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Expression, solubilization, and purification of eukaryotic borate transporters

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DAVIDSON

November 4, 2018

Dear Dr. Ronald Myers,

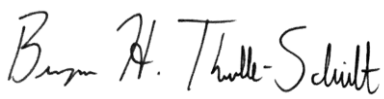
Please find enclosed our revised manuscript entitled, "Expression, solubilization, and purification of eukaryotic borate transporters." Please note that, per a reviewer comment, we have changed the title from our original submission by subtracting one word.

We were very glad to see that the comments were overwhelmingly positive and the concerns minor. We have responded to every comment line-by-line in a document we are enclosing with our revised manuscript. All of the concerns fell into the "minor" category except for one comment from Reviewer #5, who suggested we perform electron microscopy on our samples. This is an unusual comment for several reasons. First, we note that the reason given for doing this – monitoring protein homogeneity – is already evaluated using size exclusion chromatography, which is the standard used in the field of membrane protein expression studies (recent examples include JoVE papers from Robert Ford and Eric Gouaux). We have modified the text to emphasize this point. Also, 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.

In our revised manuscript we have highlighted text totaling less than 2.75 pages that we think identifies the essential steps of the protocol for the video. In sharing our expertise on membrane protein purification with our detailed protocols, we continue to think this work should appeal to a significant cross-section of researchers who rely on JoVE, especially those interested in membrane proteins, transporters, and protein expression methodologies.

Thank you for your consideration, and please do not hesitate to contact us with any questions.

Sincerely,
Bryan H. Thurtle-Schmidt



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TITLE:

Expression, Solubilization, and Purification of Eukaryotic Borate Transporters

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KEYWORDS:

Biochemistry, structural biology, membrane protein, protein purification, Bor1, transporters, bicarbonate transporters, borate transporters, SLC, SLC4, Band 3, AE1

SUMMARY:

Here we present a protocol to express, solubilize, and purify several eukaryotic borate transporters with homology to the SLC4 transporter family using yeast. We also describe a chemical cross-linking assay to assess the purified homomeric proteins for multimeric assembly. These protocols can be adapted for other challenging membrane proteins.

ABSTRACT:

The Solute Carrier 4 (SLC4) family of proteins is called the bicarbonate transporters and includes the archetypal protein Anion Exchanger 1 (AE1, also known as Band 3), the most abundant membrane protein in the red blood cells. The SLC4 family is homologous with borate transporters, which have been characterized in plants and fungi. It remains a significant technical challenge to express and purify membrane transport proteins to homogeneity in quantities suitable for the structural or functional studies. Here we describe detailed procedures for the overexpression of borate transporters in *Saccharomyces cerevisiae,* isolation of yeast membranes, solubilization of protein by detergent, and purification of borate transporter homologs from *S. cerevisiae*, *Arabidopsis thaliana*, and *Oryza sativa*. We also detail a glutaraldehyde cross-linking experiment to assay multimerization of homomeric transporters. Our generalized procedures can be applied to all three proteins and have been optimized for efficacy. Many of the strategies developed here can be utilized for the study of other challenging membrane proteins.

INTRODUCTION:

The difficulty in obtaining sufficient quantities of purified membrane protein remains a critical bottleneck in the pursuit of structural and functional studies of receptors, ion channels, and transporters. Many protocols exist for the moderately high throughput pipelines to screen and

find candidate membrane proteins that express well enough to enable subsequent *in vitro* studies[1–4]. Typically, proteins are tagged with an N- or C-terminal green fluorescent protein (GFP), and expression levels are monitored either by in-gel fluorescence or by fluorescence-detection size exclusion chromatography (FSEC)[5]. Such approaches enable the triaging of membrane protein candidates into high expressing proteins, moderate or low expressing proteins, or proteins that express poorly or not at all. This approach works well when the experimental design is to investigate large numbers of candidate genes with the intention of selecting whichever protein expresses the best. However, in some cases, an experimental approach is predicated on studying a particular membrane protein, which can be challenging when that protein's expression levels are in the moderate or low range. Additionally, sometimes in these cases, expression levels can be only minimally increased by altering the construct via truncations, thermostabilizing mutations, or codon optimization. It is, therefore, sometimes necessary to optimize membrane protein expression and purification protocols for membrane proteins that express only moderately well.

The SLC4 family of transporters includes the bicarbonate transporter Anion Exchanger 1 (also known as Band 3), the most abundant membrane protein in red blood cells[6], and a key driver of cellular respiration. The SLC4 family is homologous with borate transporters, which are critical in plants and have been shown to exhibit a similar structure to Anion Exchanger 1 as well as to sodium-coupled SLC4 transporters[7–10]. Here we report optimized protein expression and purification protocols using *S. cerevisiae* to purify three different borate transporters found in yeast and plants. We highlight in detail key steps we took to optimize the yield and efficiency of the purifications to homogeneity. Additionally, we report a chemical cross-linking assay using glutaraldehyde to monitor the multimeric assembly of these transporters in the context of a purified protein-detergent-lipid complex. The cross-linking experiment can help evaluate homolog and detergent suitability by assessing the multimeric state of oligomeric transporters after purification.

This protocol assumes that the desired transporter has been cloned into a 2 μ derived plasmid under inducible control of the GAL1 promoter for expression in *S. cerevisiae*. For these procedures, DNA was used encoding full-length wild-type borate transporters *Saccharomyces cerevisiae* Bor1 (ScBor1)[11], *Arabidopsis thaliana* Bor1 (AtBor1)[12], and *Oryza sativa* Bor3 (OsBor3)[13]. The C-terminus in each construct is appended with a 10-His-tag and a thrombin cleavage site inserted between the transporter and the 10-His-tag to enable its removal if desired. The protocol will also assume that the plasmid has already been transformed into the DSY-5 expression strain of *S. cerevisiae* on complete supplemental selective media (CSM) lacking histidine.

PROTOCOL:

1. Preparation of media and important buffers

1.1. Prepare 500 mL of 40% galactose by adding 200 g of galactose and de-ionized water to a total volume of 500 mL. Use a hot plate or microwave to increase the rate of galactose getting into

solution. Sterile filter with a vacuum filtration apparatus consisting of a 0.2 µm filter top attached to a sterile glass bottle.

NOTE: All media solutions are prepared using de-ionized water.

1.2. Prepare 500 mL of 40% glucose by adding 200 g of glucose and water to a total volume of 500 mL. Autoclave to sterilize.

1.3. Into each of the four 2 L flasks, add 0.4 g of complete supplement mixture without histidine (CSM-His), 3.35 g yeast nitrogen base with ammonium sulfate (YNB + nitrogen), and 475 mL water. Autoclave. Add 25 mL of 40% glucose to achieve a final glucose concentration of 2%.

1.4. Add, to a glass bottle, 50 g peptone and 25 g yeast extract and water to 375 mL. Autoclave. Add 125 mL of 40% galactose to give 5x yeast peptone (YP) solution containing 10% galactose.

1.5. Add, to a 250 mL flask, 0.04 g CSM-His, 0.335 g YNB + nitrogen, and water to 47.5 mL. Autoclave. Add 2.5 mL of 40% glucose to get 50 mL of CSM-His + YNB + 2% glucose.

1.6. Prepare 1 L of 1M Tris pH 7.0 by mixing 121.14 g of Tris base with 800 mL of water. Add concentrated hydrochloric acid until the pH reaches 7.0. Add water to a final volume of 1 L and pass through a 0.2 µm filter.

1.7. Prepare 500 mL of 0.5 M ethylenediaminetetraacetic Acid (EDTA) pH 8.0 by mixing 73.06 g of EDTA with 400 mL water. Add sodium hydroxide pellets until the EDTA has dissolved and the pH reaches 8.0. Add water to a final volume of 500 mL and pass through a 0.2 µm filter.

1.8. Prepare 100 mL of 100 mM phenylmethanesulfonyl fluoride (PMSF) by mixing 1.74 g of PMSF with 200 proof ethanol. Store at -20 °C.

1.9. Prepare 225 mL of 2x bead wash buffer consisting of 50 mM Tris pH 7.0, 1.4 M NaCl, 20% glycerol, and 1 mM EDTA pH 8.0.

1.10. Prepare 100 mL of the size exclusion chromatography buffer (S200 buffer) consisting of 20 mM 4-Morpholinoethanesulfonic acid hydrate (MES) pH 6.5, 100 mM NaCl, 2% glycerol, and 0.03% n-Dodecyl-beta-D-Maltopyranoside (DDM).

1.11. Prepare 100 mL of membrane resuspension buffer consisting of 50 mM Tris pH 7.0, 500 mM NaCl, and 10% glycerol.

1.12. Prepare 10 mL of a 3x SDS-PAGE loading dye by mixing 3 mL 20% sodium dodecyl sulfate (SDS), 3 mL glycerol, 2.4 mL 1M Tris pH 6.8, 0.03 g bromophenol blue, and 1.6 mL 2-mercaptoethanol.

2. Overexpression of Borate Transporter in *S. cerevisiae*

2.1. Inoculate three transformed yeast colonies in 50 mL of CSM-His + YNB + 2% glucose media and shake overnight at 190 rpm at 30 °C.

2.2. The following day use a spectrophotometer to determine the optical density at a wavelength of 600 nm (OD_{600}) and inoculate to an OD_{600} of 0.01 each of four 2 L flasks that each contain 500 mL of CSM-His + YNB + 2% glucose media.

2.3. Shake at 190 rpm at 30 °C for 30 h to allow the cells to consume all glucose and grow to a high density.

NOTE: A longer growth time results in larger cell yields, larger harvested membrane yields, and ultimately larger purified protein yields.

2.4. Induce expression by adding 125 mL of 5x YP media supplemented with 10% galactose for a final induction concentration of 2% galactose. Shake at 190 rpm at 30 °C for 16 h.

2.5. Harvest cells by spinning at 4,000 x g for 15 min. Resuspend the cells in 100 mL cold water.

NOTE: At this point cells may be frozen at -80 °C indefinitely, or the prep may continue into cell lysis and membrane harvesting. We typically harvest 40-45 g of cells.

3. Harvesting of Yeast Membranes

3.1. Add to the cell resuspension 11.25 mL of 1M Tris pH 7.0, 0.45 mL of 0.5 M EDTA, and 2.25 mL of 100 mM PMSF, the latter two of which act as protease inhibitors. Add water to a final volume of 225 mL, which results in a cell resuspension buffer of 50 mM Tris pH 7.0, 1 mM EDTA, and 1 mM PMSF.

NOTE: If using frozen cells allow cells to thaw thoroughly, about 1 h at room temperature, because if they remain partially frozen, they will resist lysis by bead beating.

3.2. Add the cell resuspension into a 450 mL metal canister for bead beating. Top off the remaining volume with cold 0.5 mm glass beads.

3.3. Assemble a bead-beating chamber with the rotor and immerse in an ice bath. Perform six 1 min pulses, separated by 2 min rest periods to prevent overheating the lysate.

3.4. Assemble a vacuum filtration apparatus by using a plastic disposable bottle top filter screwed into a glass bottle. Remove the filtration membrane, because the remaining plastic device can capture the beads while allowing lysate to pass. Separate the beads from the lysate by pouring the contents of the bead beating chamber onto the assembly while applying the vacuum.

3.5. Wash the bead beating chamber with 225 mL of a 2x wash buffer that contains 50 mM Tris

pH 7.0, 1 mM EDTA, 1 mM PMSF, 1.4 M NaCl, and 20% glycerol. Empty the contents of the chamber onto the beads to wash them.

NOTE: The wash gives a final volume of ~450 mL lysed cells and significantly increases harvested membrane yields. The final concentrations are 50 mM Tris pH 7.0, 1 mM EDTA, 1 mM PMSF, 10% glycerol, and 700 mM NaCl, and the primary purpose of elevating the salt concentration is to help dissociate peripheral membrane proteins.

3.6. Spin down the cell lysate for 15 min at 15,000 x g. Pour the supernatant into polycarbonate bottles and spin for 1 h at 135,000 x g in an ultracentrifuge to collect membranes.

NOTE: If an ultracentrifuge is not available, membranes may be collected by spinning for 3 h at 53,000 x g, a force that is achievable in floor model centrifuges.

3.7. Discard the supernatant and weigh the bottles with the membrane pellets. Resuspend the membrane pellets in approximately 35 mL membrane resuspension buffer containing 50 mM Tris pH 7.0, 500 mM NaCl, and 10% glycerol and add to a glass douncer. Weigh the empty centrifuge tubes to determine the mass of membranes harvested.

NOTE: In an efficient membrane harvest, the weight of membranes will be equal to approximately 20% the weight of cells used for that membrane harvest. This protocol gives typical cell yields of 40-45 g, and thus we likewise expect a yield of 8-9 g membranes.

3.8. Dounce homogenize the membranes, and aliquot into tubes that may now be stored indefinitely at -80 °C.

NOTE: For this experiment, typically two aliquots are made, to allow two separate protein purifications to be performed from one original cell preparation.

4. Solubilization and Purification of Protein

4.1. To a beaker add a stir bar and 150 mg of n-Dodecyl-β-D-Maltopyranoside (DDM) per g membrane being used. Keep protein on the ice or at 4 °C at all times.

NOTE: DDM is hygroscopic and should be stored in a glass jar with desiccant at -20 °C when not in use. Also, it is typical to use between 4-5 g of membranes in a purification to obtain an appreciable amount of pure protein.

4.2. Thaw membranes and bring to a final volume of 15 mL per g membrane in membrane resuspension buffer supplemented with 1 mM PMSF and 20 mM imidazole pH 8.0. Add membranes to the beaker with DDM and stir for 1 h at 4 °C.

NOTE: The DDM concentration will be 1% w/v, but for the solubilization of membrane proteins it is more critical to be aware of the mass of detergent added per g membrane.

4.3. Spin for 25 min at 135,000 x g at 4 °C to pellet non-solubilized material. Filter the supernatant through a 5 µm syringe filter.

4.4. Load the sample by the peristaltic pump set to a flow rate of 1 mL/min onto a 1 mL immobilized nickel affinity column equilibrated in 20 mM Tris pH 7.0, 500 mM NaCl, 10% glycerol, 20 mM imidazole pH 8.0, and 0.05% DDM.

NOTE: For lower expressing proteins, an improved purity and yields were observed by using a 1 mL instead of 5 mL column, and nickel instead of cobalt ions for affinity chromatography.

4.5. Wash the column with 10 column volumes of a wash buffer containing 20 mM Tris pH 7.0, 500 mM NaCl, 10% glycerol, 80 mM imidazole pH 8.0, and 0.05% DDM.

NOTE: The imidazole concentration that can be used during washes for a 10-His-tagged protein is higher than is commonly appreciated and results in improved purity.

4.6. Elute the protein in the buffer containing 20 mM Tris pH 7.0, 200 mM NaCl, 10% glycerol, 300 mM imidazole pH 8.0, and 0.05% DDM. Collect the eluant in ten 1 mL fractions and run on a 4-20% Tris-glycine SDS-PAGE gel along with solubilized lysate and wash fractions.

4.7. Pool peak fractions, usually about 5-6 mL, and concentrate to 500 µL or less volume in a 50 kDa cutoff concentrator in a benchtop centrifuge refrigerated at 4 °C.

NOTE: 500 µL is a typical maximum volume injected into size exclusion chromatography (SEC) columns. At this point the protein may be stored indefinitely at -80 °C or continue onto SEC.

4.8. Filter the protein through a 0.2 µm spin column filter and inject onto a size exclusion column (*e.g.,* Superdex-200) equilibrated in S200 buffer: 20 mM Mes pH 6.5, 100 mM NaCl, 2% glycerol, and 0.03% DDM.

NOTE: For the subsequent glutaraldehyde cross-linking assay, the buffering agent must not contain a primary amine. SEC is an opportunity to exchange buffers if necessary, as done here when exchanging Tris for Mes.

4.9. Run the peak fractions on a 4-20% Tris-glycine SDS-PAGE gel. Stain the gel, collect pure peak fractions, and concentrate in a 50 kDa-cutoff concentrator at 4 °C.

4.10. Determine the protein concentration by measuring the absorbance at a wavelength of 280 nm, and store indefinitely at -80 °C.

5. Glutaraldehyde Cross-linking Assay

5.1. Prepare a 10 µL reaction by mixing 3 µL of 0.5 mg/mL protein in S200 buffer, 5 µL of S200

buffer, 1 µL of either water or 20% sodium dodecyl sulfate (SDS), followed by 1 µL of 1.5% glutaraldehyde.

NOTE: This will give a 1x reaction of 0.15 mg/mL protein and 0.15% glutaraldehyde. Samples containing a pre-treatment of SDS should be mixed and incubated at room temperature for 5 min before adding glutaraldehyde.

5.2. Incubate the reaction for 30 min at room temperature. Terminate the reaction by adding 5 µL of 3x SDS-PAGE gel loading dye, which contains an excess of Tris buffer that quenches the glutaraldehyde.

5.3. Load all 15 µL onto 4-20% Tris-glycine SDS-PAGE gel, which will load 1.5 µg protein per lane. Run the gel at 200 V for 30 min. Stain and determine the extent of dimer cross-linking by evidence of a band that runs at twice the size of the denatured monomer.

NOTE: A glutaraldehyde titration and time course may be performed first to find the best conditions for a homomeric transporter.

REPRESENTATIVE RESULTS:

Typical gels for the eluted fractions from performing nickel affinity chromatography show all three proteins partially purified (**Figure 1A**). To the right of the ladder is the lysate, which in each case does not show a significant band corresponding to the borate transporter which is typical for proteins that do not overexpress well. The 80 mM wash lane shows minimal loss of 10-His-tagged protein despite the relatively high imidazole concentration. Bands corresponding to the borate transporters are readily apparent in eluted fractions, and fractions deemed to contain the bulk of the eluted protein are concentrated before injecting onto the S200 gel filtration column. At this stage, the proteins are insufficiently purified for many downstream applications, as indicated by extra bands in the concentrated sample before injecting onto the S200 column (**Figure 1B**). However, injecting the sample onto the size exclusion column reveals eluted fractions of high purity (**Figure 1B-C**). Chromatograms and their corresponding gels for each of the borate transporters are presented. Despite the moderate purity of the samples upon elution from affinity chromatography, the protein is highly pure upon eluting from the gel filtration column. Critically, the chromatograms reveal each protein to migrate primarily as a single monodisperse peak with little protein retained in the void volume. A stable and folded protein generally gives a monodisperse and symmetrical peak, while unstable, misfolded, or aggregated protein will generally give either multiple asymmetric peaks or a large peak in the void volume. It is typical to obtain a final yield of approximately 2 mg purified protein per L of yeast culture for ScBor1, and approximately 1 mg each of purified AtBor1 and OsBor3 per L culture. These numbers are the amount of protein purified from one aliquot of 4-5 g of membranes, which originates from one half of our original cell preparation.

The cross-linking experiment shows that purified homomeric transporters can have their assembly in solution readily assessed (**Figure 2**). The purpose of the samples containing a pre-

treatment of SDS is to show that cross-linking is dependent on a folded state in solution and that cross-linking therefore does not occur when multimerization is disrupted by a harsh detergent. The results shown distinguish that one of the three borate transporters, ScBor1, is not dimerized when purified in DDM in these conditions, while the other two, AtBor1 and OsBor3, cross-link and show dimerization. It is possible to see that not all protein may cross-link. In this example, trace amounts of OsBor3 monomer are visible, while AtBor1 cross-links to completion. The extent of cross-linking will depend on the number of lysine residues in proximity to one another, and it is possible that other cross-linking reagents may efficiently cross-link different membrane proteins.

FIGURE AND TABLE LEGENDS:

Figure 1. Nickel affinity and size exclusion chromatography purification for three borate transporters. Each vertical panel (**A**), (**B**), and (**C)** contains data for ScBor1, AtBor1, and OsBor3. (**A**) Nickel affinity column. M is a ladder of molecular weight markers with weights in kDa specified to the left. L is the crude lysate, and W is the 80 mM imidazole wash. All other numbered lanes correspond to 1 mL fractions eluted with 300 mM imidazole. Bars above fractions indicate which were selected to be combined and concentrated for injections onto the column. (**B**) P indicates concentrated protein before injecting onto the S200 column. Eluted SEC fractions are to the right, with brackets used to match gel lanes with their chromatogram fractions in (**C**). The void volume is just after 8 mL.

Figure 2. Cross-linking assay assesses purified transporters for multimeric assembly. A ladder with molecular weights is indicated on the left. Above all other lanes is shown which protein is present and whether the sample has had glutaraldehyde added or a pre-treatment of SDS before glutaraldehyde addition. Arrows indicate the positions of monomer and dimer for AtBor1 and OsBor3. ScBor1 is a smaller protein than AtBor1 and OsBor3 and runs as expected.

DISCUSSION:

Here we have shared detailed protocols that result in the purification to homogeneity of three distinct eukaryotic borate transporters. The protocols presented here are derived from other protocols for the expression of integral membrane proteins in *S. cerevisiae*[1,14], and our optimizations result in both improved purity and improved yields. The parameters optimized here include cell culture growth volumes and times, bead-beating lysis procedures, buffer composition during cell lysis and protein purification, amount of detergent used per gram membrane, metal ion identify in affinity purification, volume of the affinity column, and the implementation of a cross-linking assay to evaluate homolog and detergent suitability by assessing the multimeric state of oligomeric transporters. The protocols are successful for multiple SLC4 homologs from the plant and fungal species. A limitation of all strategies for the purification of integral membrane proteins is that there exists no universal membrane protein purification method that is guaranteed to work. It is possible that our protocols are more likely to be successful for proteins closer in sequence identity and structure to SLC4 transporters, and thus members of the SLC4, SLC23, and SLC26 families could be promising targets[15]. Likewise, the more evolutionarily distant from the borate transporters a membrane transporter might be, the

more likely the protocol will have to be different, such as by varying the expression system, detergent, or other key parameters[4].

The protocol takes advantage of the most commonly used affinity tag in protein purification, the His-tag. Despite the presence of impurities in the initial eluted fractions, the combinations of nickel affinity, extensive washing, and subsequent SEC purification results in the highly purified protein. A 10-His-tag allows for the more stringent imidazole washes and thus can remove more background binding proteins than can be removed in washes permitted by 8-His- or 6-His-tags. Our selections for pure fractions err on the conservative side of the most highly pure gel fractions, which correspond to the peak SEC fractions. Final protein yields can be increased by pooling and concentrating more fractions, albeit with the trade-off of slightly less pure protein. The methods presented here enabled the purification of AtBor1 in quantities that led to the determination of its crystal structure[7], with purification differences consisting of cutting off the His-tag and exchanging the transporter into a different detergent to improve crystal diffraction[7].

Our protocol raises important considerations for the selection of homologs and the detergent used to solubilize and purify them. DDM is a common first choice for detergent because it is relatively mild, often successful at solubilizing, and has been used in structural and functional studies of a wide variety of membrane proteins. In determining whether a detergent is a poor selection for a membrane, a common method of evaluation is whether the protein gives a single monodisperse peak on an SEC chromatogram, rather than a large peak in the void volume or a range of polydisperse peaks, which indicate misfolded or unstable protein. A more subtle consideration is raised by our purification of ScBor1. It purifies in large quantities and looks favorable and monodisperse on an SEC chromatogram, which suggests it could be an attractive target for structural studies. However, our cross-linking assay reveals that it is a monomer when purified. While it is possible that ScBor1 could natively exist as a dimer in the cell, studies indicate that SLC4 transporters and their homologs are likely to be dimers[7–10]. Our development of the cross-linking assay occurred after crystallizing and solving the structure of AtBor1. However, had we been able to evaluate ScBor1 in comparison with AtBor1 with the cross-linking assay, valuable time and research efforts could have been redirected to the pursuit of AtBor1 instead of ScBor1, the latter of which ultimately was not successful in crystallization and diffraction experiments. This assay can thus help researchers distinguish between either homologs or detergents to use when pursuing structural studies in order to prioritize conditions in which the protein maintains its suspected native conformation. Additionally, the assay can be used to probe which amino acids are critical for multimerization, in order to find amino acid substitutions that destabilize the multimerization interface and lead to obligate monomers. Such an approach has been used to probe the functional significance of homomeric assembly in membrane transporters[16–18].

A general advantage of our method is that yeast has been shown to enable the expression of many challenging membrane proteins of diverse function[1]. Additionally, its low cost and growth times promote its accessibility for many research efforts that may be less able to use expression strategies that require tissue culture or expensive growth media. The procedures presented here are relatively inexpensive and can be performed in one week which underscores the feasibility of the approach. Implementation of these protocols can help enable structural and functional

studies of other challenging membrane proteins.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Fig. 1

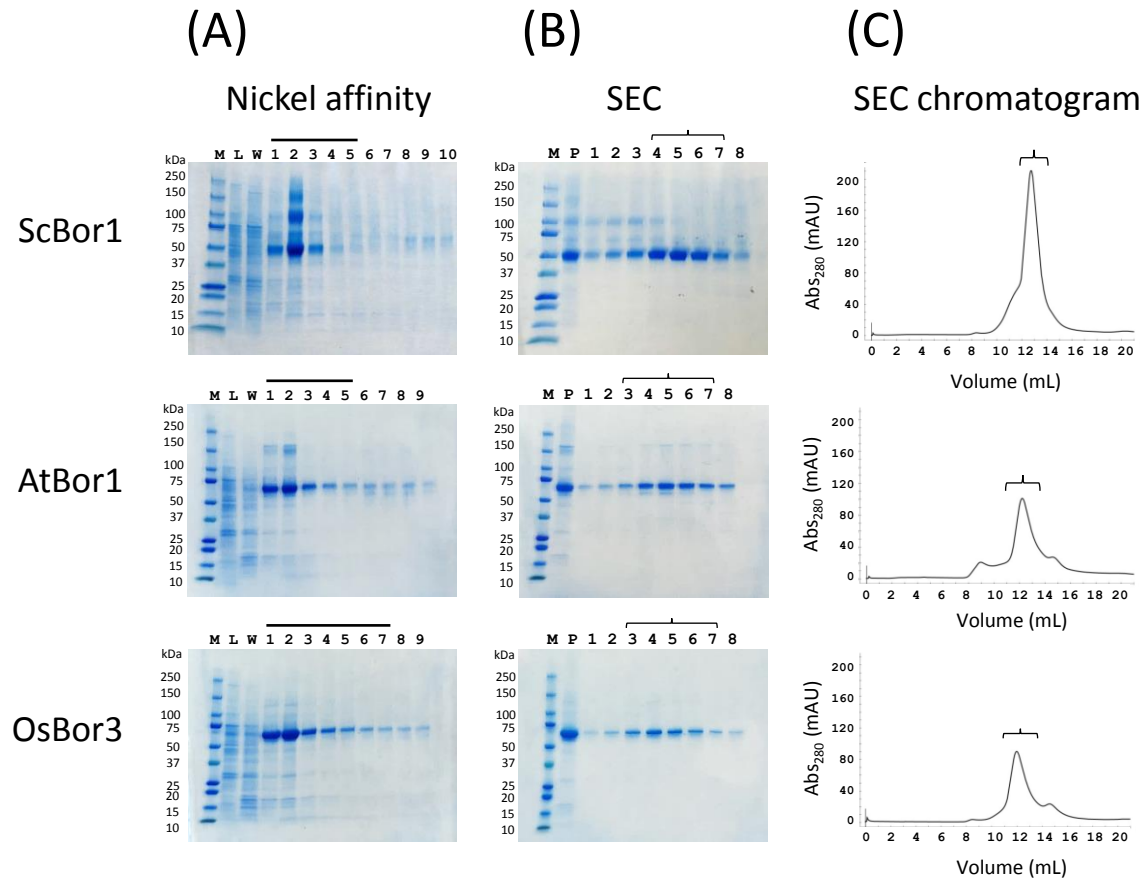
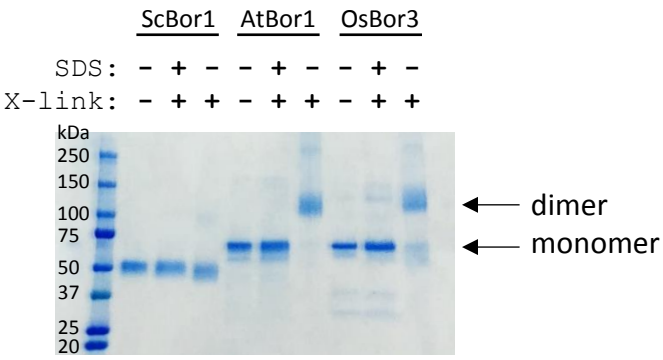


Fig. 2



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2-mercaptoethanol	Sigma-Aldrich	M3148	
4-Morpholinoethanesulfonic acid hydrate (MES)	Acros	172591000	
Äkta Pure 25 L FPLC	GE Healthcare	29018224	
Amicon Ultra 15 mL, 50 kDa MWCO concentrator	Millipore	UFC905024	for concentrating nickel col
Amicon Ultra 4 mL, 50 kDa MWCO concentrator	Millipore	UFC805024	for concentrating S200 frac
Bacto Peptone	BD Diagnostics	211677	
Bacto Yeast Extract	BD Diagnostics	288620	
Glass Erlenmeyer flask, 2L	Sigma-Aldrich	CLS44442L	
Benchtop centrifuge	Sorvall	Legend RT	
Bottle top filter	Nalgene	595-4520	the membrane is removed
Bromophenol blue	Sigma-Aldrich	114391	
Complete supplement mixture without histidine	Sunrise Science	1006-100	
D-Galactose	Sigma-Aldrich	G0750	
D-Glucose	Sigma-Aldrich	RDD016	
Ethanol, 200 proof	Pharmco-Aaper	111000200	
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich	EDS-500G	
Gel tank SDS-PAGE system	Bio-Rad	1658004	
Glass bead-beating cell disruptor	BioSpec	1107900	
Glass beads, 0.5mm	BioSpec	11079105	
Glutaraldehyde	Electron Microscopy Sciences	16019	sent as an 8% solution unde
Glycerol	Sigma-Aldrich	G7893	
HiTrap IMAC FF column, 1 mL	GE Healthcare	17-0921-02	charged with nickel
Hydrochloric acid	Sigma-Aldrich	258148	
Imidazole	Acros	12202	
Instant Blue gel stain	Expedeon	ISB1L	
JA-10 rotor	Beckman	369687	
JA-14 rotor	Beckman	339247	
Microcentrifuge tubes, 1.5mL	Thermo Scientific	3451	
Microcentrifuge, refrigerated	Fisher	13-100-676	
Mini-Protean Tris-glycine gels, 4-20%	Bio-Rad	456-1096	
Minipuls 3 peristaltic pump	Gilson	F155005	used for loading lysate onto

n-Dodecyl-beta-D-Maltopyranoside (DDM)	Inalco	1758-1350	
Nickel(II) Sulfate Hexahydrate	Sigma-Aldrich	227676	
Orbital shaking incubator with temperature control	New Brunswick	C24	
p423 GAL1 plasmid with borate transporter insert			available from authors
Phenylmethanesulfonyl fluoride (PMSF)	Acros	215740050	
Polycarbonate ultracentrifuge tubes	Beckman	355618	
Polypropylene bottles, 250mL	Beckman	356011	
Polypropylene bottles, 500mL	Beckman	355607	
Precision Plus Protein Kaleidoscope Standards	Bio-Rad	1610375	
S. cerevisiae expression strain DSY-5			available from authors
Sodium chloride	Sigma-Aldrich	S7653	
Sodium dodecyl sulfate	Sigma-Aldrich	L3771	
Sodium hydroxide	Sigma-Aldrich	S8045	
Spin column with 0.2 µm filter, 0.5mL	Millipore	UFC30GV0S	for filtering protein before i
Sterile Falcon tubes, 15mL	Lab Depot	TLD431696	
Sterile Falcon tubes, 50mL	Lab Depot	TLD431698	
Superdex 200 10/300 GL column	GE Healthcare	17-5175-01	used for SEC purification
Syringe filter, 5µm	Pall	4650	for filtering lysate before lo
Tris-base	Sigma-Aldrich	T1503	
Type 70 Ti rotor	Beckman	337922	
Ultracentrifuge	Beckman	L8-70M	
YNB+Nitrogen without amino acids	Sunrise Science	1501-500	

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and used to filter glass beads

er nitrogen

o affinity column

injecting onto S200 column

ading affinity column

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Author(s): Sebastian Flores, Andrew P Feld, and Bryan H Thurtle-Schmidt

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Date:	9/25/18

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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), 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). 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). 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.