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A Mitochondrial Reference Assay for High-Resolution Respirometry Using Freeze-Dried Baker's Yeast.

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A mitochondrial reference assay (mtRA) was designed for high-resolution respirometry, to evaluate the reproducibility of cell respiration with baker's yeast (*Saccharomyces cerevisiae*). Quality control is an integral part of quality assurance (QA), which is becoming increasingly important in high-resolution respirometry for basic and clinical research, as well as industrial applications. Quality control will ensure comparability of quantitative results obtained by different research groups.
Baker's yeast is an anhydrobiotic organism which is very well known for its persistence without water for decades. When rehydrated, it can rapidly restore active metabolism within minutes [1]. In addition, dried baker's yeast is well known for its high level of viability prepared when rehydrated at 30 to 40 °C [1,2]. In the present study, commercially available, freeze-dried baker's yeast (2 mg dry weight/ml) was rehydrated in Na-P buffer at 30 °C (pH=8.1; catalase at 140,000 Units/ml; 6 µl stock solution in MiR05 added to 3 ml Na-P buffer). Vortexing for 7 min at 2200 rpm was necessary to obtain a homogenous cell suspension, immediately prior to adding a subsample of 50 µl into the 2 ml Oxygraph-2k chamber (0.05 mg/ml final cell density; 37 °C). Since the cells sediment rapidly, it was necessary to vortex the stock cell suspension again for 1 min prior to addition into the next chamber. Time of 15-20 min is required to distribute 50 µl aliquots of yeast cell suspension to 8 chambers (4 Oxygraph-2k).
The mtRA protocol includes an initial 20 min period after addition of cells into the oxygraph chamber. ROUTINE respiration (*R*, the mitochondrial coupling state in the intact yeast cells; lot L47) stabilized within 15 min and was quantified during 5 more minutes (Fig. 1). Then glucose (20 mM) and ethanol (2%) were titrated to stimulate respiration by these substrates, and in particular to evaluate the effect of ethanol used as a carrier for the uncoupler FCCP. Step-wise addition of FCCP (carbonyl cyanide *p*-(trifluoro-methoxy) phenul hydrazone) was applied to obtain non-coupled respiration as a measure of electron transport system (ETS) capacity (*E*), Finally, cytochrome *c* oxidase was inhibited by azide-inhibition to quantify residual oxygen consumption (ROX). *R* and *E* were corrected for ROX (4.9±0.4 pmol·s^{-1}·ml^{-1}; SD; *n*=8). Average volume-specific oxygen flux in state *R* was 65±3 pmol·s^{-1}·ml^{-1}. Under these experimental conditions, O_2 concentration would change from 174 µM to anoxia within 45-60 min, making re-oxygenations necessary (Fig. 1). Glucose-stimulated ROUTINE flux was 71±3 pmol·s^{-1}·ml^{-1}, but ethanol stimulated ROUTINE respiration to 100±3 pmol·s^{-1}·ml^{-1}. ETS capacity was 119±5. Maximum non-coupled flux (*E*) was obtained at a high concentration of 75 µM FCCP. Under conditions of ROUTINE respiration stimulated by glucose and ethanol, the *R/E* ratio was 0.84±01, indicating that substrate-stimulated ROUTINE respiration

operates at 84% of ETS capacity. Endogenous ROUTINE respiration (in buffer without exogenous substrates), however, was only 55% of ETS capacity with glucose+ethanol addition.

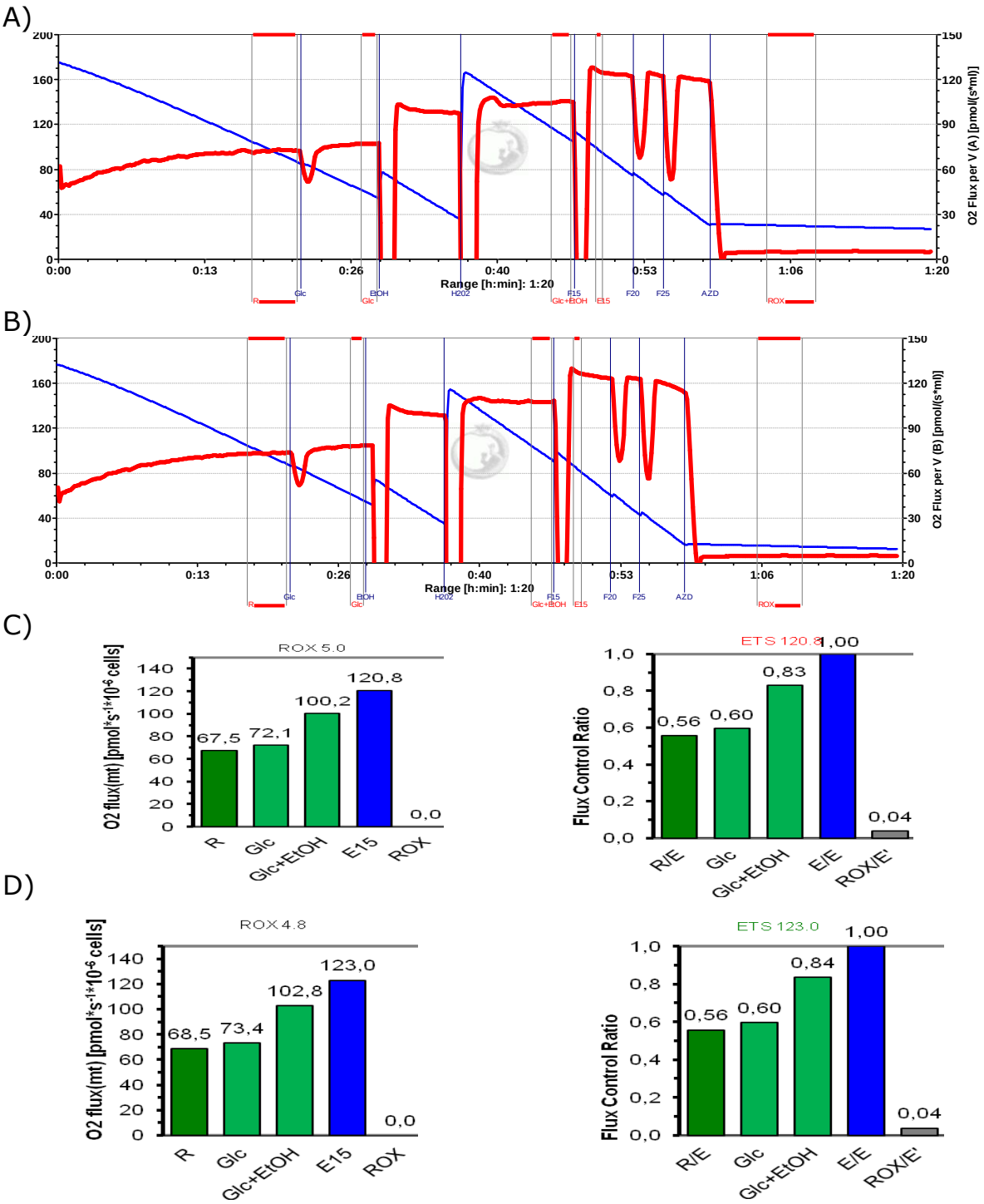

Fig. 1. Parallel replicate experiments in two O2k chambers with baker's yeast cell suspension in Na-P buffer, pH=8.1. **A** and **B**: Oxygen concentration [µM] and oxygen flux per volume [pmol·s^{-1}·ml^{-1}] corrected for instrumental background. Bars represent marked sections of the experiment for calculation of average fluxes and export to generate tables and bar graphs (**C** and **D**).

Group		R1	R2	R3	R4	R5	R6
pH 7.1, RT	*mean*	46.64	53.15	53.91	50.61	48.14	45.97
	SD	3.62	3.34	3.34	3.45	3.56	3.23
	n	3	3	3	3	3	3
		R1	R2	R3	R4	R5	R6
pH 7.1, 0°C	*mean*	48.58	55.73	57.82	54.11	52.14	49.72
	SD	1.11	1.46	2.61	3.64	3.83	3.18
	n	3	3	3	3	3	3
		R1	R2	R3	R4	R5	R6
pH 8.1, RT	*mean*	44.08	43.92	41.70	39.30	35.74	33.80
	SD	2.04	1.70	1.39	1.85	2.39	2.46
	n	3	3	3	3	3	3

Table1. ROUTINE respiration of Baker's yeast (L77) at pH 7.1, with short-term storage at RT versus ice, and at pH 8.1 with short-term storage at RT. pH 7.1 is superior, whereas temperature for short-term storage exerts a minor effect only.

For developing the mtRA assay, it was necessary to compare different batches of Baker's yeast (L47 and L77) and examine the factors that might influence respiratory activity as a result of incubation time, experimental oxygen level, and storage after rehydration.

(1) Rehydration at 30 °C followed by storage for 20-30 min and 4-5 h at RT or ice (pH 7.1), and stability of endogenous ROUTINE respiration during incubation at 37 °C for 3 hours (pH 7.1), including an intermittent aerobic-anaerobic transition and reoxygenation. The effect of storage temperature was analyzed by continuous measurement of endogenous *R* during a period of 1 h at oxygen concentrations declining from air saturation to anoxia (Fig. 2). After stabilization of flux, *R* was quantified over 15-20 min after closing the chamber *(R*1), at 100 and 50 µM (*R*2 and *R*3). Anoxia lasted for 20 min, and reoxygenation to 200 µM O_2 was achieved by titration of hydrogen peroxide (stock solution 200 mM in a.d.). ROUTINE respiration was again quantified at three specific intervals, during 15-20 min after reoxygenation (*R4*), at 100 and 50 µM O_2 (*R5* and *R6*). Short-term storage on ice versus room temperature has little effect on ROUTINE respiration, which increased from 20 to 30 min, was stable at declining oxygen levels up to 40 min, declined slightly after anoxia and reoxygenation, and short a further trend of decline towards low oxygen (Table 1). After 4-5 h storage, initial ROUTINE respiration was increased 1.3-fold (58.6±8.6 pmol·s^{-1}·ml^{-1}; *n*=3) compared to short-term storage, but declined to equal (RT) or slightly lower values (ice) after anoxia and reoxygenation.

(2) Another variable examined was the pH of the medium used for rehydration and respiration, comparing a new batch of yeast (L77) dissolved in Na-P buffer at pH=8.1 versus pH=7.1. ROUTINE respiration in L77 was lower than in L47, indicating that a mtRA must be based on a single lot. At pH 7.1, ROUTINE respiration was not only higher than at pH 8.1, but was also more stable after anoxia-reoxygenation (Tab. 1).

These experiments illustrate the complexity of factors involved to adapt the model of commercially available freeze-dried baker's yeast for a standardized mtRA. As expected, the respiratory control pattern in intact yeast cells differs from mammalian cells (for a review see ref. [3]). The benefit of this yeast model relates to its simple availability, unproblematic transport across international borders, robustness in long-term storage, and fast stabilization of respiration after storage.

References

1. Crowe HJ, Carpenter FJ, Crowe M (1998) The role of vitrification in anhydrobiosis. Annu Rev Physiol 60: 73-103.
2. Koga S, Echigo A, Nunomura K (1966) Physical properties of cell water in partially dried *Saccharomyces cerevisiae*. Biophys J 6: 665-674.
3. Pesta D, Gnaiger E (2012) High-resolution respirometry. OXPHOS protocols for human cells and permeabilized fibres from small biopisies of human muscle. Methods Mol Biol 810: 25-58.

Yeast protocol 2010-06-09

O2k-settings:

- Temp.: 37 °C ; Flux derivation N:25

Medium: Na-PBCtl (50 mM, pH 7.1); 140.000 units catalase/ml; SF = 0.95

Yeast stock suspension (2mg/ml):

- dissolve 2 mg baker's yeast (powder) in 1 ml Na-PBCtl at 30°C
- vortex for 7 min at 22000 rpm to obtain a homogenous cell suspension
- add 50 µl of yeast stock to each chamber (final conc. In the chamber = 0.05 mg/ml)
- repeat vortexing for 30 sec before each addition to the chamber

POS calibration (R1)

- Layout: 01 Calibration experiment Gr3-temperature
- After 15 min – stirrer test
- Wait for stable signal, then close the chamber and add yeast
- Open new DatLab-file
- Layout: 05 Flux per Volume corrected

Experiment

- **20 min period of ROUTINE respiration: Mark name = ROUTINE**

Titrations	Additions (µl)	Mark names
Glucose (2 M)	20	Glc
Ethanol (pure)	40	Glc-EtOH
FCCP (10 mM)	2 + 2,5 + 2,5 + (2,5)	ETS

Azide-Titration(Azd stock 4 M)

0.04 M; 100 x diluted stock 2 + 2
0.16 M; 25 x diluted stock 1 + 1 + 1 + 3
0.4 M; 10 x diluted stock **1**
4 M; stock solution 2 + 3 + 4 + 4 ROX

Note: if you need to reoxigenate, you have to do this before the azide titration because catalase is inhibited by azide!

Template for Analysis is on the Course CD: 2.Protocols > Files_Procolols > DLDemo Templates > Bakers yeast > O2k-Analysis yeast.xls