**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orthologs, which may go some way to addressing the concern of the referee (Riordan, J., Urbatsch, I and Kappes, J., unpublished data).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.