**Thank you for getting back to us with comments. We are delighted to have received positive feedback on our manuscript, and further thrilled to see that the specific comments overwhelmingly fall into minor considerations. To address the most common comment, we are creating a new, first section of the protocol that defines and gives specific instructions for making all media and several other key solutions. Below, we will respond line by line to all feedback, with the original comment in italics and our response below in bold text:**

**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.”*

**We have done so.**

*“2. Please define all abbreviations before use.”*

**As far as we can tell, we have now defined all abbreviations before use.**

*“3. 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. See examples below.”*

**We have added more details throughout the protocol, both by responding to the comments below as well as adding extra details throughout the protocol.**

*“4. 1.1: Please provide the composition of CSM-His + YNB + 2% glucose media.  
5. 1.4: Please provide the composition of YP media.  
6. 1.5: Please specify the type of water (deionized?).”*

**The above three comments are now addressed in the new, first section.**

*“7. 3.5: Please specify the flow rate during loading.”*

**We use 1mL/min, and this is now noted in the text.**

*“8. 3.7: What volume of buffer is used to elute the protein?”*

**We typically collect ten 1-mL fractions, and have changed the text to note this.**

*“9. 3.12: What wavelengths are measured?”*

**We measure absorbance at a wavelength of 280 nm, and have changed the text to note this.**

*“10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.”*

**We have combined many of our shorter protocol steps.**

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

**Done.**

*“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.*

*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.*

*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.”*

**We have highlighted 2.75 pages of text we think identifies the essential steps.**

*“15. Figures: Please add the unit for the fragment sizes.”*

**Done.**

*“16. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.”*

**Our discussion addresses these items, and in replying to the other comments from reviewers and modifying the text it does so even more.**

*“17. References: Please do not abbreviate journal titles.”*

**Done.**

*“18. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Material/ Equipment.”*

**Done.**

**Reviewers' comments:**  
  
*“Reviewer #1:  
  
Manuscript Summary:  
The authors have provided a clear and detailed protocol for the expression and purification of the borate members of the SLC4 family of anion transporter membrane proteins and a simple cross linking assay for the homo-oligomeric structure.  
  
Major Concerns: None  
  
Minor Concerns: None”*  
  
**We are thrilled to receive positive feedback from Reviewer #1.**  
  
**------------------**

Reviewer #2:  
  
The manuscript describes a protocol for the expression and purification of SLC4 family membrane transporters. The results of the study are clearly presented. I have only couple of minor comments regarding the protocol.  
  
*“As an experimental protocol, I would suggest the authors to include an additional section to provide detailed recipes for the solutions, and even the steps to make the solutions if necessary. Although the contents of some solutions were given in the protocol, I see some undefined solutions/reagents (no recipe or no commercial supplier). Here are some examples:  
  
CSM-His + YNB + 2% glucose media in line 86.  
YP media in line 95  
S200 buffer in line 200  
Laemmli loading dye in line 206  
Tris buffer in line 207 etc.  
  
Even if a solution is pre-made by a commercial supplier, I would suggest to include a detailed recipe for the solution, because the specific supplier (and thus the associated document for the solution) might disappear sometime in future.”*

**We agree and have introduced a new, first section of the protocol that gives detailed recipes for all media and key solutions like those indicted above.**

*“Is "Mini-Protean TGX Gels, 4-20%" on Page #14 the same as "4-20% Tris-glycine SDS-PAGE gel" in the text? If yes, please keep the term consistent or clarify them appropriately.”*

**They are the same, and we have updated the text to reflect this.**

*“On the last two pages (Pg# 15 and 16), I saw some weird text.”*

**This is merely the result of bleeding over of text from the Excel sheet used to create the table of reagents, and that some chemical names and descriptions are longer. Our understanding is that the table is displayed on the web interface and not in the pdf of JoVE articles, and thus should not display as in our compiled document, but we will change it if deemed necessary.**  
  
  
**-----------------**  
Reviewer #3:  
  
Manuscript Summary:  
The manuscript described the growth and isolation of Borate transporters that are homologous to the human SLC4 family of proteins. The text also describes a simple but efficient gluteraldehyde cross-linking assay that can be useful to identify if a purified sample is a dimer or monomer.  
  
The protocol is well written and easy to follow in most places. The manuscript is brief and to the point, but it seems to be efficient and it will be of interest to other researchers in the field. I recommend publication after a some minor textual revisions as described below.  
  
Major Concerns:  
No major concerns.  
  
Minor Concerns:  
  
*“Title and elsewhere:  
  
1) Define SLC first time it is used.”*

**We have taken the SLC out of our title, which means the term is now defined in its first use**

*“2) SLC, by definition (*[*https://en.wikipedia.org/wiki/HUGO\_Gene\_Nomenclature\_Committee*](https://en.wikipedia.org/wiki/HUGO_Gene_Nomenclature_Committee)*), is a gene nomeclature only used for \_human\_ membrane transporters. The manuscript should be rewritten everywhere SLC is mentioned to clarify this. There is no such thing as a plant or yeast SLC4. Those would be SLC4 homologs. For instance, the title word use of "eukaryotic SLC4" is not meaningful.”*

**We agree and have made changes in the text in each case SLC or SLC4 is mentioned. Regarding the title, we propose the following alternative:**

**Expression, solubilization, and purification of eukaryotic borate transporters**

*“3) line 71: detergent suitability. This aspect is almost not touched upon in the rest of the manuscript (except line 296). An example of a 'bad' detergent eg. as an extra figure (leading to only the monomer visible in the gluteraldehyde assay) would be very beneficial. At the very least this aspect should be better discussed in the final discussion. One is tempted to speculate that a poor detergent choice, generally, would lead to aggregation, not a monomeric species?”*

**This is a terrific question and we agree it merits discussion. To the first point, we would argue that the pre-incubation with SDS qualifies as an example of what a “bad” detergent would do. To be sure, those who purify membrane proteins probably know not to use SDS as the detergent to solubilize and purify membrane proteins, but those lanes serve as a negative control that we believe addresses this first point. To the latter point, we find that a choice of a “bad” detergent (“bad” of course being relative only for the protein of interest) can result in either aggregation – often readily detectable in the quality of an SEC trace – or non-native multimeric state, which is less easily detectable on SEC traces and therefore justifies our simple but direct cross-linking assay. We have expanded on these subtleties in our results and discussion sections.**

*“4) Line 87 and elsewhere: "O/N". this and several other words are not defined the first time they appear in the text.”*

**We have changed the text, both for O/N and several other instances.**

*“5) Line 89: OD600: define.”*

**Done.**

*“6) line 100: cells may be frozen: After resuspension in 100 ml water? I would normally assume the cell pellet was frozen without resuspension in water?”*

**Yes. The 100mL is the total volume used to resuspend all the pellets, which in our case is spread out over 6 centrifuge bottles. We make this transfer in order to avoid having to store our centrifuge bottles in the freezer, and because using liquid to resuspend the cells avoids losing cells from trying to scrape the pellets out without the help of resuspension.**

*“7) line 101: mention the expected yield (in grams of cells) from this type of growth. This would be very useful downstream to compare numbers.”*

**We typically harvest 40-45g of cells, and have now noted in the text.**

*“8) line 104: Explain why you use EDTA and PMSF. Also, should these be defined? Maybe not as they are so in the final list.”*

**We have defined the terms in a new earlier section of the protocol, and have changed the text here to indicate why they are used (i.e. as protease inhibitors).**

*“9) Line 116: the apparatus and filter top assembly is very hard to imagine based on the text alone. A picture or better description would be useful.”*

**We have added more detail to our description of the setup. We believe a video will help clarify the matter further, too.**

*“10) line 132ff: point 2.9 to 2.11 is overly explained. Perhaps 2.11 could be combined with 2.9.”*

**We have combined these steps but have chosen to leave the text mostly intact for one key reason: in comparing the weight of the centrifuge bottles with membranes and without, we are measuring the latter not by measuring clean and perfectly empty tubes, but rather by measuring the weight of the tubes after transferring the membranes to the douncer. In this state, the tubes often have a bit of remaining fluid and/or membrane debris, which makes them heavier than when weighing a perfectly clean and dry tube. In measuring it this way and in this order, we therefore get a more accurate determination of the amount of membranes we harvested. Others may systematically overestimate their membrane yields by as much as a gram by avoiding this detail, so we wish to leave it in as written.**

*“11) line 195: what is considered a pure peak fraction? See comment 16) for figures as well.”*

**In addressing the points raised in comment #16 below, we have re-worked figure #1, and its caption, and discuss fractions were selected.**

*“12) line 200. S200 buffer is not defined, but I assume it is the buffer from line 187?”*

**They are the same. We have modified the text to indicate as much, and have included buffer recipes in our first, new section of the protocol.**

*“13) line 206: Laemmli loading dye is not defined. Either write the composition or give a reference for this.”*

**We have included our gel loading dye in our list of recipes.**

*“14) line 231f: the yield is giver per L of culture. But the protocol is coming from 4-5 g of membranes. How does this correlate? What is the expected yield per purification (ie 3-4 g membrane) and the expected yield per gram of cells harvested?”*

**This is a terrific question, especially in conjunction with the earlier question #7 about cell yields. Our prep normally gives 40-45g of cells, which because of our 20% yields in going from cells to membranes results in a total of 8-9g of membranes harvested. We typically split that into two tubes to freeze, which is why in the protocol we say we use 4-5g of membranes per protein purification. These numbers will be made more explicit, both here and earlier in the text.**

*“15) line 297: The manuscript would benefit if it explained that atBor1 turned out to be a dimer in the crystal packing. Thus the chase for a dimer in the gluteraldehyde assay is justified 'post-mortem' for this particular case.”*

**This is true, and we will indicate that the glutaraldehyde assay was developed after this observation. We will likewise include in our discussion that knowledge that AtBor1 was a dimer whereas ScBor1 did not purify as a dimer (and was a poor crystallization target) which – if known because of this assay – could have saved valuable time in deciding which proteins to target for structural studies. This lesson underscores the value of this assay as a simple diagnostic.**

*“16) Fig 1. This figure need some rework. Please label all lanes of the gel, as well as all bands of the marker. Make the numbers of the SEC trace readable, and show the collected fractions and how they correlate with the SDS-PAGE gel. re. 11) use the figure to help you explain what is considered a 'pure' peak fraction. For me all of these fractions in the gel look very pure. Do you use them all?”*

**These are excellent suggestions, and we have reworked the figure to incorporate all of them. As explained in the new figure caption, lines above the first gels indicate which we selected to collect, while brackets above the SEC gels and the SEC traces correspond to one another. We also include in our discussion considerations for which fractions we select and why. In general, we err on the conservative side of peak fraction selection, but add in our discussion that increased yields may be attained with only marginal sacrifices in purity.**  
  
  
**-----------------**  
Reviewer #4:  
  
Manuscript Summary:  
*“The protocol describes methods to express and purify borate transporters from plants in Saccharomyces cerevisiae for structural biology. I agree that the protocol is helpful for researchers who deal with various membrane proteins.”*

**We are glad to hear this positive feedback.**

Minor Concerns:  
*“General, It is better to describe Buffer and media compositions although they are standard ones. CMS, YNB, YP, S200 byfferm 1.5% glutaraldehyde, 3X Laemmli loading dye.”*

**We agree with this excellent suggestion and have introduced a new, first section that lists the compositions for key media and buffer recipes. We indicate in our table of materials that glutaraldehyde is purchased as an 8% solution, and we do not think that describing how to dilute that to 1.5% is an appropriate use of space.**

*“1.3. The growth duration is very long. I think it is usual to start induction in the log phase. Please describe the merit to grow longer.”*

**The longer growths result in higher yields of cells, consequently higher yields of membranes, and in turn larger yields of purified protein. This is encapsulated in our discussion when we write “The parameters optimized here include cell culture growth volumes and times…” To address this point more explicitly, we have inserted a note after this item in the protocol to reflect this consideration even earlier in the text.**

*“1.5 Are all pellets from 4 flasks resuspended in 100 ml cold water and used in step 2?”*

**Yes. The 100mL is the total volume used to resuspend all the pellets, which in our case is spread out over 6 centrifuge bottles. We make this transfer in order to avoid having to store our centrifuge bottles in the freezer, and because using liquid to resuspend the cells avoids losing cells from trying to scrape the pellets out without the help of resuspension.**

*“2.2 Could you describe how to prepare such a huge amount of glass beads. Are they acid washed?”*

**We first purchase the beads as indicated from BioSpec. To re-use beads, we extensively wash them with water in the vacuum filter apparatus, and later autoclave them before re-using. Per the manufacturer’s directions, we do not acid wash them (see here:** [**https://biospec.com/beads-guide-lines/cleaning-your-beads**](https://biospec.com/beads-guide-lines/cleaning-your-beads) **). Nowhere else in our protocol do we indicate directions for cleaning and re-using equipment, and our inclination is to keep that consistent in this case, as we are merely following the directions from the manufacturer and not introducing any new methodologies or variations.**

*“2.6 Could you explain the reason to use wash buffer with high concentrations of NaCl and glycerol?”*

**Glycerol is a small osmolyte known to stabilize many different proteins. The value of higher concentrations of salt are to help dissociate peripheral membrane proteins. Also, we note that the concentrations stated in the text are at 2X. Our final concentration of 700mM NaCl in the lysate is in line with what many other labs use, and if anything might be considered within the medium range. For example, the Raymond Stevens lab traditionally uses a 1M salt wash to help dissociate peripheral membrane proteins during a membrane preparation (**[**https://commonfund.nih.gov/sites/default/files/JCIMPT\_MembranePrep.pdf**](https://commonfund.nih.gov/sites/default/files/JCIMPT_MembranePrep.pdf) **). We have inserted a note after the protocol to address these considerations.**   
  
  
**------------------**  
*“Reviewer #5:  
  
Manuscript Summary:  
The manuscript describes the purification of borate transporter orthologs from S. cerevisiae, Arabadopsis and Oryza.  
  
Major Concerns:  
It would be helpful to at least do scanning EM to characterize the homogeneity and quality of the protein particles visually.  
  
Minor Concerns:  
None”*

**Reviewer #5 raises an interesting point regarding the homogeneity and quality of the protein. Size-exclusion chromatography is rich in this information, and we have updated text in the Representative Results section to reflect the ability of the method to assess this information. We have not performed electron microscopy, because it is not a typical part of our, or most other labs’, approaches to routine protein expression and purification. Moreover, there is no electron microscope at Davidson College. Part of the advantage of our protocol is that it uses relatively ubiquitous equipment that can be found at a diverse array of institutions with varying degrees of resources, including all-undergraduate institutions such as Davidson College.**