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Selected media and chemicals for respirometry with mitochondrial preparations

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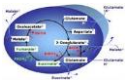

Section **Page**

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 (HRR) with isolated mitochondria, permeabilized cells, muscle fibers 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.

1. Introduction

High-resolution respirometry (HRR) 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 fibers 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 fiber 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. BIOPS for preparation of permeabilized muscle fibers

(Veksler et al 1987; Letellier et al 1992)

BIOPS

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.
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		3.180 g**	Sigma A 2383, -20 °C
$MgCl_2 \cdot 6\ H_2O$	6.56 mM	203.3		1.334 g	Scharlau MA 0036, RT
Taurine	20 mM	125.1		2.502 g	Sigma T 0625, RT
Na_2Phosphocreatine	15 mM	255.1*		3.827 g***	Sigma P 7936, - 20 °C
Imidazole	20 mM	68.1		1.362 g	Fluka 56750, RT
Dithiothreitol (DTT)	0.5 mM	154.2		0.077 g	Sigma D 0632, 4 °C
MES hydrate	50 mM	195.2		9.76 g	Sigma M8250, RT

*Anhydrous

**Changed since 2016-08-25 from 3.141 g to 3.180 g because of a calculation mistake. This change shouldn´t have an effect on biological experiments.

***Changed since 2016-08-25 from 4.097 g to 3.827 g. 4.097 g is the calculated weight for Na_2Phosphocreatine monohydrate and 3.827 g is the calculated weight for Na_2Phosphocreatine anhydrous. The Sigma product is hygroscopic and can absorb an undefined amount of hydrate over time. Store in a desiccator.

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 M KOH) at 0 °C. Divide into 20 mL portions. Store BIOPS and K_2EGTA / CaK_2EGTA solutions at -20 °C in plastic vials.

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 fiber 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 20 µL saponin stock solution to 2 mL BIOPS.

2.2. Mitochondrial Preservation Medium: MiP03

Use MiR06 (MiPNet14.13 Medium-MiR06**) and add the following:**

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

Storage Store 40 mL aliquots at -20 °C.

2.3. Isolation of mitochondria from liver and 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.

2.4. 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.

2.5. Isolation of mitochondria from heart

Stock solution	Conc.	FW	Addition to 1 litre final volume
D-Mannitol	0.5 M	182.17	91.085 g
Sucrose	0.5 M	342.30	171.150 g
EGTA*	0.1 M	380.35	38.350 g

*Neutralize with Tris to pH 7.4

Isolation Medium

	Final conc.	Addition to 200 mL final volume
D-Mannitol	225 mM	90 mL
Sucrose	75 mM	30 mL
EGTA, pH 7.4	1 mM	2 mL

Prepare fresh daily and keep at 4 °C.

3. Mitochondrial respiration media (MiR)

» www.bioblast.at/index.php/List_of_media_for_respirometry

3.1. MiR05(Cr), MiR06(Cr)

MiR05 (Gnaiger et al 2000).
MiR06(Cr) = MiR05(Cr) + Catalase: see separate protocol (MiPNet14.13_Medium-MiR06).

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 M 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

» www.bioblast.at/index.php/List_of_substrates_and_metabolites

Substrate	FW	Stock soln. Conc [mM]	Stock Soln. Amount	Comments	Source, product code and storage
G: L-Glutamic acid, sodium salt, $C_5H_8NO_4Na$	169.1	2000	3.382 g/ 10 mL H_2O	Neutralize with 5 M KOH, check pH. Divide into 0.5 mL portions. Store at -20 °C.	Sigma, G 1626, RT
M: L-Malic acid, $C_4H_6O_5$	134.1	400	268.2 mg/ 5 mL H_2O	Neutralize with 5 M KOH, check pH. Divide into 0.5 mL portions. Store at -20°C.	Sigma, M 1000, RT
M: L-Malic acid, $C_4H_6O_5$	134.1	50	-	Dilute 0.625 mL of 400 mM stock solution with 4.375 mL of H2O (final volume 5 mL). Divide into 0.25 mL portions. Store at -20°C..	Sigma, M 1000, RT
P: Pyruvic acid sodium salt, $C_3H_3O_3Na$	110.0	2000	44 mg/ 0.2 mL H_2O	Prepare everyday fresh.	Sigma, P 2256, 4°C
S: Succinate disodium salt, hexahydrate,	270.1	1000	2.701 g/ 10 mL H_2O	Check pH and adjust if necessary to 7.0 with 1 N HCl.	Sigma, S 2378, RT

$C_4H_4O_4Na_2$ x 6 H_2O				Divide into 0.5 mL portions. Store at -20 °C.	
Oct: DL-Octanoyl-carnitine-HCl, $C_{15}H_{30}NO_4Cl$	323.85	100	32.4 mg/ mL H_2O	Store at -20 °C.	TOCRIS Bioscience, No. 0605, RT, desiccate
Pal: Palmitoyl-DL-carnitine-HCl, $C_{23}H_{45}NO_4$·HCl	436.1	10	8.72 mg/ 2 mL H_2O	Store at -20 °C.	Sigma P 4509, -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 ~ 6 with ascorbic acid (a 137.6 mg mL^{-1} solution of pH ~ 2). Divide into 0.2 mL portions. Store at -20 °C. Light sensitive.	Sigma, A4034, RT
Tm: TMPD *N,N,N',N'*-Tetramethyl-p-phenylenediamine dihydrochloride, $C_{10}H_{16}N_2$·2 HCl	237.2	200	47.4 mg/ 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, T3134, RT
c: Cytochrome *c*	12500	4.0	50 mg/ mL H_2O	Divide into 0.2 mL portions. Store at -20 °C.	Sigma, C7752, -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 M 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)	Cal-biochem, 117105, 4°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 M 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).	Sigma, A 2383, - 20 °C

4.2. Uncouplers for SUIT protocols

» www.bioblast.at/index.php/List_of_uncouplers

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 ethanol	Store at -20 °C	Sigma C 2759
DNP: 2,4-	184.1	10	3.7 mg/	Neutralize with 1 M	

Dinitrophenol, $C_6H_4O_5N_2$			2 mL H_2O	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, 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

» www.bioblast.at/index.php/List_of_inhibitors

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, -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, 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, RT
Cat: Carboxy-atractyloside, potassium salt	939.1	5	4.7 mg/ 1 mL H_2O	Divide into 0.2 mL portions. Store at -20 °C. Toxic.	Calbiochem 216201, - 20°C
Kcn: Potassium cyanide, KCN	65.12	1000	13 mg/ 0.2 mL H_2O	Prepare everyday fresh. The pH of the solution may be very alkaline; adjust with HCl. Photosensitive. Hygroscopic. Very toxic.	Fluka, 60178
Mna: Malonic acid	104.06	2000	0.0208 g/ 100 µL	Dissolve in 75 µl 5 M KOH, check pH, titrate small amounts (2 µL) of 5 M KOH until you reach a pH of 6.0, add H_2O to 100 µL.Prepare fresh	Sigma Aldrich, M129-6, 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, 4°C
Omy: Oligomycin	800	4 mg/mL =5 mM	4 mg/ 1 mL ethanol	Divide into 0.2 mL portions. Store at -20 °C. Very toxic.	Sigma, O 4876, -20 °C
Oua: Ouabain	728.8	10	7.3 mg/	Divide into 0.2 mL portions.	

(G-Strophanthin) octahydrate, $C_{29}H_{44}O_{12}.8\ H_2O$			1 mL H_2O	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 M 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 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

» www.bioblast.at/index.php/List_of_permeabilization_agents

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, RT
Sap: Saponin	-	5 mg/mL	5 mg/1 mL BIOPS	Prepare fresh everyday.	Sigma, S7900, RT

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.

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