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O2k-Protocols

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Selected media and chemicals for respirometry with mitochondrial preparations

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Summary: Different media for tissue preparation and respiration are used in investigations of mitochondrial function. Initial decisions on the composition of media and chemicals are decisive for long-term studies and crucial for comparability of results. As a guideline, we summarize an update of our experience with media and chemicals for high-resolution respirometry with isolated mitochondria, permeabilized cells, muscle fibres and tissue homogenates. Whereas optimization is necessary for specific experimental protocols, standardization will improve the comparability of results obtained in different laboratories. Such efforts towards standardization are important for the advancement of mitochondrial physiology and mitochondrial medicine.

Contents

1. Introduction

High-resolution respirometry provides the basis for a detailed analysis of mitochondrial function (OXPHOS analysis). Incubation media contain compounds such as sucrose, mannitol, potassium chloride, potassium-MES, to achieve physiological osmolarity. Additional components are added to preserve mitochondrial integrity. Mitochondrial media, therefore, have different ionic strengths, pH and ionic compositions.

The list of **media** is organized according to the major applications, including isolation of mitochondria, preparation of muscle fibres and incubation media for respirometry, with emphasis on **MiR06** (MiR06 = MiR05+Catalase; MiPNet14.13) as our most advanced respiration medium. The list of **chemicals** contains mitochondrial substrates, inhibitors, uncouplers and agents for cell permeabilization. The preferred concentrations and solvents are shown for stock solutions, and storage conditions are recommended.

Finding a compromise between dynamic optimization of SUIT protocols and adherence to a fixed standard represents a well-known problem in the development and application of strategies for scientific investigation. Improvement of standard methods requires cooperation and feedback. Therefore we appreciate any comments and suggestions directed towards improved and more generally acceptable standards in mitochondrial physiology.

2. Media for muscle fibre preparation and isolation of mitochondria

Higher respiratory capacities are observed when integrating a preservation strategy in the formulation of isolation media (such as addition of antioxidants). Improvement of the quality of isolation media may be limited by the increasing cost when preparing large volumes. The media for isolation of mitochondria (Section 2.2 and 2.3) are minimum media without concerns on preservation strategies.

2.1. Preparation of permeabilized muscle fibres - BIOPS

(Veksler et al 1987; Letellier et al 1992)

The relaxing and biopsy preservation solution BIOPS contains 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1.

BIOPS Total volume = 1 litre

	Final conc.	FW	Stock solution	Addition to 1 litre final	Source and product code
CaK_2EGTA*	2.77 mM		100 mM	27.7 ml	
K_2EGTA*	7.23 mM		100 mM	72.3 ml	
Na_2ATP	5.77 mM	551.1		3.141 g	Sigma A 2383 (5 g)
$MgCl_2 \cdot 6\ H_2O$	6.56 mM	203.3		1.334 g	Scharlau MA 0036 (250 g)

Taurine	20 mM	125.1		2.502 g	Sigma T 0625 (25 g)
Na_2Phosphocreatine	15 mM	255.08*		4.097 g	Sigma P 7936 (5 g)
Imidazole	20 mM	68.1		1.362 g	Fluka 56750 (100 g)
Dithiothreitol (DTT)	0.5 mM	154.2		0.077 g	Sigma D 0632, (1 g)
MES	50 mM	195.2		9.76 g	Sigma M8250 (250 g)

BIOPS contains the following ion concentrations:

Ca^{2+} free	0.1 µM
Mg^{2+} free	1 mM
MgATP	5 mM
Ionic strength	160 mM

Adjust the pH to 7.1 (with 5 N KOH) at 0 °C. Divide into 20 ml portions. Store BIOPS and K_2EGTA / CaK_2EGTA solutions at -20 °C in plastic vials.

* Anhydrous; preparation of stock solutions K_2EGTA and CaK_2EGTA:

K_2EGTA Mix 100 mM EGTA (Sigma, E 4378, 25 g) and 200 mM KOH (Sigma, P 1767, 1 kg) (dissolve 7.608 g EGTA and 2.3 g KOH in 200 ml H_2O, adjust the pH to c. 7.0 with KOH).

CaK_2EGTA Dissolve 2.002 g $CaCO_3$ (Sigma, C 4830; 100g) in 100 mM hot (80 °C) solution of EGTA (7.608 g / 200 ml) while stirring continuously, add 2.3 g KOH, adjust the pH to c. 7.0.

KH_2PO_4 ATP will be hydrolyzed at least partially during fibre storage, thus generating mM levels of inorganic phosphate. It has not been reported if addition of 3 mM phosphate (Veksler et al 1987; Skladal et al 1994) exerts any effect on preservation quality.

Saponin solution: for muscle permeabilization, prepared fresh everyday:

1. Saponin stock solution: add 5 mg saponin (Sigma S 2149; 25 g) to 1 ml BIOPS.
2. For permeabilization in saponin solution, add 21 µl saponin stock solution to 2 ml BIOPS.

2.2. Isolation of mitochondria from liver, heart, placenta

Medium A1 Total volume 1 litre

	Final conc.	FW	Addition to 1 litre final volume
Sucrose	250 mM	342.3	85.6 g
Na_2EDTA	0.5 mM	372.2	0.186 g
Tris	10 mM	121.1	1.211 g

Adjust the pH to 7.4 (HCl) at c. 0 °C. Store at −20 °C in 100-200 ml plastic vials.

Medium B1: take 500 ml of medium A1 and add:

BSA	1 g/l		0.5 g/500 ml

Store at -20°C in 100-200 ml plastic vials.

2.3. Isolation of mitochondria from skeletal muscle

Medium A2 Total volume 1 litre

	Final conc.	FW	Addition to 1 litre final volume
KCl	180 mM	74.55	13.42 g
Na_2EDTA	0.5 mM	372.2	0.186 g
Tris	10 mM	121.1	1.211 g

Adjust the pH to 7.4 (HCl) at c. 0 °C. Store at −20 °C in plastic vials.

Medium B2: take 500 ml of medium A2 and add:

BSA	1 g/l		0.5 g/500 ml

Store at -20 °C in plastic vials.

3. Mitochondrial respiration media (MiR)

3.1. MiR06

MiR05: Isolated mitochondria (liver and cardiac), and permeabilized endothelial cells (Gnaiger et al 2000).

Preparation of **MiR06** (= MiR05 + Catalase): see separate Protocol (MiPNet14.13).

3.2. Oxygraph medium for cytochrome *c* test

The high concentration of KCl favours dissociation of cytochrome *c* from the inner mitochondrial membrane and cytochrome *c* release upon injury of the outer mitochondrial membrane. Respiratory flux is reduced with cytochrome *c* depletion, and can be restored after addition of 10 µM cytochrome *c* (Saks et al 1992, 1995; Gnaiger and Kuznetsov 2002; Kuznetsov et al 2004).

	Final conc.	FW	Addition to 1 litre final
EGTA	0.4 mM	336.2	0.134 g
$MgCl_2.6\ H_2O$	3 mM	203.3	0.61 g
KH_2PO_4	5 mM	136.1	0.68 g
Dithiothreitol	0.3 mM	154.2	0.046 g
KCl	125 mM	74.55	9.32 g
HEPES	20 mM	238.3	4.77 g

Cytochrome *c* medium contains the following ion concentrations:

Ca^{2+} free	0.0 µM
Mg^{2+} free	2.51 mM
EGTA free	0.36 µM
Ionic strength	142 mM

Adjust the pH to 7.1 (5 N KOH) at 25 °C. Divide into 20 ml portions. Store at -20 °C in plastic vials.

4. Chemicals for mitochondrial SUIT protocols

Calculation of concentrations: MiPNet09.12_O2k-Titrations.xls.

4.1. Substrates for SUIT protocols

Substrate	FW	Stock soln. Conc [mM]	Stock soln. Amount	Comments	Source, product code and storage
G: L-Glutamic acid, sodium salt, $C_5H_8NO_4Na$ (contains 1 mol/mol H_2O)	187.1 169.1 anhy-drous	2000	3.742 g/ 10 ml H_2O	Neutralize with 5 N KOH, check pH. Divide into 0.5 ml portions. Store at -20 °C.	Sigma, G 1626 (100 g) RT
M: L-Malic acid, $C_4H_6O_5$	134.1	400	536 mg/ 10 ml H_2O	Neutralize with 10 N KOH, check pH. Divide into 0.5 ml portions. Store at -20 °C.	Sigma, M 1000 (100 g) RT
P: Pyruvic acid sodium salt, $C_3H_3O_3Na$	110.0	2000	44 mg/ 0.2 ml H_2O	Prepare everyday new.	Sigma, P 2256 (25 g); 4°C
S: Succinate disodium salt, hexahydrate, $C_4H_4O_4Na_2$. 6 H_2O	270.1	1000	2.701 g/ 10 ml H_2O	Adjust pH to 7.0 with 37% HCl. Divide into 0.5 ml portions. Store at -20 °C.	Sigma, S 2378 (100 g) RT
Oct: DL-Octanoyl-carnitine-HCl, $C_{15}H_{30}NO_4Cl$	323.85	100	32.4 mg/ 1 ml H_2O	Store at -20 °C.	TOCRIS Bioscience, No. 0605 (50 mg), RT, desiccate
Pal: Palmitoyl-DL-carnitine-HCl, $C_{23}H_{45}NO_4 \cdot HCl$	436.1	10	8.72 mg/ 2 ml H_2O	Store at -20 °C.	Sigma P 4509 (100 mg) -20 °C
As: Ascorbate sodium salt, $C_6H_7O_6Na$	198.1	800	1.584 g/ 10 ml H_2O	To prevent autooxidation, adjust pH to ca 6 with ascorbic acid (a 137.6 mg ml^{-1} solution of pH ca 2). Divide into 0.2 ml portions. Store at -20 °C. Light sensitive.	Sigma, A 4034 (100 g) RT
Tm: TMPD *N,N,N',N'*-Tetramethyl-p-phenylenediamine dihydrochloride, $C_{10}H_{16}N_2 \cdot 2$ HCl	237.2	200	47.4 mg/ 1 ml H_2O	To prevent autooxidation add 0.8 M ascorbate to a final concentration of 10 mM. Divide into 0.2 ml portions. Store at -20 °C.	Sigma T 3134 (5 g) RT
c: Cytochrome *c*	12500	4.0	50 mg/ 1 ml H_2O	Divide into 0.2 ml portions. Store at -20 °C.	Sigma, C 7752 (50 mg) -20 °C

D: ADP** (Adenosine 5'diphosphate, $C_{10}H_{15}N_5O_{10}P_2K$, potassium salt, contains 1 mol/mol H_2O)	501.3	500	0.501 g/ 2 ml H_2O	Neutralize with 5 N KOH (approx.450 µl), check pH. Divide into 0.2 ml portions. Store at -80 °C. ** To keep [Mg^{2+}] constant during respiration measurement mix ADP with $MgCl_2$ (0.6 mol/mol ADP)	Calbio-chem 117105 (1 g) 4°C or Sigma A 5285 (1 g) -20 °C
T: ATP** (Adenosine 5'-triphosphate, $C_{10}H_{14}N_5O_{13}P_3Na_2$, disodium salt, contains 3.5 mol/mol H_2O)	614.1 3.5 mol/ mol H_2O 551.1 anhy-drous	500	0.614 g/ 2 ml H_2O	Neutralize with 5 N KOH (approx. 400 µl), check pH. Divide into 0.2 ml portions. Store at -80 °C. ** To keep [Mg^{2+}] constant during respiration measurement mix ATP with $MgCl_2$ (0.8 mol/mol ATP).	Calbio-chem 1191 -20 °C or Sigma A 2383 (5 g)

4.2. Uncouplers for SUIT protocols

Uncoupler	FW	Stock soln. Conc. [mM]	Stock soln. Amount	Comments	Source, product code and storage
U CCCP: $C_9H_5ClN_4$	204.62	1.0	1.02 mg in 5 ml DMSO	Store at -20 °C	Sigma C 2759 (100 mg)
DNP: 2,4-Dinitrophenol, $C_6H_4O_5N_2$	184.1	10	3.7 mg/ 2 ml H_2O	Neutralize with 1 N KOH, check pH. Store at –20 °C. Toxic.	
F (FCCP): Carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone $C_{10}H_5F_3N_4O$	254.2	1.0	2.54 mg/ 10 ml ethanol	Divide into 0.5 ml portions. Store in glass vials at −20 °C.	Sigma C 2920 (10 mg) 4 °C
TTFB: 4,5,6,7-Tetrachloro-2-trifluoromethyl-benzimidazole	323.94	1.0	3.24 mg/ 10 ml ethanol	Divide into 0.5 ml portions. Store at -20 °C.	

4.3. Inhibitors for SUIT protocols

Inhibitor	FW	Stock soln. Conc. [mM]	Stock soln. Amount	Comments	Source, product code and storage
Ama: Antimycin A	540	5.0	11 mg/ 4 ml ethanol	Divide into 0.2 ml portions. Store at -20 °C. Very toxic.	Sigma A 8674 (25 mg) -20 °C
Amy: Amytal (Amobarbital) sodium salt, $C_{11}H_{17}N_2O_3Na$	248.3	200	0.497 g/ 10 ml 50% ethanol	Divide into 0.5 ml portions. Store at -20 °C. Light sensitive. Toxic.	

Atr: Atractyloside dipotassium salt, $C_{30}H_{44}O_{16}S_2K_2$ (2.5 mol/mol H_2O)	803.0	50	40.2 mg/ 1 ml H_2O	Dissolves better in warm water. Store at -20 °C. Toxic.	Sigma A 6882 (250 mg) RT
Azd: Sodium azide, NaN_3	65.01	4000	260 mg/ 1 ml H_2O	Divide into 0.5 ml portions. Store at –20 °C. Very toxic.	Sigma S 2002 (25 g) RT
Cat: Carboxy-atractyloside, potassium salt	802.99 free acid	5.0	4.02 mg/ 1 ml H_2O	Divide into 0.2 ml portions. Store at -20 °C. Toxic.	Sigma C 4992 (2 mg) -20°C
Kcn: Potassium cyanide, KCN	65.12	1000	13 mg/ 0.2 ml H_2O	Prepare everyday new. The pH of the solution may be very alkaline; adjust with HCl. Photosensitive. Hygroscopic. Very toxic.	Fluka 60178 (100 g)
Mna: Malonic acid	104.06	2000	0.0208 g/ 100 µl	Dissolve in 35 µl H_2O+65 µl of KOH 5 N, check pH, prepare fresh	Sigma Aldrich M129-6 (5 g) RT
Myx: Myxothiazol	487.7	1.0	1.0 mg/ 2.05 ml ethanol	Divide into 0.2 ml portions. Store at -20 °C. Very toxic.	Sigma T-5580 (1 mg) 4°C
Omy: Oligomycin	800	4 mg/ml	4 mg/ 1 ml ethanol	Divide into 0.2 ml portions. Store at -20 °C. Very toxic.	Sigma O 4876 (5 mg) -20 °C
Oua: Ouabain (G-Strophanthin) octahydrate, $C_{29}H_{44}O_{12}.8\ H_2O$	728.8	10	7.3 mg/ 1 ml H_2O	Divide into 0.2 ml portions. Store at -20 °C. Light sensitive. Toxic.	
Pep: p5-Di (adenosine -5') penta-phosphate sodium salt, $C_{20}H_{29}N_{10}O_{22}P_5$ (5 mol/mol Na, 1.5 mol/mol H_2O)	1058.4 916.4 free acid	50	52.91 mg/ 1 ml H_2O	Neutralize with 5 N KOH, check pH. Divide into 0.2 ml portions. Store at -20 °C. Toxic.	
Rot: Rotenone, $C_{23}H_{22}O_6$	394.4	1.0 [a]	3.94 mg/ 10 ml ethanol	Difficult to dissolve. Store at -20 °C. Light sensitive. Very toxic.	Sigma R 8875 (1 g) RT
Rut: Ruthenium red (ammoniated ruthenium oxychloride)	551.22	10	5.5 mg/ 1 ml H_2O	Store at -20 °C.	

[a] Rotenone is added at a high final concentration (0.5 µM), based on a 1.0 mM stock solution. Since 0.1 µM may be fully inhibiting some mitochondrial preparations, a lower concentration may be used (0.2 mM stock, 0.1 µM final), to reduce the problem of rotenone retention in the O2k-chamber.

4.4. Agents for cell permeabilization

Substance	FW	Stock sol. Conc.	Stock solution Amount	Comments	Source, product code and storage
Dig: Digitonin	1229.3	8.1 mM	10 mg/1 ml DMSO	Store at -20 °C. Toxic.	Fluka 37008 (1 g) RT
Sap: Saponin	-	5 mg/ml	5 mg/1 ml H_2O	Prepare everyday new.	Sigma S 2149 (25 g)

5. General comments

5.1. Solutions stored at low temperature: Mix carefully after re-warming, since phase separation may occur and compounds may precipitate in cold solutions. During the course of the experiment, keep stock solutions on ice.

5.2. Solutions containing ethanol: there may be a problem of evaporation and subsequent increase of concentration of stock solutions.

5.3. Chemicals dissolved in ethanol or DMSO: To check the influence of ethanol or DMSO on mitochondrial function and experimental sensors (ion selective electrodes), the same additions of pure solvents should be used in carrier control experiments.

5.4. For all stock solutions of mitochondrial substrates, inhibitors, and uncouplers; the total volumes of solutions are indicated.

5.5. Store chemicals as indicated by the suppliers. The storage conditions of prepared solutions are indicated in the comments.

5.6. Aliquots of stocks for rotenone, succinate, glutamate, malate, and oligomycin can be refrozen for later use, since these chemicals are stable.

6. References

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