**Response to Reviewers**

We thank the reviewers for their helpful and constructive comments. Below we have addressed point by point all issues raised by the reviewers. The newly introduced/modified text is underlined in the resubmitted manuscript.

**Reviewer #1:**

*The manuscript by Kreiter et al. deals with the elaboration of the electrophysiological technique for the reconstitution and functional characterization of the reconstituted carrier proteins. The results are sound, the manuscript is well written and quite interesting.*

We thank the reviewer for the positive feedback.

*1) I have however, several comments which can potentially improve the impact of the work. 1). I did not find the description of the reference electrode preparation in the manuscript. It seems that the authors did not use it. I think that it is necessary to test the effect of the agar bridge presence on the reference electrode on the measurements of reverse potentials. Since the micro agar salt bridge improved substantially the results of the measurements, the presence of the agar bridge on the reference electrode may also be helpful.*

We thank the reviewer for addressing this point. Indeed, if substrates would be added directly to the buffer reservoir containing the reference electrode, the usage of the salt bridge would balance substrate-related diffusion potential. However, we used buffer solution which is chloride-free to exclude the putative possibility that uncoupling proteins transport chloride ions. For the adjusting of pH we used Tris or MES. The electrode potential in the absence of substantial concentration of chloride is primarily dependent on chloride impurities in the buffer solution. As its composition is unchanged during the experiments it will simply result in a constant offset potential. However, for a different situation where it is required to obtain an absolute potential difference between the two electrodes, a simple agar salt-bridge system (Ag/AgCl 3 M KCl) could also be used for the reference electrode.

We made a corresponding comment in the revised manuscript (underlined).

*2). The manuscript would benefit if the authors describe in more details the background and the meaning of the equations which transform the measurable parameter, a reverse potential, into another parameter, i.e. proton turnover rate. For example, it is hard to understand the*

*relationship between the proton turnover rate and delta_T which is a time parameter of the triangular alternating voltage applied.*

We thank the reviewer to mention this point. The more detailed deviation of the conversion of the measurable parameter, reverse potential, to the turnover number is described in Urbankova et al (2003) JBC and Beck et al (2007) FASEB. We adjusted the symbols to $\Delta T_{ramp}$ to indicate the time constant of the voltage ramp in step 3 in order to distinguish it from the time unit of the substrate turnover rate.

*3). I suggest to run control measurements with a protonophore (i.e. CCCP), K+-carrier (i.e. nonactin), and H+/K+-channel (gramicidin) which can validate the approach.*
We appreciate the suggestion of the reviewer. Our main goal in this study was to develop a salt bridge for the rapid and stable determination of the substrate turnover number and we therefore did not focus on reverse potential measurements and conversions to substrate turnover rates per se, as it was already established in Urbankova et al. 2003. Proposed experiments will substantially confirm the method, but are out of the scope for this work.

**Reviewer #2:**
*This manuscript clearly demonstrates that using such a 'novel micro agar salt bridge electrode configuration' gives vastly superior results compared to the large inaccuracies that resulting from the use of a simple Ag/AgCl electrode as the test electrode for analysing proton turnover rate of membrane proteins reconstituted in the lipid bilayer membrane. The problem with the use of a simple Ag/AgCl electrode results from the fact that its electrical potential in a salt solution is highly dependent on the Cl- activity in the solution (e.g., Eqs. 1 & 2 in Raynauld & Laviolette, 1987 paper) and that in the absence of a significant Cl- concentration, the electrode potentials are poorly defined and unstable. This creates a major problem at the internal membrane interfacial solution in the absence of a significant Cl-, and where the solution composition is being altered. The problem is essentially overcome with the use of this novel 3M KCl micro-agar electrode, as described in this JoVE manuscript.*
Thank you for the positive comment.

*There are some relatively minor suggestions, comments and questions, which the authors should take note of:*

*1. With respect to the Ag/AgCl electrode in the large external Cl- -free buffer solution, there should also be some small comment indicating that while its electrode potential is poorly defined and primarily dependent on chloride impurities in that solution, as its composition is unchanged during the experiments, it will simply result in a constant offset potential, during an experiment. However, for a different situation where it was required to obtain an absolute potential difference between the two electrodes, a simpler Ag/AgCl 3 M KCl agar salt-bridge system could also be used for the reference electrode.*

We appreciate the advice of the reviewer and introduced the following comment to the text of the article.

Since uncoupling proteins were suggested to transport chloride ions, chlorid- free buffer solution was used and the pH was adjusted using Tris or MES. The electrode potential in the absence of substantial concentration of chloride is primarily dependent on chloride impurities in the buffer solution. As its composition is unchanged during the experiments it will simply result in a constant offset potential. However, for a different situation where it is required to obtain an absolute potential difference between the two electrodes, a simple agar salt-bridge system (Ag/AgCl 3 M KCl) could also be used for the reference electrode.

*2. With respect to the micro agar salt bridge on Line 77, I was surprised that the agarose concentration used was only 1%, as the more standard concentration in most salt-bridges 3-4% (w/v) agarose is used (Barry et al., 2013, Eur Biophys J 42-631-646; cp Kleene, 1993, who even used 5% agarose for his salt bridges) and I wondered if the salt-bridge in this JoVE manuscript is sufficiently gelled to retain the agar in the salt bridge for a reasonably long time.*

We thank the reviewer for addressing this point. Indeed, the concentration of agarose in the salt bridge is lower than compared to the before mentioned references. Since our measurements are relatively short (less than 5 minutes) and we prepare new salt bridges for each new measurement, the usage of 1 % (w/v) agarose is enough to gel the salt bridge in this short period. Generally we suggest using agar in a concentration range of 1 – 5% (w/v), depending on the time scale of the

measurements and how often the salt bridge is reused. We also introduced the recommended citations.

*3. I found the statement that the 'salt ions K+ and Cl- have equal mobility in liquid' (Lines 316f) was strictly inaccurate and misleading with respect to the role of 3 M KCl at such junctions. The relative mobilities of the Cl- and K+ ions uCl/uK = 1.04, and 3 M KCl microelectrodes or salt-bridges are useful for minimizing liquid junction potentials (LJPs), because of the high concentration of the 3 M KCl and because the mobilities of the Cl- and K+ ions are similar [u(Cl)/u(K) = 1.04] BUT NOT EQUAL , so that the 3 M KCl LJP contribution swamps the effects of the diffusion of the solution ions into the 3 M KCl agar salt bridge and will be relatively solution independent. If the Cl- and K+ mobilities had been equal, there would be no contribution from the 3 M KCl salt -bridge and the solution ions alone diffusing into the salt-bridge would by themselves contribute to the LJP at that junction, so that the LJP would be solution-dependent and could even be large.*

We apologize for the inaccuracy regarding the mobilities of the ion species. The values are of course not equal but similar. We have corrected the phrases in the manuscript (underlined) to avoid incorrect statements.

*4. Fig. 1a. With respect to the precise construction of the Microload pipette, it is difficult to see from Fig. 1A, the precise relationship between the chlorided part of the Ag wire and the part of it in contact with the 3 M KCl agar-Ag/AgCl region (compare Fig. 1 in Shao & Feldman). It could also help to replace 'Agar - Salt' by 'Agar - 3 M KCl' in Fig. 1A).*

We have made changes to Fig. 1 A so that the contact side of the chloride part of the Ag wire and the 3 M KCl agarose solution is outlined more clearly.

*5. Section 3.1 (Lines 191-195). It would be more accurate to define ΔT as "...and the duration of each ramp as ΔT, calculate…" especially, as 'time constant' tends to have a well-defined usage in electrophysiology and electricity. I also take it that Umax = 100 mV?*

We have defined the parameters of the voltage ramp - the maximum voltage $U_{max}$ (50 mV) and the time of the ramp $\Delta T_{ramp}$ - more clearly.