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Purification of the Cystic Fibrosis Transmembrane conductance Regulator protein expressed in Saccharomyces cerevisiae.

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Abstract:	We describe two methods for the purification of CFTR from a novel heterologous expression system, S. cerevisiae. Unlike prokaryotic systems, S. cerevisiae can traffic and post-translationally modify large membrane proteins, but can be rapidly grown in the lab at low cost. The purification protocol yields up to 80 µg CFTR per litre of culture at 90 % purity. In addition, we present a comparison of the purification of CFTR in two different detergents, dodecyl-β-D-maltoside (DDM) and 1-tetradecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (LPG-14). Protein purified in DDM by this method shows ATPase activity in functional assays. Protein purified in LPG-14 shows high purity and yield, can be employed to study post-translational modifications, and can be used for structural methods such as small-angle X-ray scattering and electron microscopy. However it displays significantly lower ATPase activity.
Author Comments:	Dear Nandita Sorry about the formatting of references in the last paragraph of the Introduction. This has now been put right. best wishes

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Dear Nandita

We published a JoVE protocol with you last year **doi:10.3791/3860** on the expression of a clinically important membrane protein in yeast. It has had over 5400 views since then. The original article gave an example of a purification gel, but no detailed protocol was shown. In addition there was little discussion of the activity of the protein. We are therefore keen to extend the application of the original video and article by extending the protocol to show the purification of the protein.

best wishes

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Purification of the Cystic Fibrosis Transmembrane conductance Regulator protein expressed in *Saccharomyces cerevisiae.*

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SHORT ABSTRACT: Heterologous expression and purification of the cystic fibrosis transmembrane conductance regulator (CFTR) are significant challenges and limiting factors in the development of drug therapies for cystic fibrosis. This protocol describes two methods for the isolation of milligram quantities of CFTR suitable for functional and structural studies.

LONG ABSTRACT:

Defects in the cystic fibrosis transmembrane conductance regulator (CFTR) protein cause cystic fibrosis (CF), an autosomal recessive disease that currently limits the average life expectancy of sufferers to <40 years of age. The development of novel drug molecules to restore the activity of CFTR is an important goal in the treatment CF, and the isolation of functionally active CFTR is a useful step towards achieving this goal.

We describe two methods for the purification of CFTR from a eukaryotic heterologous expression system, *S. cerevisiae*. Like prokaryotic systems, *S. cerevisiae* can be rapidly grown in the lab at low cost, but can also traffic and post-translationally modify large membrane proteins. The selection of detergents for solubilization and purification is a critical step in the purification of any membrane protein. Having screened for the solubility of CFTR in

several detergents, we have chosen two contrasting detergents for use in the purification that allow the final CFTR preparation to be tailored to the subsequently planned experiments.

In this method, we provide a comparison of the purification of CFTR in dodecyl-β-D-maltoside (DDM) and 1-tetradecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (LPG-14). Protein purified in DDM by this method shows ATPase activity in functional assays. Protein purified in LPG-14 shows high purity and yield, can be employed to study post-translational modifications, and can be used for structural methods such as small-angle X-ray scattering and electron microscopy. However it displays significantly lower ATPase activity.

INTRODUCTION:

Cystic fibrosis (CF) is the most common genetic disorder in Europe and North America with an incidence of about 1 in 2500 live births. CF occurs when mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein cause loss of its function at the plasma membrane of epithelial cells[1]. The most serious consequence of this defect is irreversible lung damage, which shortens the life expectancy of sufferers to < 40 years of age [2,3].

CFTR is an ATP-binding cassette (ABC) transporter that has evolved to become an ion channel [1,4]. Despite its quite altered function in the plasma membrane of cells, it still retains significant sequence homology with other ABC transporters. Intriguingly, the specialized parts of CFTR (i.e. its regulatory region and its N- and C-termini) share no significant sequence similarity with other metazoan ABC transporters, hence there are no clues as to the origins of these sequences in CFTR. On the basis of its primary structure, CFTR is classified as a C-family member of the ABC transporter family, but there is no strong evidence for a residual functional linkage to this sub-family. There have been some reports of glutathione transport activity for CFTR [5-7], which would be consistent with the roles of other C-family members[8,9], although other reports suggest that reduced glutathione may inhibit the CFTR ATPase activity, rather than showing the substrate-induced stimulation that characterize the ABC transporters[10]. Measurement of ion conductance is sufficiently sensitive to allow the channel activity of single CFTR molecules to be studied[1] and CFTR channel properties have been monitored as a function of time, temperature, ATP concentration, membrane potential and phosphorylation state, as well as in the presence of a host of small molecule inhibitors, potentiators and modifiers. These studies have also added significantly to our knowledge of how ABC transporters function. Nevertheless, expression of CFTR in significant amounts and its subsequent purification has proven to be particularly challenging and success has been limited to a few laboratories [10-13].

The need to develop more effective drugs is pressing, yet this process has been hindered by the lack of purified CFTR for screening small molecules. Solving the CFTR expression and purification problem would enable high-throughput drug screening aimed at correcting the primary defect in CF and would also open up a route for high-resolution structural studies to inform rational drug design. Moreover, even relatively basic biochemical characteristics of the protein, such as its functional oligomeric state, interacting proteins and ATPase activity remain poorly characterized. We have previously reported a protocol for the large-scale

expression of GFP- and His-tagged murine CFTR in *S. cerevisiae* [14] and now further describe protocols for the purification of CFTR. We have used these methods to purify five orthologues of CFTR, and present data for the purification of chicken CFTR as an example. The selection of detergents for solubilization and purification is a critical step in the purification of any membrane protein. Having screened for the solubility of CFTR in several detergents, we have chosen two contrasting detergents for use in the purification. Dodecyl-β-D-maltoside (DDM) is a non-ionic detergent that has been extensively used for both structural and functional studies of membrane proteins[15-21]. The ionic detergent 1-tetradecanoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol) (LPG-14) is highly efficient in the solubilisation of CFTR and has previously been used in the purification of functional membrane proteins[10,22,23], including purification of CFTR from *S. cerevisiae*[24].

PROTOCOL:

1. Preparation of buffers

1.1. To make the 100 x stock of protease inhibitor (PI) cocktail dissolve 96 mg AEBSF, 3.5 mg chymostatin, 10 mg E64, 16.5 mg leupeptin, 16.5 mg pepstatin, 348 mg PMSF and 4 mg bestatin in 20 ml DMSO. Make 1 ml aliquots and store at -20 ^{o}C. To make a 100 x stock of benzamidine, dissolve 720 mg in 20 ml ultrapure water (ddH_2O) and store in 1 ml aliquots at -20 ^{o}C. This quantity is sufficient for one purification. In all buffers, PI and benzamidine stocks are used at a 1 in 100 dilution.

1.2. Prepare 'mPIB' (0.3 M Tris pH 8, 0.3 M sucrose, 2 mM DTT) and 'CFTR' (50 mM Tris pH 8, 20 % (v/v) glycerol, 1 mM DTT) buffers and chill to 4 ^{o}C. Before use, add 1:100 of the protease inhibitor cocktail and 1:100 benzamidine according to the volume of mPIB used to resuspend the cell pellet (e.g. use 3.5 ml PI and 3.5 ml benzamidine in a total volume of 350 ml mPIB).

1.3. Prepare solubilization buffers. Lyso-phosphatidyl glycerol-14 (LPG) solubilization buffer (50 mM Tris pH 8, 10 % (v/v) glycerol, 50 mM NaCl, 1 mM DTT, protease inhibitors (PIs) and 4 % (w/v) LPG) and dodecyl maltoside (DDM) solubilization buffer (50 mM Tris pH 8, 20 % (v/v) glycerol, 1 M NaCl, 1 mM DTT, protease inhibitors, 4 % (w/v) DDM). Buffer can be sonicated in a sonicator bath (35 W, 40 kHz) to assist with dispersal of the detergent, but avoid vortexing the mixture, as this creates bubbles. Chill to 4 ^{o}C before use.

1.4. CFTR purification buffer for the LPG purification is 50 mM Tris, 10 % (v/v) glycerol, 50 mM NaCl, 1 mM DTT, 0.1 % (w/v) LPG-14 and protease inhibitors. Prepare 350 ml of this buffer, and 150 ml of the same buffer plus 1 M imidazole. Adjust pH of both buffers to 8.

1.5. The buffer for purification in DDM consists of 50 mM Tris pH 8, 20 % (v/v) glycerol, 1 M NaCl, 1 mM DTT, 0.1 % (w/v) DDM. Prepare 350 ml of this buffer, and 150 ml of the same buffer plus 1 M imidazole. Adjust pH of both buffers to 8.

1.6. For gel permeation chromatography (GPC) buffer containing LPG, prepare 50 mM Tris pH 8, 10 % (v/v) glycerol, 50 mM NaCl, 1 mM DTT, 0.05 % (w/v) LPG-14. For GPC using DDM prepare a buffer of 50 mM Tris pH 8, 20 % (v/v) glycerol, 1 M NaCl, 1 mM DTT, 0.1 %

(w/v) DDM. All buffers and ddH_2O used on the GPC column should be filtered (0.2 µm filter) and degassed before use.

1.7. SDS-PAGE sample buffer (2 x the working concentration): 50mM Tris-HCl pH 7.6, 5 % (v/v) glycerol, 5 mM EDTA, 0.02 % (w/v) bromophenol blue. Make 700 µl aliquots and store at -20 ^{o}C. Before use, add 200 µl of 20 % (w/v) sodium dodecyl sulfate (SDS) and 100 µl of fresh 0.5 M DTT. Incubate for at least 10 min with sample at room temperature before loading on gel. Do not heat; this will denature the GFP and may cause CFTR to aggregate.

1.8. To make lipid stocks for reconstitution, dissolve a 4:1 (w/w) mixture of *E. coli* lipids and cholesterol in chloroform and methanol (2:1 v/v), and dry in a glass vial under N_2 gas for 2 h to form a lipid film. Add GPC buffer (with no NaCl) to a lipid concentration of 40 mg/ml and use repeated vortexing and sonication (35 W, 40 kHz) to clarify the solution.

1.9. For the ATPase assay, prepare 100 x stocks of ATPase inhibitors by dissolving sch28080 to 1 mM in DMSO, NaSCN to 1M in ddH_2O and oligomycin to 2.5 mM in 100 % (v/v) ethanol. Store in aliquots at -20 ^{o}C. Make 100 ml of ATPase buffer with 50mM Tris pH 7.4, 150mM NH_4Cl, 5mM $MgSO_4$ and 0.02% (w/v) NaN_3. This can be stored at room temperature and used for several assays. Prepare a 5 mM ATP stock immediately prior to use and keep on ice. (N.B. Use Na_2ATP to prevent excessive background signal from phosphate in the assay). Prepare the SDS stop solution (12 % (w/v) SDS in ddH_2O).

1.10. For the Chifflet detection prepare buffer A (3 % (w/v) ascorbate, 0.5 % (w/v) ammonium molybdate, 0.5 M HCl) immediately before use and buffer B (2% (w/v) sodium citrate, 2% (w/v) sodium meta-arsenite, 2% (v/v) acetic acid).

2. Isolation of yeast microsomes.

2.1. *S. cerevisiae* expressing chicken CFTR are grown as described in O'Ryan et al (2012). Store the material from a 20 L fermentation in two aliquots at -80 ^{o}C for up to 6 months.

2.2. Defrost one aliquot of cells rapidly and resuspend in 3 ml chilled mPIB per gram of cells.

2.3. Disrupt cells in a bead mill using glass beads of 425-600 µm diameter. Use 5 x 1 min periods of cell disruption separated by 1 min rest periods. (The rest periods are essential to ensure that the cells are not heated during disruption.)

2.4. Monitor cell disruption by centrifugation of a 1 ml sample of the cell lysate from the bead mill. Centrifuge (12 000 x *g*, 4°C, 5 min) in a bench top centrifuge. Dilute the supernatant to 1:50 with mPIB in a cuvette and measure the A_{380}. If A_{380} > 0.1, or has stopped increasing despite several repeated bead-beating cycles, proceed to the following step. If not, repeat 2.3-2.4.

2.5. Centrifuge the total cell lysate (12 000 x *g*, 4 ^{o}C, 20 min). Retain the supernatant. Discard the pellet (containing unbroken cells and mitochondria), but if there is any doubt about the efficiency of cell breakage (see 2.4), then retain the pellet also.

2.6. Centrifuge the supernatant from the previous step (200 000 x *g*, 4 °C, 1.5 h). Discard the supernatant and resuspend the pelleted microsomal membranes in CFTR buffer. **If the microsomes are intended for purification using DDM, supplement the CFTR buffer with 1 M NaCl**.

2.7. Repeat the centrifugation of the resuspended membrane fraction (100 000 x *g*, 4 ^{o}C, 1 h) and discard the supernatant.

2.8. Resuspend the pelleted microsomes in a minimum volume of CFTR buffer (final volume 5 - 15 ml, total microsomal protein 70-200 mg). A Bradford assay may be used to determine the total concentration of microsomal proteins [25]. In addition the fluorescence emission spectrum of the membranes should be measured (excitation = 485 nm, emission = 500-600 nm) and should have a distinct GFP fluorescence peak (maximum at 512 nm). CFTR can be specifically detected on an SDS-PAGE gel, scanned under GFP fluorescence conditions (Figure 1).

2.9. Flash-freeze the resuspended microsomes by plunging into liquid nitrogen and store at -80 °C, or continue to Step 3.

3. Solubilization of microsomes

3.1. If frozen, defrost microsomes immediately before use in a water bath set to 10 ^{o}C.

3.2. For the solubilization of membranes, dilute the microsomes with an equal volume of the relevant solubilization buffer (1.3) to give a final detergent concentration of 2 % (w/v) and a microsomal protein concentration 5 mg/ml. Incubate this mixture for 1 h at 4 ^{o}C with agitation (tube rotator). Retain 200 µl for analysis.

3.3. Centrifuge the mixture (100 000 x *g,* 4^{o}C, 45 min). Remove the supernatant containing the solubilized membrane proteins, pass it through a 0.45 µm syringe filter and store on ice. Measure the fluorescence of the supernatant (as in step 2.8).

3.4. Resuspend the insoluble fraction in 1 % (w/v) SDS solution to a volume equal to the soluble fraction. Measure the fluorescence in this fraction and retain an aliquot of 50 µl for SDS-PAGE analysis.

4. Nickel-affinity purification of CFTR

4.1. Link two 5 ml nickel sepharose columns in series. Wash with 2 column volumes (CV) 20 % (v/v) ethanol, followed by 2 CV ddH_2O, then wash the column with 2 CV of solubilization buffer (1.4-1.5), containing 1 M imidazole. Repeat with 2 CV of solubilization buffer lacking imidazole.

4.2. Add imidazole to a final concentration of 5 mM to the solubilized material (2.8) and manually load the material onto the column or into a sample loop if using an automated liquid chromatography device.

4.3. Load the solubilized material onto the column at a flow rate of 0.5 ml/min, and wash with 2 CV of imidazole-lacking buffer at the same flow rate to remove unbound material. Collect fractions in 50 ml Falcon tubes.

4.4. For the first wash, use 3 CV of purification buffer with 40 mM imidazole at a flow rate of 1 ml/min. Collect 2 ml fractions.

4.5. For the second wash, use 3 CV of purification buffer with 100 mM imidazole. Collect 2 ml fractions.

4.6. Elute CFTR from the HisTrap column with 3 CV of purification buffer with 400 mM imidazole. Collect 2 ml fractions.

4.7. Monitor fluorescence in eluted fractions (2.8).

4.8. Retain aliquots of peak fractions for SDS-PAGE analysis. Flash freeze remaining peak fraction samples and store at -80 ^{o}C, or continue to the next purification step.

5. Gel permeation chromatography (GPC) purification of CFTR

5.1. Equilibrate the column (Superose 6 10/300 GL) with 1.2 CV ddH_2O followed by 1.2 CV GPC buffer.

5.2. During step 5.1, concentrate the Ni-affinity purified fractions with the highest GFP fluorescence using a 100 000 MWCO centrifugal filter at 4 °C. If purifying in DDM, avoid concentrating the sample above a protein concentration of 0.3 mg/ml protein as this will cause significant sample loss. Remove the retentate from the concentrator and centrifuge at 100 000 x *g* for 30 min at 4 °C to pellet large particles.

5.3. Inject this sample onto the column and elute with an isocratic gradient of 1.2 CV GPC buffer. Collect 0.5 ml fractions.

5.4. Measure GFP fluorescence as in section 2.8 to identify those fractions containing CFTR. Retain a small volume (e.g. 50 µl) of each for analysis by SDS-PAGE.

5.5. Freeze fractions in liquid nitrogen and store at -80 ^{o}C.

6. Reconstitution of CFTR

6.1. Add lipids (1.8) to the purified CFTR at lipid-to-protein ratio 100:1 (w/w) and incubate at 4 °C for 1 h. Similarly set up a lipid-only control, substituting the purified protein with the same volume of GPC buffer.

6.2. Remove detergent from the protein/lipid mixture using hydrophobic adsorbent beads. Wash adsorbent beads in 5 CV ddH_2O, 5 CV 70% (v/v) ethanol, 5 CV ddH_2O and 5 CV GPC buffer lacking the detergent. Add 200 mg of washed adsorbent beads per ml of purified

protein and incubate at 4 °C overnight with gentle agitation.

6.3. Collect the reconstitution sample from the adsorbent beads into a fresh tube using a thin-ended pipette tip.

7. Measurement of ATPase activity

7.1. Determine the rate of CFTR-specific ATPase activity using a modified Chifflet assay [26,27] in a 96-well plate format. With sodium phosphate stock solution (0.65 mM) prepare 0 – 20 nmol phosphate in a final volume of 50 µl as standards. Use a 1:1 mixture of CFTR buffer and ATPase buffer to dilute the phosphate stock.

7.2. Incubate both reconstituted CFTR and blank liposomes with 1:100 (v/v) ATPase inhibitors (1.9) on ice for 10 min. Use at least 5 µg of reconstituted CFTR .

7.3. Add ATP (1.9) to a final concentration of 2mM and incubate at 25 °C for 1 h. Stop the reaction by adding 40 µl of 10 % (w/v) SDS (1.9) to each well (including the standards).

7.4. Add 100 µl of buffer A (1.10) and incubate for 10 min. Add 100 µl buffer B (1.10) to each well and measure the absorbance at a wavelength of 800 nm in a 96-well plate-compatible UV/Vis spectrophotometer.

7.5. Convert absorbance at 800 nm into an amount of liberated phosphate using the phosphate standards. Calculate the rate of ATP hydrolysis after subtracting background signal (liposome-only wells) .

7.6. For non-reconstituted CFTR follow the same protocol using CFTR buffer for the background readings.

REPRESENTATIVE RESULTS:

The protocol described above is an efficient means to isolate CFTR-enriched microsomes, with almost complete recovery of CFTR during the cell breakage and preparation of the crude microsomes (Figure 1). Other cell breakage methods may also be employed effectively. We have utilized a French pressure cell, and other high-pressure/cavitation devices (also in combination with impacting against a ruby target) with equal efficiency. For convenience and low initial cost of the equipment, we find the bead-beating method the best.

Using LPG to solubilize and purify CFTR yielded 80 µg protein/L culture at >90 % purity (Figure 2). The high yield was due to efficient solubilization of CFTR by LPG (compare Figure 2b, lanes 2 and 4). In addition, efficient and tight binding to the column resulted in minimal loss of CFTR in the unbound fraction and the absence of CFTR in the wash fractions (Figure 2, lanes 3, 5 and 6). The eluted protein had a purity of >90 %, estimated by Coomassie-stained SDS-PAGE gels and using densitometry of the CFTR and contaminant bands. Gel

permeation chromatography (GPC) separated LPG-purified CFTR from low-molecular weight contaminants (Figure 4, lower panel).

The protocol for CFTR purification using DDM gives purity of about 60 % and yield of roughly 50 µg/L (Figure 3). Electron microscopy (EM) of negatively stained fractions from the GPC eluting at about 10 ml (Figure 4) showed that DDM-purified CFTR contains aggregates of 20-30 nm diameter as well as smaller particles of 10 nm diameter (data not shown). It is possible that the small aggregates can reversibly associate and dissociate as ultrafiltration with a 1 MDa cut-off filter failed to remove the EM-detectable aggregates. LPG-purified material did not adsorb to a glow-discharged grid, hence was studied by cryo-EM of unstained fractions. This showed a very homogeneous particle population of a relatively small size (6-8 nm diameter, data not shown).

Finally, the ATPase activity of the purified proteins was measured (Figure 5). As a member of the ABC protein family, CFTR has two nucleotide-binding domains (NBDs) capable of binding and/or hydrolyzing ATP. The data indicate that the purified protein was not able to hydrolyze ATP in the LPG-solubilized state and showed weak ATPase activity in the presence of DDM (Figure 5, unfilled bars). After the addition of lipids, and detergent removal, ATPase activity was 4-fold higher for samples that had been purified in DDM (13 nmol ATP/min/mg protein). The addition of lipids and removal of LPG similarly restored activity to CFTR that had been isolated using LPG, but with a final lower rate (1.5 nmolATP/min/mg protein) than the DDM-purified and reconstituted material.

Figures and Tables

Figure 1: Monitoring levels of chicken CFTR in cell lysate (CL), supernatants (S) and pellets (P) during various centrifugation steps used for microsome isolation and washing. SDS-PAGE gels were visualized using the in-gel fluorescence of the GFP tag. The supernatant after cell breakage and centrifugation at 14 000 x *g* contains virtually all the CFTR (including degradation products). Ultracentrifugation at 200 000 x *g* sediments all the full-length CFTR leaving some fragments in the supernatant. Ultracentrifugation at 100 000 x *g* of salt-washed microsomes pellets nearly all the CFTR with the removal of some further fragments.

Figure 2: Purification of chicken CFTR in LPG by immobilized metal ion affinity chromatography. Fractions were analyzed by SDS-PAGE followed by Coomassie staining (upper panel) and fluorescence detection of the GFP tag (lower panel). *Tracks:* (1) Microsomes. (2) LPG-solubilized microsomes. (3) Unbound material. (4) Insoluble material. (5) & (6) 40 and 100 mM imidazole washes. (7) Material eluted with 400mM imidazole.

Figure 3: Purification of chicken CFTR in DDM by immobilized metal ion affinity chromatography. Fractions were analyzed by SDS-PAGE followed by Coomassie staining. The left hand panel shows fractions prior to elution. Several consecutive elution fractions are shown in the right hand panel with CFTR indicated by the arrow. Later fractions are enriched in a 40kDa contaminant, which has been identified by mass spectrometry as ribosomal protein L3.

Figure 4: Purification of chicken CFTR by gel permeation chromatography. CFTR purified by Ni-affinity chromatography was concentrated and applied to a GPC column. The elution profile for CFTR (upper panel) purified in buffer containing LPG-14 (solid line) or DDM (dashed line) are overlaid. SDS-PAGE (lower panel) revealed that CFTR eluted between 8 and 11 ml.

Figure 5: ATPase activity of purified chicken CFTR fractions. Protein purified in DDM or LPG was assayed using a modified Chifflet assay [26] in the presence of a cocktail of ATPase inhibitors to eliminate any background ATPase activity from F-, P- and V-type ATPases (unfilled bars). The rate of ATP hydrolysis was also measured after detergent removal and lipid addition (filled bars). The plot shows the mean and standard deviation (*n=3*). Differences between mean values for ATPase activity in presence and absence of lipid, and difference between activity in DDM and LPG are significant to $p<0.05$.

DISCUSSION:

We have previously described a method for the overexpression of murine CFTR [14]. Since the publication of that protocol, we have expressed and purified several different orthologs of CFTR using the same system. All orthologs tested so far purified well in the LPG detergent, whilst the DDM purification showed more variation across different orthologs (data not shown). This flexibility illustrates the strength of the yeast approach: it is possible to screen many constructs with relative rapidity in order to select one for a particular purpose.

Washing the yeast microsomes with buffer containing 1 M NaCl prior to solubilization with DDM results in a cleaner microsome preparation and reduces contaminants at later stages. This step is unnecessary in the LPG protocol as the final CFTR sample is >90 % pure without the microsome wash. Furthermore, purification in DDM requires several alterations to the buffers for solubilization and purification, namely the addition of extra glycerol and salt. Together, these additions considerably increased the binding of the DDM-solubilized protein to the column.

The DDM purification methodology has scope for improvement, in particular the removal of a 40 kDa major contaminant that, judged by mass spectrometry, is due to the yeast ribosomal subunit L3, which appears to have an inherent affinity for the nickel resin. There is no obvious polyHis sequence in the L3 protein, but examination of its 3D structure when bound to the ribosome (PDB= 1FFK) shows that the folded L3 subunit has a potential polyHis cluster. That this band is less problematic in LPG-purified material may be due to the harsher LPG detergent.

Though the purification in DDM appears to be poorer than that in LPG, milder detergents such as DDM may be more compatible with functional and structural analyses and have already been used in several X-ray crystallographic studies of membrane proteins [15-21]. Furthermore, our results indicated that the use of LPG leads to loss of ATPase function in CFTR relative to purification in DDM. Hence we would recommend the LPG-based purification protocol for the generation of CFTR where the purity is crucial, for example in applications such as the characterization of post-translational modifications, or in the generation of antibodies, the LPG-based protocol would be chosen. On the other hand in

applications where the activity and fully native state of the protein is essential, we would propose the DDM-based protocol as a better option.

To conclude, this protocol describes a reproducible method for the isolation of CFTR in the zwitterionic detergent LPG-14 or the non-ionic detergent DDM. As such it indicates a greater range of purification conditions for CFTR than have previously been reported[10-13]. In addition milligram quantities of purified CFTR can be obtained using these procedures when combined with a high volume yeast growth system such as a 20 L fermenter and a high capacity cell harvesting system such as a 6 L low speed centrifuge rotor. The CFTR obtained has a cleavable GFP tag which allows easy monitoring of the protein in various biochemical and biophysical assays.

The reagent described in this manuscript (chicken CFTR–containing plasmid or frozen yeast cells) can be obtained through the Cystic Fibrosis Foundation (USA).

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DISCLOSURES

The authors have no competing financial interests nor other conflicting interests with respect to this work.

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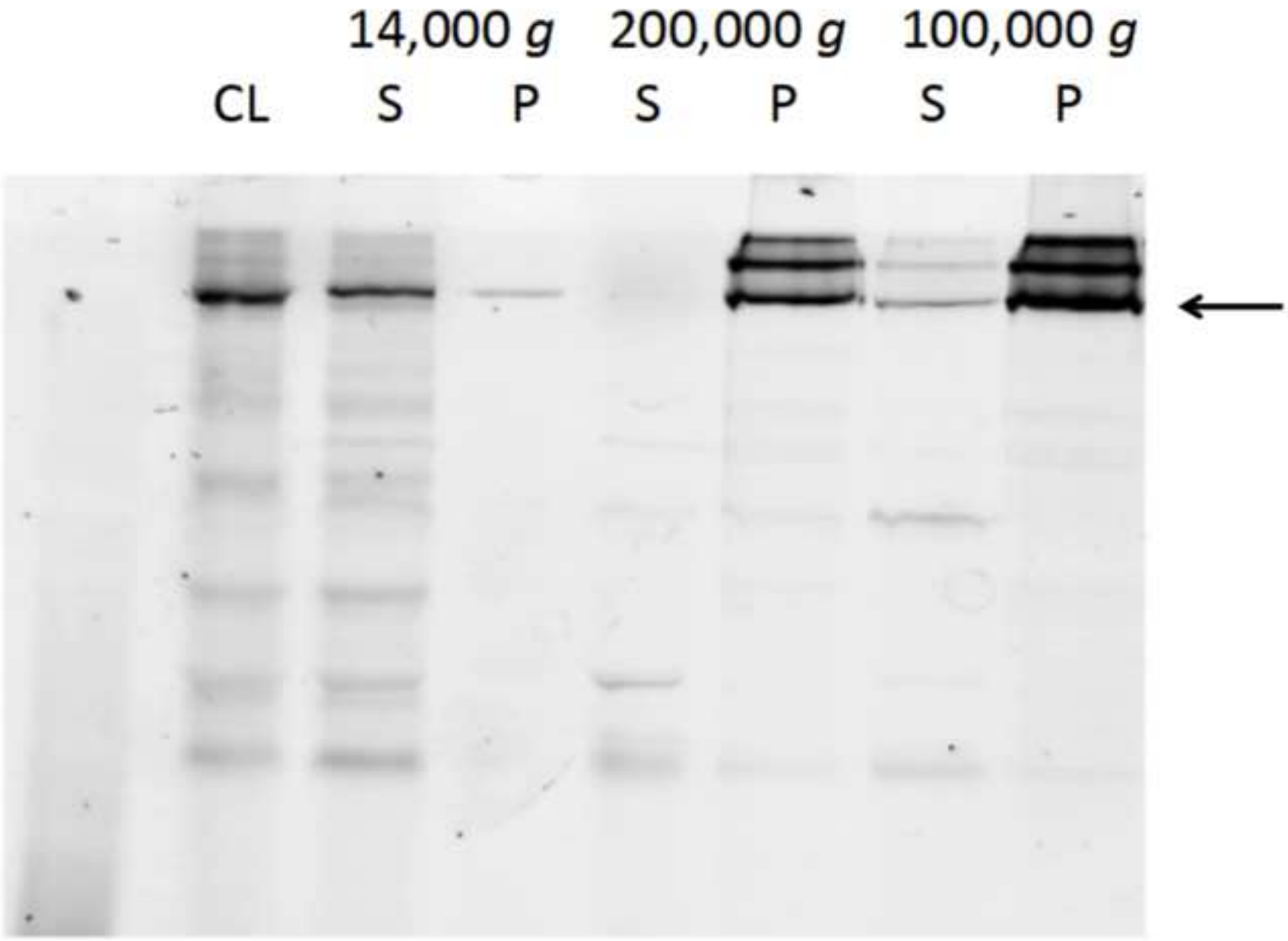

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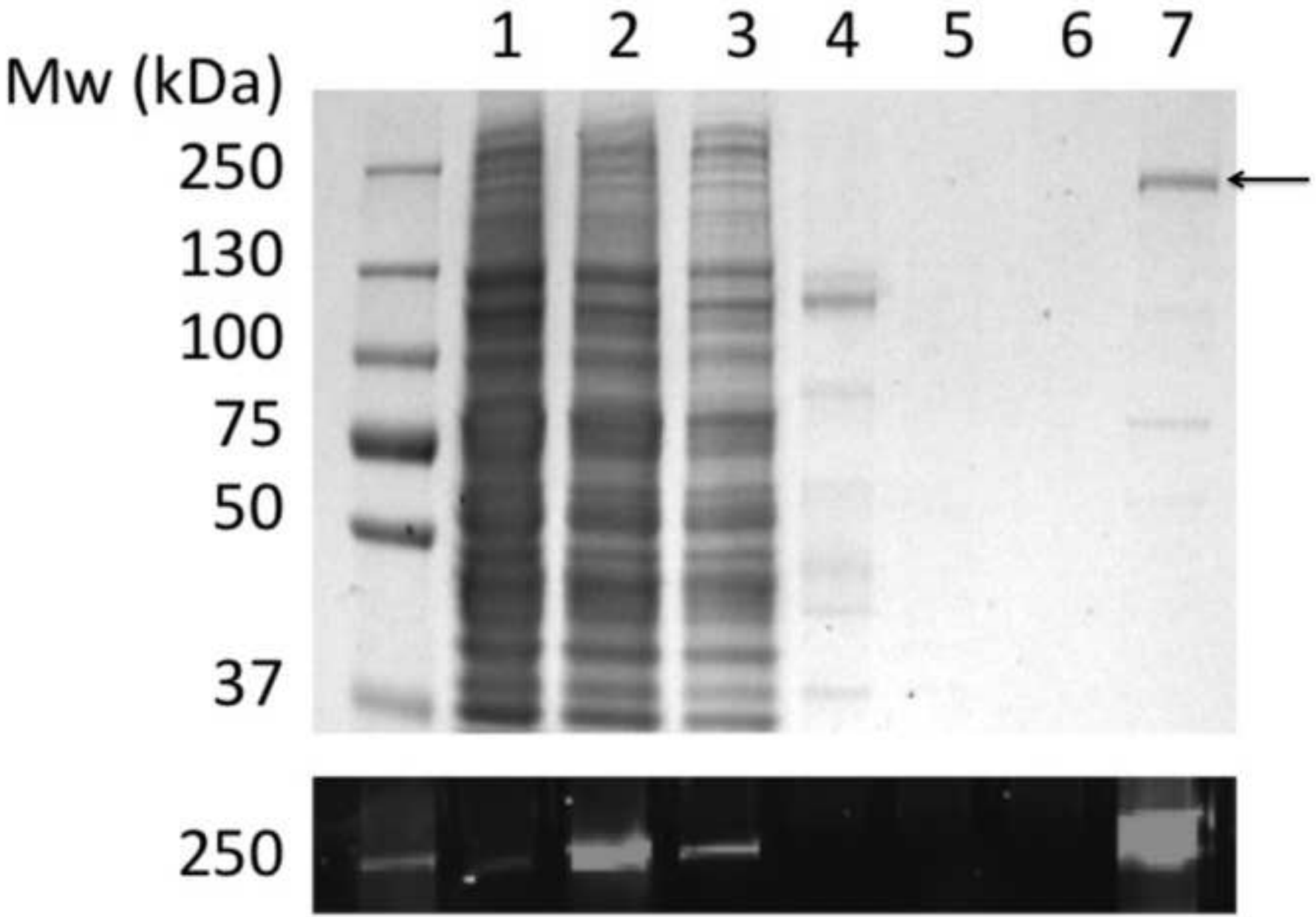

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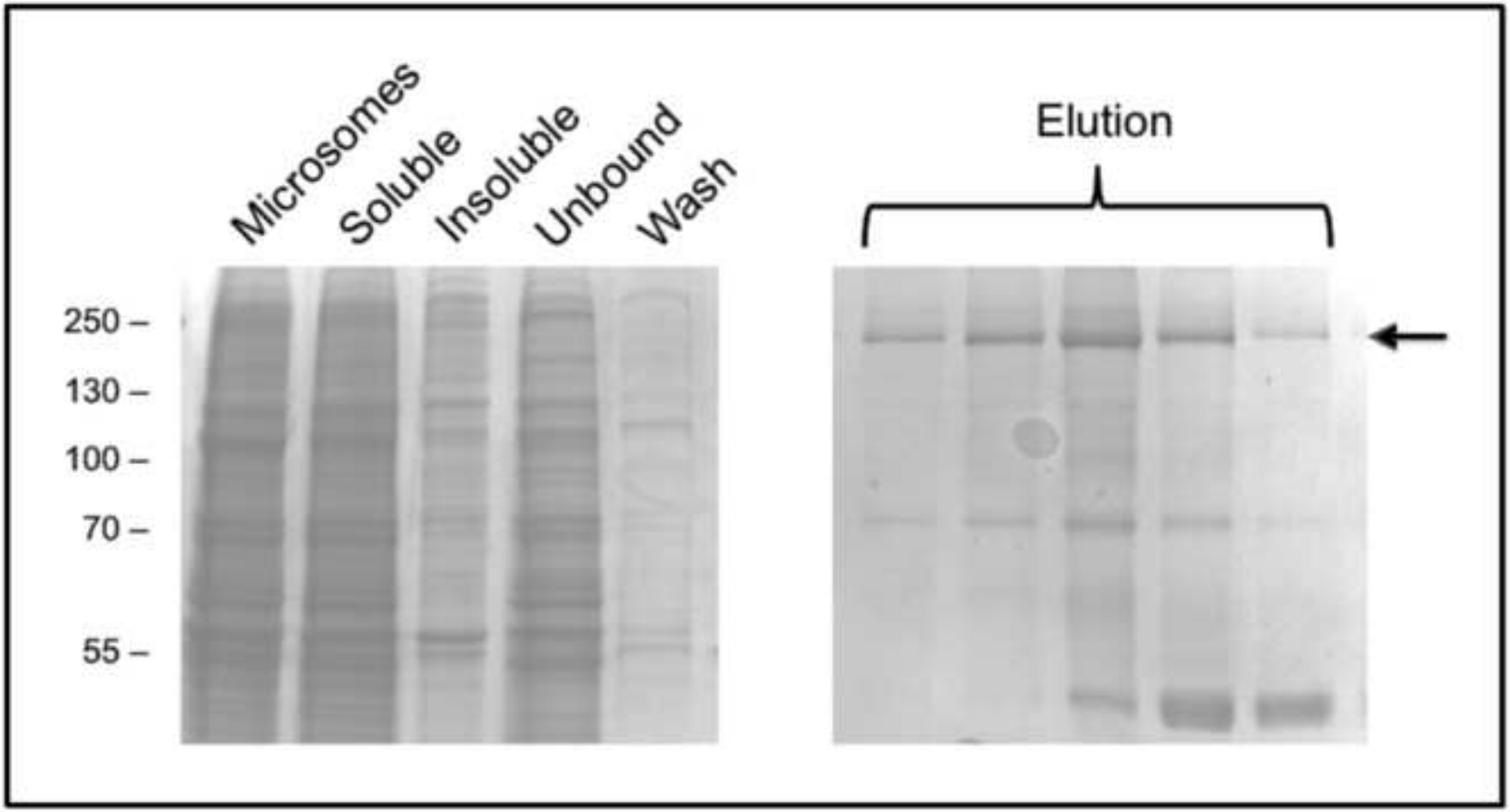

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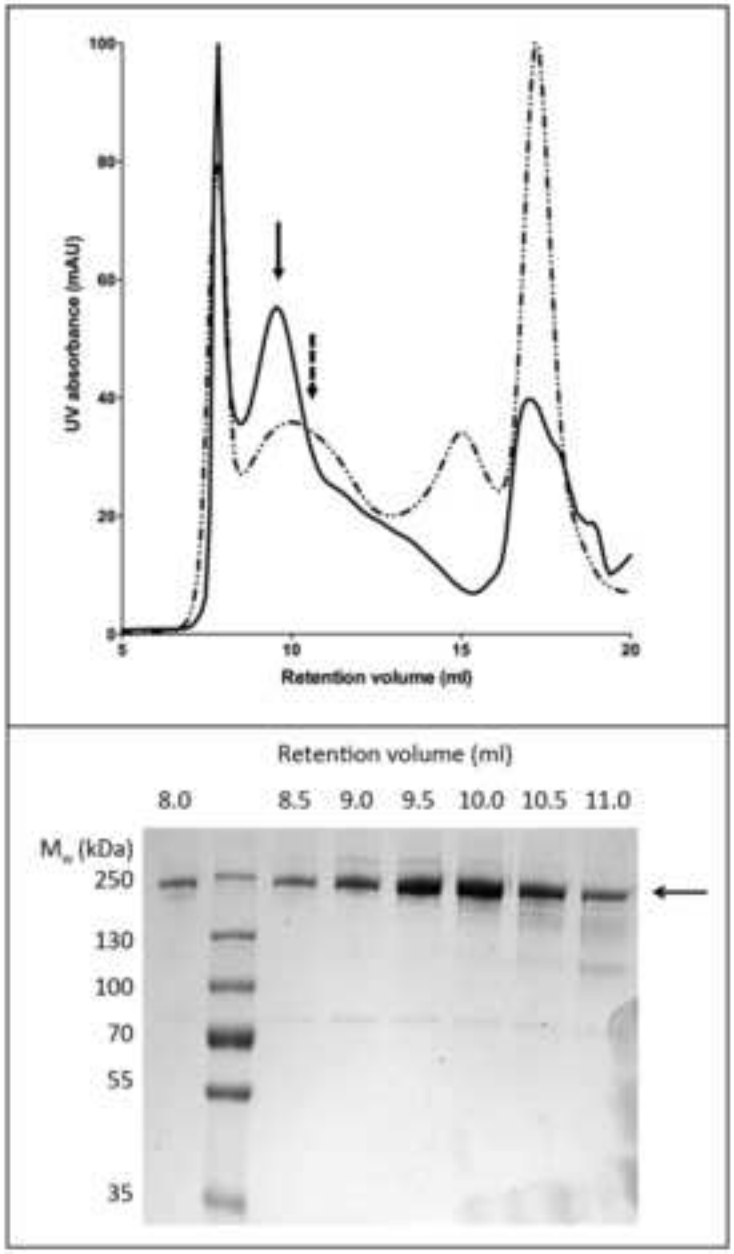

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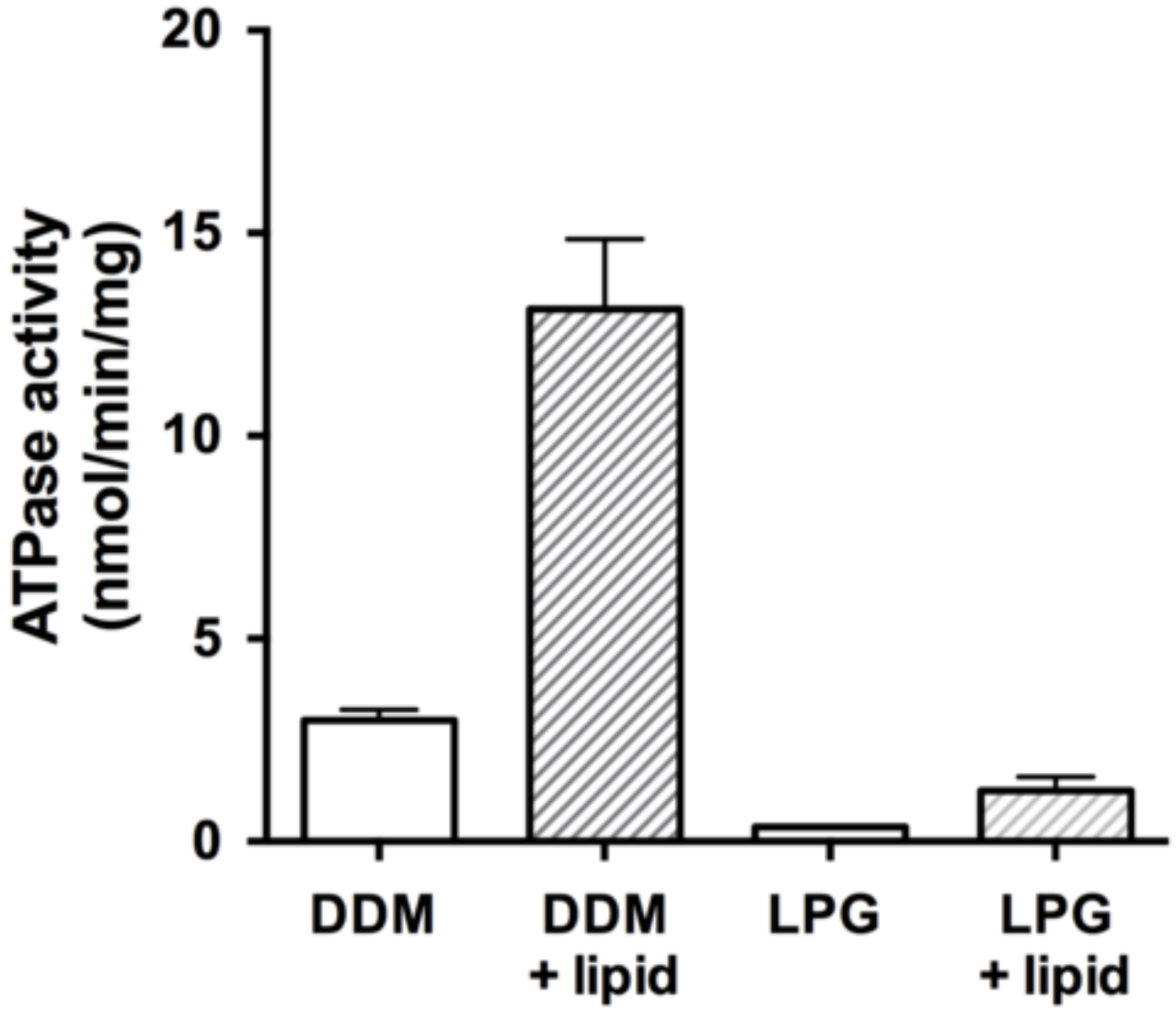

*Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: CFTR_Purification_JoVE_Materials-3.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.2µM syringe filter	Sartorius	FC121	
100 kDa MWCO centrifugal concentrator (PES membrane)	Vivaspin	VS0641	
2ml microfuge tubes	Sarstedt	72.695	
40Ti rotor	Beckman Coulter	337901	
50ml Sterile Falcon Tubes	Sarstedt	62.547.254	
Adenosine triphosphate disodium salt (Na_2ATP)	Sigma-Aldrich	A26209	
Liquid chromatography system	GE Healthcare	28-4062-64	
Aminoethylbenzenesulfonyl fluoride (AEBSF)	Sigma-Aldrich	A8456	
Glass bead-beating cell disrupter	BioSpec	1107900	
Benchtop centrifuge	HERMLE	Z300	
Benchtop centrifuge	Eppendorf	5417R	
Benchtop microfuge	Fisher	13-100-511	
Benzamidine hydrochloride	Sigma-Aldrich	434760	
Hydrophobic Beads SM-2 Adsorbent	BioRad	152-3920	
Bromophenol blue	Sigma-Aldrich	114391	
Centrifuge tubes	Beckman Coulter	357000	
Gel imaging system	BioRad	170-808	
Cholesterol	Sigma-Aldrich	C8667	
Chymostatin	Sigma-Aldrich	C7268	
Dimethylsulfoxide (DMSO)	Sigma-Aldrich	D8418	
Dithiothreitol (DTT)	Sigma-Aldrich	43815	
E.coli total lipid extract	Avanti lipids	100500	

Epoxysuccinyl-leucylamido-butane (E-64)	Sigma-Aldrich	E3132
Glass beads, acid washed	Sigma	G8772
Glycerol	Fisher	65017
HisTrap HP columns (5 ml)	GE Healthcare	17-5247-05
Rapid Coomassie Stain	Novexin	ISB1L
Centrifuge JA-17 rotor	Beckman Coulter	369691
Leupeptin	Merck	108975
Lyso-phosphatidyl glycerol-14 (LPG)	Avanti lipids	858120
$MgSO_4$	Sigma-Aldrich	M7506
Gel tank SDS-PAGE system	BioRad	165-8006
n-dodecyl-β-D-maltopyranoside (DDM)	Affymetrix	D310S
NaCl	Sigma-Aldrich	S6191
NaN_3	Sigma-Aldrich	S2002
NH_4Cl	Sigma-Aldrich	A9434
Oligomycin	Sigma-Aldrich	75351
Ultracentrifuge	Beckman Coulter	392050
Prestained protein standards	Fermentas	SM1811
Desalting columns (Sephadex G-25)	GE Healthcare	*17-0851-01*
Pepstatin A	Sigma-Aldrich	P4265
Phenylmethanesulfonylfluoride (PMSF)	Sigma-Aldrich	P7626
Sch28080	Sigma-Aldrich	S4443
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	L37771
Sodium thiocyanate (NaSCN)	Sigma-Aldrich	251410
Gel filtration 10/300 GL column	GE Healthcare	17-5172-01
Tris-base	Formedium	TRIS01

Ultracentrifuge tubes	Beckman Coulter	355618
Vortex mixer	Star Labs	N2400-0001
Ultrasonic water bath	Ultrawave	F0002202
Multimode plate reader	BioTek	BTH1MF

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Clarification of Changes and Responses to Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript entitled "Purification of Cystic Fibrosis conductance Regulator" is a very clearly presented protocol for preparation of very pure CFTR produced in S. Cerevisiae (90% purity). This protocol will be very useful in the field to prepare antibodies against the whole protein and to perform some structural studies.

I have some remarks that I would like the authors to answer.

Major Concerns:

1. Along the manuscript it is unclear which CFTR orthologs are presented. This should be mentioned at least in the figure legends.

The figure legends have been changed to address this concern.

2. The functional assay used to validate CFTR purification is an ATPase assay. Did the authors try to check the Cl- channel activity? For example, any assays using valinomycin as "voltage-clamp " could help (in the presence of a chloride gradient with vesicles containing reconstituted CFTR, PKA-dependent fluxes or PKA-dependent volume regulation could be evaluated). Alternatively, bi-layer experiments could be used.

We have been able to reconstitute the CFTR into lipid vesicles for ATPase measurements, but the vesicles are, so far, too leaky to be able to measure channel activity/ion flux as suggested by the referee. This may be a case of optimizing the lipid composition and reconstitution protocol, but until now we have been focused on purification of the protein. However the single channel gating properties of the CFTR construct (ie codon optimized and with GFP and Sumo tags) have been measured by collaborators using fusion of microsomes containing CFTR (about 1% total membrane protein) to a black lipid membrane system. The CFTR gating properties are very similar to the non-codon optimized and untagged versions of the CFTR protein orthologs, which may go some way to addressing the concern of the referee (Riordan, J., Urbatsch, I and Kappes, J., unpublished data).

Minor Concerns:

3. On which basis were detergents presented? A figure showing solubilization levels with other detergent could be informative for the reader. I would propose to add also a Table with the properties of tested detergents, including structure, CMC values, and other parameters.

A short paragraph describing the rationale for choosing the two detergents is now included (lines 111-117).

4. Point 1.3 : How much PI needs to be added to solubilization buffers (in µL)?

This detail has now been added (line 126). All PI additions are 1:100 v/v dilution into buffer for solubilisation or purification steps.

5. Point 1.3 : what type of sonicator was employed? Please add this information to the Table of specific equipment.

This detail has been added to the table.

6. Point 1.6: it seems that GPC is not defined in the text.
GPC is now defined (line 378)

7. Point 1.10: Did the authors try to use MgATP? If yes, please include the information why Na2ATP is used.

This is now explained (line 188) – MgATP is less pure and has phosphate contamination that will overwhelm the Chifflet assay. Hence one uses Na2ATP, which is purer and adds MgCl2 separately to the assay.

8. Point 2.8: What is the minimum volume? Provide the range, please.

This is now indicated (line 241).

9. Point 6.3: It is not clear for a non-experienced reader why Biobeads are used.

Changed to absorbent beads and explained what these are doing (line 328).

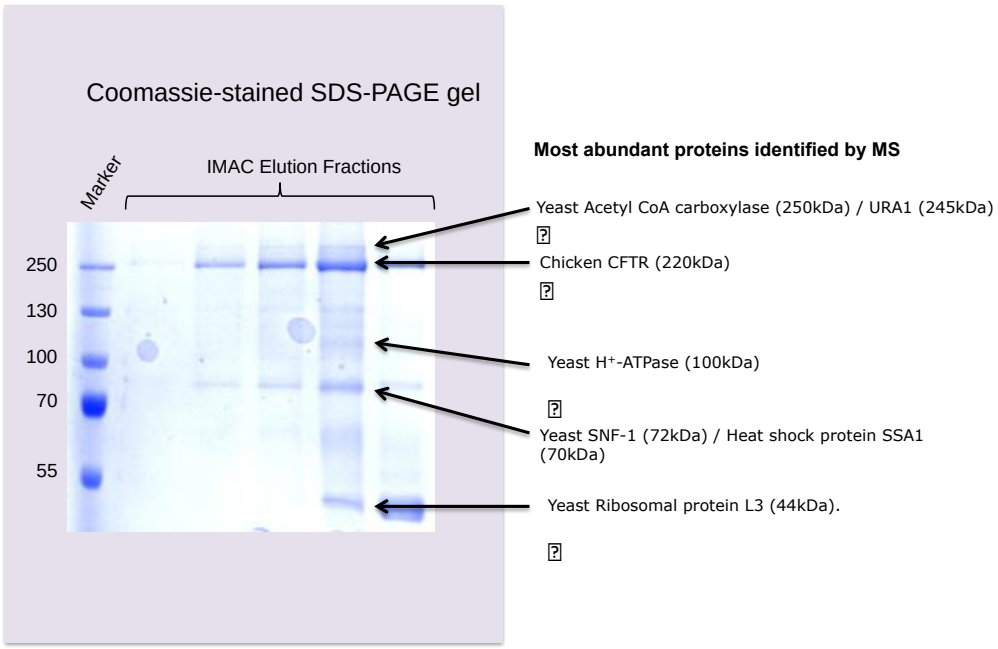
10. "Chiffled assay" should be described.`

A step-by-step protocol is now included (line 345 onwards). Although the Chifflet assay is long since published, the adapted protocol is for a 96 well plate format, so it is worth including.

11. The statement about the chicken ortholog is not supported by the data in the manuscript.

12. Have the authors used orbitrap or a mass spectrometer of equivalent quality to check how many proteins there are in the "CFTR" band?

We have employed MS to check for contaminating proteins and to confirm the identity of the 220kD Coomassie band. Mass spectrometry data when searched against the chicken genome identifies CFTR as the only hit. The figure below summarises the MS data for the DDM-based purification procedure. When searched against the yeast genome, MS data has identified ribosomal subunit L3 as the major 40kDa band contaminating the DDM-purified CFTR material (line 491) along with some other yeast proteins (as below). The L3 protein is also present in the much purer LPG – purified CFTR preparations, but with much lower abundance (as judged by number of peptide fragments detected as well as the relative intensity of the band on a Coomassie stained gel).



13. Figure 1: "Kg" labeling is misleading: "kilograms"? Please, write "14000 g" as it is written in the legend.

This has now been changed throughout.

14. Figure 2 and 3: have the authors done mass spectrometry analysis on lanes 1 to 7 in fig 2 and in the "elution" bands? If not, I would suggest to do it.

See above explanation. It has been done.

15. Figure 4: please indicate with an arrow the CFTR peak.

This has now been added to the Figure.

16. Figure 5: please indicate the number of experiments, indicate if bars are SD or SE, and provide statistics.

This has been added to the legend (line 469).

17. Please precise in the discussion what are the differences in the protocol as compared to the previous publication (ref 3).
A short additional discussion is added. We assume the referee meant ref13 rather than 3 (which is a review article in Lancet).

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
The manuscript by Pollock et al describes the detailed purification procedure of the mammalian ABC transporter CFTR. Cystic fibrosis is a lung disorder and links with mutations in the CFTR ABC transporter gene have been identified. The development of drugs specific to CFTR require pure, active protein for functional characterisation and structural studies. The authors provide a very detailed step-by-step procedure of their lab's purification procedure in two structurally different detergents (DDM and LPG14); subsequently they show that their protocol results in pure and active protein in both detergents (with DDM more active than LPG-14) as determined by the ATPase assays. This protocol is the natural follow up paper from the original expression paper published in JOVE by the same lab (O'Ryan, L. et al 2012). The two papers together will be of great help for researches working on the CFTR field, as well as groups on other mammalian ABC transporters. In addition, the combination of the yeast expression system with this well defined purification protocol will mainstream the production of CFTR for structural and functional studies.

Major Concerns:
Line 49: A brief comment on why the authors used LPG-14 or a reference on its successful use with other membrane proteins.

This has been included + two extra references (line 115).

Line 277: " The rate of CFTR-specific ATPase activity can determined using the Chifflet assay 17,18..." : Since this is a detailed protocol for the production of CFTR for structural and functional studies, it will be very helpful to provide a detailed protocol for the Chifflet assay. Even though the assay is well referenced by the authors (references 17,18) , it will be handy to have the functional characterisation procedure in this manuscript, as a complete protocol.

This was also requested by referee 1 and we agree it is important and have added detail for this.

Line 378: "Hence we would recommend the LPG-based purification protocol for the generation of CFTR where the purity is crucial but the activity of the protein is less important." : I think this statement should be removed or amended. Even though the authors comment that the less active protein could be used for antibody production or post-translational modification studies, the sentence does not read well in a methods paper in its current format. Activity is always crucial and most researches would put great effort to gain active protein over inactive. They also need to specify if these antibodies will be used for structural

work or detection in a westerblot. If the antibodies to be raised are for structural work, they will obviously want to use CFTR that is active.

Agreed. LPG-purified material still displays activity, just less activity than DDM-purified material. It is plausible that this reflects a harsher detergent, but it is also possible that CFTR is better conserved in LPG and displays a more regulated ATP hydrolysis activity. (line 423)

Minor Concerns:
Line 128: "...with approximately the equivalent amount of microsomes." : It is not very clear if the authors mean volume of concentration. It is better to state the amount of microsomes in mg.

This has been changed (line 241)

Line 131: "For GPC buffer containing..." : What does GPC abbreviate for?
This has now been defined (line 304)
Line 177: "If the microsomes are intended for purification using DDM, supplement the CFTR buffer with 1 M NaCl" and line 184: "...(or CFTR buffer plus 1M NaCl if DDM will be used later)": Not necessary since it is repetition from paragraph 1.5
Agreed, now removed, line 242.
Line 203: "Decant the supernatant containing soluble proteins...": The sentence should read: "Decant the supernatant containing the solubilized membrane proteins..."
Changed as suggested, line 261.

Line 291: "...Superose6 GPC column in a discrete peak...": Rewrite sentence. What do the authors mean by discrete peak?

This has been clarified/simplified (line 470).

Line 294-302: Have the authors performed cryo EM on the DDM solubilized CFTR? It is not uncommon for membrane proteins to aggregate on the EM grids upon addition of the negative stain?

We have attempted cryo EM with DDM-purified CFTR, however the protein concentrations achievable in this detergent are currently too low for cryo EM specimens. The referee's insight is useful though, and we will attempt a comparison between unstained and stained CFTR in the future.

Line 305: "...capable of binding and/or hydrolysis of ATP..." : Bad grammar, should be: "...capable of binding and/or hydrolysing ATP...

Agreed, and changed. Line 422

Line 392: "The chicken CFTR reagent can..." : I suppose the authors mean the gene, please rewrite. Also provide an accession number for the gene or plasmid associated with the Cystic Fibrosis Foundation.

This is now clarified, line 537.

Figure 4: Use an arrow or asterisk to show CFTR peak.

Now included in the figure.

Correct abbreviations for references following JOVE guidelines.

Additional Comments to Authors:
N/A

Reviewer #3:
Manuscript Summary:
The manuscript describes the method of purifying CFTR from yeast. Description is detailed enough to be reproduced in any laboratory. Results are convincing, and invite others to do experiments with this preparation.

Additional Comments to Authors:
Disruption of yeast cells is done with a bead mill. This instrument is not available in all labs. It would be interesting to suggest alternatives, as well as mention those disrupting methods that cannot be used - for example, sonication could be a good method, but not all proteins resist it.

We have added a few lines on this subject. Sonication has not been useful in our experience with yeast-expressed CFTR, but may well work with alternative sonicators or settings.