refernces that show better sensitivity, specificity…. for the EV method compared against a PCR nose swab; I do not agree that this method can be used as an alternative to detect SARS-CoV2. I can buy the argument that it may be used as confirmation/add-on prior to lung-transplant. Please, make the necessary corrections in the manuscript.
11.This added paragraph does not make sense "…… In the case of exosomes, proteins enclosed within intact vesicles may not be accessible to the reagent; therefore, without lysis, primarily surface-associated proteinsare detected, resulting in limited color development. The optical density measurement at 562 nm confirms that a color change occurred, indicating the presence of accessible proteins and supporting the conclusion that lysis was necessary for total protein quantification….." What are these 'surface associated proteins?' and where do they originate? Please perform a thorough literature analysis or contact the vendor to confirm that the BCA reagents have the ability to lyse exosomes. All the literature I have seen uses RIPA buffer to lyse exosomes prior to BCA. see for example: https://doi.org/10.1177/0300060520957541 . I do agree that the assay must have access to some proteins for a color change, however if not expressely designed to lyse, may lyse different size distributions differently? Therefore, your objective of "…… differences in band intensity reflect true differences in the proteins of interest, rather than variations in sample loading….." is not met.

Answer to Reviewer's Comments

We would like to thank the reviewer for their comments to help us improve the manuscript. We have highlighted all the edited and added sections in yellow in the revised manuscript.

1. Your control concentration in the BCA assay is 39.1 ug/uL (I am assuming that this is the case, since your heading in table says "Conc/uL)". Your standard max concentration is 20 ug/ul ? (don't know because your X and Y axes have neither headings nor units??, axes headings and units go with the figure.) What is this "Control" sample and why is it needed? Its concentration is outside the range of standard concentrations.

Answer to reviewer's comment; Yes, the standard max concentration is 20 µg/µL. I have updated Figure IV to include all axes heading and units. The control sample is used to make sure we are performing the BCA assay correctly and is used as a baseline. We use the same process for the negative control that we use for the SARS-CoV-2 positive samples to ensure there are no additional variables leading to differences in the control and the samples.

2. you state "….SARS-CoV-2 positive samples was 18.47 µg/µL, with a standard deviation of 7.30 µg/µL." I input the same numbers into a SD calculator which returned an SD value of 6.44. Please double-check the figures.

Answer to reviewer's comment; I have rechecked the figures and changed the Standard Deviation value to 6.44 µg/µL. Thank you for your patience and I apologize for the error.

3. Please replace the block black colored columns in figure 7B with only the 5 points and remove the additional columns that you have created.

Answer to reviewer's comment; I have replaced the block black colored columns in 7B with the 5 individual points. I have removed the additional columns that were created.

4. The p-Key rectangle is not necessary, simply state that *:p<0.05 and p<0.0001 in the legend. Similarly, the C, M, P1…. should go in the legend.

Answer to reviewer's comment; I have removed the p-key rectangle. I have added the following caption to Figure VII in the Figure legends word doc: Western blot and densitometry analysis of sEV proteins from lung transplant recipients (LTxRs) with confirmed SARS-CoV-2 infection and non-infected controls. Panel A shows representative Western blots of sEV protein samples, with C representing a non-infected control, M denoting the molecular weight marker (protein ladder), and P1–P5 corresponding to samples from patients 1 through 5 with positive SARS-CoV-2 infection. Panel B presents the corresponding densitometry analysis of the protein bands. Statistical significance is indicated as follows: $p < 0.05$ (*), $p < 0.0001$ (****).

5. Remove Table 1, it is too confusing and the headings are cryptic. Instead, present all the concentrations from P1 to P5, then present average and SD.

Answer to reviewer's comment; I have removed Table 1 and added the following sentences to the revised manuscript: The total protein concentrations were as follows: P1 – 16.73 µg/µL, P2 – 20.02 µg/µL, P3 – 21.24 µg/µL, P4 – 26.96 µg/µL, and P5 – 7.40 µg/µL. The average protein concentration across all samples was 18.47 µg/µL, with a standard deviation of 6.44 µg/µL.

6. An ideal write up on any instrument would follow "NTA measurement with Nanosight NS300 (Malvern, UK)" in https://doi.org/10.1080/20013078.2019.1596016. I tried to find the manufacturer's directions on how to perform EV analysis using the machine, but could not. Please provide a link on where this information may be obtained. Also, if you did not calibrate this machine with polystyrene sphere standards with known size, please include this omission and/or limitation in the manuscript.

Answer to reviewer's comment; I could not find specific manufacturer's process on how to perform EV analysis. However, I found a reference that used Nanosight NS300 (Malvern, UK) to perform EV analysis in the same way we did. I have attached the link here. https://www.mdpi.com/1422-

0067/23/4/2310. The reference you have listed is also very similar to the procedure we used. This is a link below from the company's website detailing how the machine works. Please let me know if you would like me to detail the procedure I used and write it in the manuscript.
https://www.malvernpanalytical.com/en/products/technology/light-scattering/nanoparticle-tracking-analysis#howntaworks

Yes, we did calibrate the machine with polystyrene sphere standards.

7. Is Figure 1 reproduced from a public domain source? Please obtain permission to use figures. Merely citing a source is not enough. Please do not violate copyright.

Answer to reviewer's comment; Figure 1 was reproduced from Transplantation Journal, Extracellular Vescicles in Transplantation: Friend or Foe. I have attached the permission from Walters Kluwer as an additional document in the submission portal. The remaining figures are only made for this manuscript.

8. Need manufacturer, model, operational settings for "…..Power Blotter system….."

Answer to reviewer's comment; I have added this to the materials section of the revised manuscript: Power Blotter System (Invitrogen, Cat# PB0012).

9. "….secondary Abs conjugated with horseradish peroxidase (HRP), specific to the primary Abs, were employed….." need vendor, cat.#

Answer to reviewer's comment; I have added this to the materials section of the revised manuscript: Pierce™ Horseradish Peroxidase (Thermo Fisher Scientific, Cat# 31490).

10. In the absence of references that show better sensitivity, specificity…. for the EV method compared against a PCR nose swab; I do not agree that this method can be used as an alternative to detect SARS-CoV2. I can buy the argument that it may be used as confirmation/add-on prior to lung-transplant. Please, make the necessary corrections in the manuscript.

Answer to reviewer's comment; I have also added the following line to the revised manuscript: Exosomes may offer improved sensitivity and specificity as diagnostic tools (14). They may serve as confirmation of a SARS-CoV-2 infection prior to a lung transplant.

11. This added paragraph does not make sense "…… In the case of exosomes, proteins enclosed within intact vesicles may not be accessible to the reagent; therefore, without lysis, primarily surface-associated proteins are detected, resulting in limited color development. The optical density measurement at 562 nm confirms that a color change occurred, indicating the presence of accessible proteins and supporting the conclusion that lysis was necessary for total protein quantification….." What are these 'surface associated proteins?' and where do they originate? Please perform a thorough literature analysis or contact the vendor to confirm that the BCA reagents have the ability to lyse exosomes. All the literature I have seen uses RIPA buffer to lyse exosomes prior to BCA. see for example: https://doi.org/10.1177/0300060520957541 . I do agree that the assay must have access to some proteins for a color change, however if not expressly designed to lyse, may lyse different size distributions differently? Therefore, your objective of "…… differences in band intensity reflect true differences in the proteins of interest, rather than variations in sample loading….." is not met.

Answer to reviewer's comment; I tried my best to find literature that says the BCA reagents have the ability to lyse exosomes but I could not find anything. I attempted to contact the vendor multiple times but they are not getting back to me. It has been the common practice in the lab I completed this research at to use the BCA reagents to perform the BCA assay and that the lysis of the exosomes is a part of that process. They have published many studies using this process but they do not have any explicit references that state the BCA reagents have the ability to lyse exosomes.

Listed below is some references I have found that use the Thermo Fisher micro bca kit and don't mention an explicit lysing procedure, but I do acknowledge it is not a conclusive justification. I have added the kit website information and instruction manual. I have removed the added paragraph since it is not making sense or aiding in this justification.

https://arizona-ua.primo.exlibrisgroup.com/discovery/fulldisplay?context=PC&vid=01UA_INST:01UA&docid=cdi_pangaea_primary_oai_pangaea_de_doi_10_1594_PANGAEA_973194
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4730153/?pq-origsite=primo_ra
https://www.thermofisher.com/order/catalog/product/23225
https://assets.thermofisher.com/TFSAssets/LSG/manuals/MAN0011237_Micro_BCA_Protein_Asy_UG.pdf

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Thank you for addressing my comments. Accepted. Please thoroughly check the attached galley proof for errors. Note that I have added content in the Limitations section and elsewhere for clarification. I still do not understand the 20 uL start, then the 18 uL loading, then ultimately analyzing 15 uL ? Can you explain this in your subsequent communication?
