BEC BIOENERGETICS COMMUNICATIONS Bioenerg Commun 2021.4.r1 doi: 10.26124/bec:2021-0004.r1
Open peer review 1 Open Access

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Facts and artefacts on the oxygen dependence of hydrogen peroxide production using Amplex UltraRed

Authors: Timea Komlódi, Ondrej Sobotka, Erich Gnaiger

Manuscript submitted 2021-11-09, 2021-12-17 (revision)

Manuscript accepted 2021-12-17
doi:10.26124/bec:2021-0004

Published online 2021-12-21

Reviewer 1

Alba Timon-Gomez

Department of Neurology, University of Miami Miller School of Medicine, Miami, US

Manuscript reviewed 2021-11-27
doi.org/10.26124/bec:2021-0004.r1

Reviewer 1

"This paper collects in a well-organized and logical sequence important experimental evidence on the advantages of the respiratory medium MiR05 when performing the Amplex UltraRed (AmR) assay, compared to other media. The authors studied the background fluorescence slope of the AmR assay in different media, and the oxygen dependence of H_2O_2 flux, using yeast as a model system. They showed a hypoxic peak in the fluorescence signal only when yeast cells were incubated in DPBS or KCl-medium, but not in MiR05; suggesting that this peak was a medium-dependent background artefact. Furthermore, H_2O_2 production decreased following a linear function with oxygen concentration. These results allowed to apply an accurate background fluorescence correction in MiR05. The paper is well written and the conclusions are supported by a number of independent, well-designed experiments, displayed in the figures and supplementary material.

The manuscript might be improved adding the number of technical and biological repeats in the figure legends. Besides, in Figure 5, when assessing the fluorescence slope in the absence and presence of yeast, it was not clear when the experiments were performed in the presence of yeast. It would be very helpful to state it in the figure legend, to facilitate the comprehension of the reader without going back and forward in the text. Also, it is confusing in Figure 5 when comparing DPBS (Fig. 5a) vs Mir05 (Fig.5c and 5e) why results were in one case shown at 3 different constant oxygen concentrations (Fig. 5a) whereas in the other it is not clearly indicated which are the oxygen concentrations (If it is as Figure 2f, the experiment is only performed in two oxygen concentrations). A line explaining the rationale of this experimental design (and

the logic of the differences between both media) would improve the understanding of the reader."

Authors

1.1.- We added the number of technical repeats *n* to the figure legends. We added to the Methods section: "Independent preparations are indicated as separate experimental days in the figure legends."

1.2.- We split Figure 5 into two separate figures (new Figures 5 and 6). The new Figure 5 shows background only. The absence (background) or presence of yeast is indicated in the new Figure 6.

1.3.- In the new Figure 5a and b, the background fluorescence slope was measured at constant oxygen concentration in DPBS (see new legend Figure 5 a and b), while in the new Figure 5c and d the oxygen concentrations were changed within each technical repeat (representative trace in Figure 4c, new legend Figure 5c).

1.4.- The rationale of the experimental design is now more explicitly stated in an introductory sentence to section 3.2.