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Mitochondrial Respiration Medium - MiR06

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Section

1. Respiration: Mitochondrial Respiration Medium MiR06

MiR06 = MiR05 + Catalase (see Gnaiger et al 2000; MiPNet08.05)
Total volume = 1 litre.

Compounds in MiR05/MiR06	Final conc.	FW	Addition to 1 litre final volume	Source, product code
EGTA	0.5 mM	380.4	0.190 g	Sigma, E 4378 (25 g)
$MgCl_2 \cdot 6\ H_2O$	3 mM	203.3	0.610 g	Scharlau, MA 0036 (250 g)
K-lactobionate	60 mM	358.3 free acid	120 ml of 0.5 M K-lactobionate stock*	Aldrich, 153516 (100 g)
Taurine	20 mM	125.1	2.502 g	Sigma, T 0625 (25 g)
KH_2PO_4	10 mM	136.1	1.361 g	Merck, 104873 (1 kg)
HEPES	20 mM	238.3	4.77 g	Sigma, H 7523 (250 g)
Sucrose	110 mM	342.3	37.65 g	Roth, 4621.1 (1 kg)
BSA, essentially fatty acid free	1 g/l		1 g	Sigma, A 6003 fraction V (25 g)

Weigh given amounts of the listed compounds (except BSA and lactobionic acid) into a 1000 ml glass beaker, disrupt big lumps mechanically, ad 800 ml H_2O and dissolve on a magentic stirrer at 30 °C. Add 120 ml of

K-lactobionate stock solution; adjust the pH to 7.1 (5 N KOH; Sigma P 1767; 1 kg) at 30 °C. Adjust volume with H_2O to a final volume of 1 litre. Dissolve BSA separately in a subsample of the solution (recommended to prevent foam building) and transfer back to the final solution, while stirring continuously but gently. Check pH again and adjust if necessary. Store frozen at -20 °C in plastic vials.

***** Preparation of K-lactobionate stock solution:

Dissolve 35.83 g lactobionic acid in 100 ml H_2O, adjust pH to 7.0 with KOH, adjust volume to 200 ml with H_2O.

MiR06 contains the following final concentrations:

Ca^{2+} free	0.0 µM
Mg^{2+} free	2.1 mM
K^+	90 mM
Na^+	0
EGTA free	0.46 mM
Osmolarity	330 mOsm
Ionic strength	95 mM

The ionic strength increases with the addition of substrates and adenylates, particularly in multi-substrate/inhibitor titrations.

EGTA A general chelator for heavy metals, with high affinity for Ca^{2+} but low affinity for Mg^{2+}.

Mg^{2+} Activation by ATP due to ATPase activity is Mg^{2+} dependent. The high quality of mitochondrial preparations cannot be tested in the absence of Mg^{2+}. Physiological Mg^{2+} concentration is in the range of 1-3 mM. Several enzyme systems depend on free Mg^{2+}.

P_i The K'_m is up to 1 mM in the ADP-activated OXPHOS state in heart mitochondria with glutamate/malate; 90% of maximum flux are reached at 10 mM. Is flux *measurably* higher at 15 mM?

K-lactobionate The intracellular K^+ concentration is high (>100 mM), adding significantly to the ionic strength. In many previous studies of isolated mitochondria, KCl was used for this reason, but a high Cl^- concentration is unphysiological and inhibitory on mitochondrial creatine kinase (and possibly on other enzymes in the intermembrane space). K-MES or K-methanesulfonate have been used successfully. Lactobionate is well established in (extracellular) organ preservation solutions (University of Wisconsin solution).

Taurine Biological membrane stabilizer and ROS scavanger. 20 mM intracellular concentration in heart.

Histidine 20 mM histidine was added in MiR04 (as in MiP02), as an imidazol-based buffer, with temperature dependence of p*K* identical to that of water (α-stat pH buffer). No effect was observed when adding histidine to MITOMED1 with unpermeablized endothelial cells. Omitted in MiR05 and MiR06 owing to increased autooxidation of TMPD and ascorbate in MiR04.

HEPES Well established buffer with pK close to 7.

Sucrose Impermeant and oxygen radical scavenger.

BSA Bovine serum albumine is a membane stabilizer, oxygen radical scavenger, and binds Ca^{2+} and free fatty acids, hence the rather expensive essentially free fatty acid free BSA is required.

Glutathione No effect was observed with glutathion added to MITOMED1 with unpermeabilized or permeabilized endothelial cells (tEC); but background oxygen flux is significantly higher. This complication is avoided by not adding glutathion to the respiration medium.

98-03-30/04-02 Autooxidation of glutathion in MiR04 (with histidine) was variable between experiments, with *a*°' of −1.7 (range −2.1 to 0.3, *N*= 6), and *b*°' of 0.053, 0.065 and 0.11 in three oxygraphs (two chambers each).

Catalase is an antioxidant enzyme at high intracellular activity, and improves the antioxidant quality of physiological respiration medium beyond MiR05. In the presence of high catalase activity, hydrogen peroxide, H_2O_2, is titrated into the O2k-chamber to increase oxygen levels by up to 200 µmol/l, e.g. from 200 µM to 350 µM. MiR06 is stored like MiR05, or can be prepared by adding catalase stock solution (dissolved in MiR05) directly into the closed O2k-chamber filled with MiR05 at the start of an experiment.

Compound	Final conc.	FW	Stock solution	Addition to 2 ml final volume	Source and product code
Catalase lyophilized powder, 2,000-5,000 units/mg protein*	280 u/ml*		112000 u/ml,* dissolve in MiR05	5 µl	Sigma C9322
H_2O_2		34.01	200 mM in H_2O, adjust to pH 6		Sigma Aldrich 516813 17.6 M, 50% w/w

* Units of enzymatic activity (u) in µmol/min; assay used by Sigma Aldrich: '*One unit will decompose 1.0 µmole of H_2O_2 per min at pH 7.0 at 25 °C, while the H_2O_2 concentration falls from 10.3 to 9.2 mM, measured by the rate of decrease of A_{240}.*'

H_2O_2 Small volumes (µl) of H_2O_2 are injected into the O2k-chamber filled with MiR06, to increase oxygen levels. A typical H_2O_2 stock concentration in the syringe is approximately 200 mM. Maintain the pH in the stock solution acidic to minimize autoxidation. Manual injection: Fill a 10 µl syringe with the H_2O_2 solution, inject a small volume, observe the oxygen level displayed by DatLab and inject further H_2O_2 until the desired oxygen level (Δc_{O2} ≤200 µmol/l) is reached. Alternatively, the "Oxystat" setup of the TIP2k may be used to reach a desired oxygen level and then maintain the oxygen concentration automatically between set limits in the oxystat-titration mode.

Creatine **MiR06Cr**: MiR06Cr = MiR06 plus 20 mM creatine (Sigma 27900, 100g). MiR06Cr has to be prepared fresh every day.

2. Preservation: Mitochondrial Preservation Medium MiP03

Take MiR06 and add the following before freezing:

Compound	Final conc.	MW	Addition to 20 ml final volume	Company, product code and storage
Histidine	20 mM	155.2	62.1 mg	Sigma, H8000, RT
Vitamin E succinate	20 µM	530.8	200 µl (2 mM stock)	Sigma, T3126, RT
Glutathion	3 mM	307.3	18.4 mg	Sigma, G4251, 4 °C
Leupeptine	1 µM	463.0	20 µl (1 mM stock)	Sigma, L9783, -20 °C
Glutamate	2 mM	169.1	40 µl (1 M stock)	Sigma, G1626, RT
Malate	2 mM	134.1	40 µl (1 M stock)	Sigma, M1000, RT
Mg-ATP	2 mM	614.1	80 µl (500 mM stock)	Sigma, A2383, -20 °C

MiP03 preservation medium has the following final concentrations:

Ca^{2+} free	0.0 µM
Mg^{2+} free	2.1 mM
K^+	90 mM
Na^+	4 mM
EGTA free	0.46 mM
Osmolarity	340 mosM
Ionic strength	108 mM

Adjust the pH to 7.1 (5 N KOH) at 30 °C.

Vitamin E D-α-Tocopherol succinate is soluble in chloroform (50 mg/ml) or ethanol, it is practically insoluble in water and it is unstable in alkaline conditions. Solutions of D-α-Tocopherol are stable at 4 °C (light protected) for several months. 20 µM intracellular concentration in liver.

Leupeptine Soluble in water. The aqueous solution is stable for a week at 4 °C and for at least 6 months as frozen aliquots at -20 °C.

Oxygen solubility factor in MiR05 [1] at 30 °C and 37 °C is 0.92 [3]. The same solubility is valid for MiR06 and MiR06Cr.

3. References

Gnaiger E, Kuznetsov AV, Schneeberger S, Seiler R, Brandacher G, Steurer W, Margreiter R (2000) Mitochondria in the cold. In: Life in the Cold. (Heldmaier G, Klingenspor M, eds) Springer, Heiderlberg, Berlin, New York, pp. 431-442.

Rasmussen HN, Rasmussen UF (2003) Oxygen solubilities of media used in electrochemical respiration measurements. Analyt Biochem 319: 105-113. *The manuscript* (Gnaiger et al 2000) *was sent to Dr. H. Rasmussen, and we appreciate that the oxygen solubility of MiR05 was then determined and published* (Rasmussen 2003). *Surprisingly, no reference was made in ref.* (Rasmussen et al 2003) *to the original publication on MiR05* (MiPNet08.05).

MiPNet08.05 Mitochondrial Respiration Medium - MiR05 (2003).
MiPNet03.02 Selected media and chemicals for respirometry with mitochondria and permeabilized cells.
MiPNet06.06 Oxygraph assay of cyt *c* oxidase activity: Chemical background correction.

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Author contributions
Gnaiger E with collaboration of Kuznetsov AV was responsible for the development and testing of MiR05 (Gnaiger et al 2000). All authors contributed to various details in the development of MiR06.