Amit Krishnan, Ph.D.
Review Editor,
Journal of Visualized Experiments
Manuscript ID: JoVE63028
TITLE: Cell-free scaled production and adjuvant addition to a recombinant major outer membrane protein from Chlamydia muridarum for vaccine development

Dear Dr. Krishnan,
Please consider our revised manuscript titled “Cell-free scaled production and adjuvant addition to a recombinant major outer membrane protein from *Chlamydia muridarum* for vaccine development” for publication in the Journal of Visualized Experiments. We thank the reviewers for their thorough assessment and helpful comments, and we appreciate the opportunity to revise our manuscript in oreder to address concerns raised by the reviewers. Below please find our responses to the reviewers’ comments (which have been bolded for clarity). We have also made other minor modifications to the text to enhance the clarity of the overall message.
Kindest Regards,

Matthew Coleman, PhD
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Editor's comments:

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**
   All authors have reviewed the manuscript and we have corrected any errors found.

2. **Please provide an institutional email address for each author.**
   Done.

3. **Please reword the lines to avoid previously published works:191-193, 194-195, 197-199, 330-333. Please refer to the iThenticate report attached.**
   We have reviewed the report and made an effort to avoid overlap of text with previous works.

4. **Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).**
   We have reduced the use of pronouns in the manuscript.

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.**
   Use of commercial language has been removed.

6. **Line 45-53: Please include the lines as paragraph.**
   Corrected.

7. **Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution (Please move the lines 293-296)**
   We have moved the ethics statement to the correct position.

8. **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. 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." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.**
   The protocol section has been edited to use the imperative tense when possible.

9. **Line 95-96: The Protocol should contain only action items that direct the reader to do something. Consider revising the lines to direct the reader to do something.**

We have changed this step to direct the reader:

"1.1. All material used in producing vaccine-grade formulations for animals are endotoxin-free. To prepare glassware for this process, bake cleaned glassware in an oven at 180ºC for four hours."

10. **For SI units, please use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2**
    Corrected.

11. **For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks**
    Corrected.

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. Please add more details to your protocol steps. 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. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) 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.**

13. **Line 297-298: Please specify the volume injected.**
    We have added this information:

    "Total volume per injection is 50 µL.**"**

14. **Line 299-300; How much was the booster dose.**
    We have added this information:

    "11.2. Four weeks after the initial vaccination (prime), animals are vaccinated a second time (boost) with 10 µg of MOMP in the form of MOMP-tNLP adjuvanted with 5 µg of CpG and 1 µg FSL-1. Total volume per injection is 50 µL."

15. **Line 301: Please specify the volume of blood drawn. What was used to draw blood.**
    We have added this information:

"11.6. On day 56 after the initial vaccination, collect blood to assess antibody titers. Using a micro-hematocrit capillary tube, puncture the retro-orbital plexus. Collect 100 μL blood in a microcentrifuge tube."

16. **Being a video based journal, JoVE authors must be very specific when it comes to the humane treatment of animals. Regarding animal treatment in the protocol, please add the following information to the text:**
    a) **Please include an ethics statement before all of the numbered protocol steps indicating that the protocol follows the animal care guidelines of your institution.**
    b) **Please specify the euthanasia method, if any.**
    c) **Please mention how animals are anesthetized and how proper anesthetization is confirmed.**
    d) **Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.**
    e) **For survival strategies, discuss post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.**
    f) **Discuss maintenance of sterile conditions during survival surgery.**
    g) **Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.**
    h) **Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.**
    i) **Please do not highlight any steps describing euthanasia.**

    We have added in the pertinent details to section 11 of the protocol.

17. **Please highlight up to 3 pages 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. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.**
    We have indicated the essential sections.

18. **Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.**
    Corrected.

19. **As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:**
    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**

We have revised the discussion section to address these points.

20. **Please do not use the &-sign or the word "and" when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.**
Corrected.

21. **Use Uppercase letters to label the figures in a multipaneled image (replace "a, b, c" by "A, B,C")**
Corrected.

22. **Figure 1: Please label the figure to make it more informative**
Corrected.

23. **Graphical abstract: Please add the figure as a schematic (e.g., Figure 1) and include a title and description in the Figure Legends.**
We have added a description in the legends.

24. **Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.**
Done.

<u>Reviewer #1:</u>

1. What is the novelty of the current work compared to the previous ones? For example, a similar work has already been published in journal of biochemical chemistry, 2017, J Biol Chem. 2017 Sep 8;292(36):15121-15132. doi: 10.1074/jbc.M117.784561, Cell-free production of a functional oligomeric form of a Chlamydia major outer-membrane protein (MOMP) for vaccine development cited as ref.5.

We concur that similar work has been published with a focus on production, function and immunogenicity of the Chlamydia major outer-membrane protein (MOMP). However, those previous publications did not focus on replicating and visually illustrating the minute details related to the methodology for co-translation with ApoA1 in the presence of lipids solubilized by the telodendrimer using the continuously fed cell-free lysate. Publication of these details in JOVE will reach a far broader audience and allow other groups to apply these methodologies to wider range of membrane bound proteins.

2. If the goal was to enhance the yield of protein production, 1.5mg/ml reported by

others suffice for vaccine purposes. Also, the yield of protein production as the novelty of current work is an important factor yet is not mentioned throughout the paper, more specifically in the abstract part.

We totally agree with the reviewer and have tried to further highlight the scale of the protein production from a few mLs of cell free lysate. We have added text to the abstract and discussion to further make this point.

3. Another key issue is that subunit vaccines are incapable of eliciting full protection against all serotypes/serovars due to the nature of C.t. as an intracellular bacterium and the complicated β-barrel transmembrane structure of MOMP. Thus using a subunit vaccine in the current work compared to other recent attempts deems fruitful. see the recent study https://www.nature.com/articles/s41541-021-00312-9 which is much better that the current work and also ref.5.

Our current manuscript is focused on presenting the collated details for a cell-free protein production protocol that can produce a fully functional beta-barrel such as the MOMP pore (He et al., 2017) that is capable of being protective in an animal challenge study (Tifrea et al., 2021). The methods we have detailed for JOVE cover several other factors regarding lipids, adjuvants and other possible additives that may improve expression as well as potentially protection in future studies. We cannot comment on the reviewers suggested work given the antigen, adjuvants, experimental methods as well as animal model systems are very different.

Reviewer #2:

P3, L135: "w/o" change to "without"
Corrected.

P3, L141: "w/o" change to "without"
Corrected.

P5, L183: How much amount of eluted MOMP-tNLP?
We now specify 5-15 µl of the eluted MOMP-tNLP is loaded.

"Aliquots of the eluted MOMP–tNLPs, washes, flowthrough, and total lysate are mixed with 4X SDS-PAGE sample loading buffer."

P6, L211: Information about PVDF could be added to the table of materials
The PVDF transfer stacks are listed in the table of materials, catalog number IB24001.

P6, L218: "5 minutes" change it to "5 minutes each"
Corrected.

P6, L222: "5 minutes" change it to "5 minutes each"
Corrected.

P7, L244: "DI water" Please provide the full name (i.e., deionized water) before using the acronym
Corrected.

P7, L246: "at on dry ice" change to "on dry ice"
Corrected.

P8, L288: Detected at specific wavelength?
We now specify the wavelengths of 214 and 280 nm.

"Incorporation is assessed through comparing absorption of the adjuvanted particles to the unadjuvanted particles at 214 and 280 nm."

P9, L310: "previous results" Is this referring to the previous study or this study? This is referencing a previous article by He et al. We have added the reference number here.
This refers to a previous article and we have added the reference number here.

P9, L316-318: This info could be added to the protocol.
We have made a note to follow the instructions provided by the manufacturer for protein quantitation.

"The elution fractions containing MOMP-tNLPs were pooled and the total protein concentration were determined using a fluorescence-based quantitation device, or a device that measures concentration through absorbance at 280 nm, following manufacturers instructions for protein quantitation.**"**

P9, L328: To include DTT in the table of materials.
The DTT solutions used are purchased pre-made and these are listed as catalog number NP0009.

P11, L326-328: The DTT was the one added in the sample buffer? What was the concentration? (10X was the stock?) I wonder this experiment should also be described in the protocol?
We have added the diluted concentration of DTT for clarity.

"To test the oligomeric state of MOMP, we analyzed MOMP-tNLP in the presence and absence of both heat and the reducing agent DTT (50 mM, Figure 3a)."

P9, L339: Since authors are referring to the "Previous studies", perhaps authors could provide at least 2 or more references here.
We have added additional references supporting this claim, see references 12-15.

P12, L407: If this is the authors' previous paper, please include the citation.
We have inserted references 3 and 19 here clarifying this.

P11, L399: Perhaps authors could also discuss or compare their findings with other similar studies and highlight the amount of purified MOMP produced from this study?
We described the MOMP yield in the introduction, but reiterate it here.
"Detailed here is a validated cell-free co-expression method that produces oligomeric MOMP formed within tNLP nanoparticle as a vaccine, with yields of approximately 1.5 mg of purified MOMP per 3 mL of lysate.**"**

Figure 1a: Image without color? Is this for GFP color?
We have replaced this with a color image that shows the GFP color more clearly.

Reviewer #3:
1. Lane 108 in the protocol section, is the DMPC lipid can be used for different membrane antigens?
Yes, DMPC can be used to prepare other membrane protein formulations, but there may be a benefit from screening other lipids. We have made a note of this in the discussion:

"Expression of other candidate antigens from bacteria is feasible, although parameters such as expression temperature, lipid choice, and type of expression system may all need to be explored in order to achieve optimal yields."

2. Lane 111 in the protocol section, the authors need to specify the temperature of the centrifugation.
Corrected.

"Remove any contaminant metal from the probe by centrifugation at 13k for 2 mins at 22°C and then transfer the solubilized lipid to a new 1.5 mL endotoxin-free tube.**"**

3. Lane 156 in the protocol section, what kind of shaker has been used? The shaking speed (to be included) and the temperature (30°C) are suitable for the production of the chlamydial MOMP antigen but are they appropriate for the overexpression of other membrane proteins?
The shaker is listed in the materials as being from New Brunswick. We make a note that conditions may need to be optimized for other proteins.

*"*Place the reaction in a shaker at 300 RPM, 30°C for up to 18 hours. These conditions, particularly temperature, may need to be optimized for expression of other membrane proteins.*"*

4. Lane 164, please mention the reference of the chromatography column.
These columns are listed in the materials table as "Disposable, polypropylene fritted columns 10 ml capacity".

5. Lane 301, please give the protocol for obtaining the whole blood from vaccinated mice.
We have added additional detail for this procedure:

*"*11.5. On day 56 after the initial vaccination, collect blood to assess antibody titers. Begin by anesthetizing mice by injecting i.p. a solution of Xylazine (0.3mg/20g body weight) and Ketamine (3.0 mg/20g body weight). Front and hind legs are pinched to make sure no jerking occurrs. Apply vaseline jelly around the eyes to prevent eye dryness during anesthezia.

11.6. Using a micro-hematocrit capillary tube, puncture the retro-orbital plexus. Collect 100 μL blood in a microcentrifuge tube.*"*

6. Figure 1, several contaminants are visible in the elution fractions. At an industrial scale, it would be interesting to know whether these contaminants are presents in the different batches of the recombinant vaccine preparations and the potential toxicity of these contaminants. How can the authors may characterize these contaminants? A specific protocol to remove these contaminants has to be added in the protocol section.

We have made a note at the end of the discussion that size exclusion chromatography can be used to further purify formulations if desired purity is not achieved after nickel affinity chromatography:

"While not described here, additional purification following nickel affinity chromatography may be necessary if many contaminants are observed in subsequent analysis steps such as through SDS-PAGE. This could be accomplished with SEC, but conditions may require optimization on a formulation by formulation basis."

Reviewer #4:

1. How to prepare the highly insoluble recombinant MOMP protein.

Our protocol is designed to produce a scaffold supported MOMP that is soluble without the need for the use of detergents in a single step, where co-translation produces both the MOMP and ApoA1 protein in the presence of lipids. Another approach is the use of preformed NLPs added to cell-free reaction with plasmids encoding MOMP (Katzen et al., 2008). The protocol could also potentially work with insoluble recombinant MOMP by including detergents and lipids as a means to solubilize MOMP. The process could then just focus on the translational system for expressing ApoA1 protein to form the supporting nanodisc scaffold within the reaction.

2. What micropipette and pipette tips are used.

We have added the manufacturer and catalog information for the pipettes and tips used to the materials table.

3. What type of glass vials were used for lyophilization.

Polypropylene tubes were used as containers during lyophilization and this is now specified in the text:

"Make note of the final volume and aliquot into endotoxin-free 15 mL or 50 mL polypropylene tubes as desired."