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O2k-Protocols

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SUIT reference assay for OXPHOS analysis by high-resolution respirometry

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Contents

Summary: A substrate-uncoupler-inhibitor titration (SUIT) reference assay (SUIT-RA) is developed to provide a common baseline for comparison of mitochondrial respiratory control in a large variety of species, tissues and cell types, mt-preparations and laboratories, for establishing a database on comparative mitochondrial phyisology. The SUIT-RA will be applied in the MitoFit proficiency test with HEK 293T cells. It includes a large number of chemicals used in various specific SUIT protocols, such that these chemicals are under quality control in the MitoFit proficiency test.

The SUIT-RA consists of two coordinated SUIT reference protocols (SUIT-RP). These are harmonized such that they can be statistically evaluated as replicate measurement for carefully selected respiratory states, while additional information is obtained when the two protocols are conducted in parallel. Therefore, the two SUIT-RP are complimentary with their focus on specific respiratory coupling and substrate control aspects, extending previous strategies for respirometrc OXPHOS analysis.

SUIT reference protocol: RP1(pre02)
PM + mt + D + c + (NADH) + U + Oct + G + S + Rot + Gp + Ama + AsTm + Azd

SUIT reference protocol: RP2(pre02)
D + mt + Oct + M_{tit} + P + c + (NADH) + G + S + U + Gp + Rot + Ama + AsTm + Azd

1. SUIT_RP1: CI-coupling/substrate control

PM + mt + D + c + (NADH) + U + Oct + G + S + Rot + Gp + Ama + AsTm + Azd

	CI	CI&FAO	CI&FAO	CI&II&FAO	CII	CII&GpDH	CIV
E	**PM**	**PMOct**	**PGMOct**	**PGMSOct**	**S**	**SGp**	**CIV**
P	**PM**						
L	**PM**						
	PM	Oct	G	S	Rot	Gp	Ama+AsTm+Azd

RP1 spotlights

- CI-linked linear coupling control: *L* – *P* - *E*, thus separating coupling control (CI-linked) and substrate control (in the ETS state).
- CI_E, $CI\&II\&FAO_E$ and CII_E are measured. If $CI\&II\&FAO_E \approx CI\&II_E$, then this sequence allows calculation of the additivitiy index of CI- and CII- linked ETS capacity (related to supercomplex-channeling). This criterium is tested in the step (+Oct), evaluating the effect of Oct on PM_E.
- Oct is added before G and S to compare RP1 with RP2: harmonization between protocols in states (1) $PGMSOct_E$, (2) SGp_E, (3) CIV_E.
- If Oct is without effect on PM_E (expected in many types of mt), then additional harmonization between protocols is obtained in state PM_P = $PMOct_P$.
- If Oct is without effect on PM_E, then we can conclude that it is also without effect on PGM_E and $PGMS_E$. On the other hand, if Oct added after $PGMS_E$ is without effect, then it is not clear if it would be without effect on PM_E and PGM_E.
- Harmonization with many previous protocols up to the step (+Rot).

Limitations:

- Depletion of endogenous substrates with D is not possible, to obtain CI_L without an inhibitor of ATP synthase (Omy).
- $PGMSOctGpDH_E$ is not obtained (substrate combination for maximum ETS capacity), in favour of measuring CII_E. This reference state has to be calculated using the $PGMSOct_E$/$PGMSOctGpDH_E$ ratio from RP2.

Substrate type: CI-CII-FAO-CGpDH-CIV

Substrate state category:
CI + CI&FAO + CI&II&FAO + CII + CII&GpDH + CIV

SUIT protocol name:
PM(LP)+c(PE)+Oct+G+S+Rot+Gp+Ama+AsTm+Azd

RP1-Pfi

Add	State	Comment
+PM		CI-linked substrates are added to the medium before the mt-preparation (mt). The state without added substrates is not well defined, slightly higher than ROX due to the presence of some endogenous substrates (shown by a slight decline of respiration and mt-membrane potential upon inhibition by Rot; Krumschnabel et al 2014).
+mt	PM_L	Incubation for about 20 min to allow stabilization of flux at high oxygen and during slow exhaustion of endogenous substrates, to obtain CI_L.
+D	PM_P	OXPHOS coupling efficiency (*P-L* or ≈*P* control factor), $j_{\approx P}$ = ≈*P*/*P* = (*P*-*L*)/*P* = 1-*L*/*P*, is measured in the CI-linked substrate state, with defined coupling sites (CI, CIII, CIV) and at high flux compared to FAO (human skeletal muscle: compare with FAO(*LP*) and CII(*PL*); Gnaiger et al 2015).
+c	PMc_P	Quality control test early in the SUIT protocol. In pathological states with c release, early addition of c provides information on the FCF_c = 1-CI/CIc, and separates the FCF_c from other injuries in the subsequent respiratory states. All subsequent states contain c, which is not explicitely written in the following substrate states.
(NADH)		NADH is titrated only in case of a high cytochrome *c* control factor, FCF_c>0.1, to check for the presence of inverted mitochondrial particles and permeability of the inner mt-membrane. If FCF_c<0.1, then the high intactness of the outer mt-membrane provides an indication for intactness of the inner mt-membrane, and thus NADH does not have to be added.
U	PM_E	CCCP is titrated stepwise to maximum flux, to evaluate limitation of OXPHOS by the phosphorylation system, expressed as the apparent excess *E-P* capacity factor (*E-P* coupling control factor), j_{ExP} = (*E*-*P*)/*E* = 1-*P*/*E*. If j_{ExP}>0, then the ETS coupling efficiency rather than the OXPHOS coupling efficiency is the proper expression of coupling, $j_{\approx E}$ = ≈*E*/*E* = (*E*-*L*)/*E* = 1-*L*/*E*.
+Oct	$PMOct_E$	FCF_{Oct} = 1-CI/CI&FAO low or zero in many mt-types. Inhibition is observed at higher FA

		concentrations. Then also state PM_P is identical to $PMOct_P$ in RP2, and may thus further link the two protocols in the SUIT reference assay for statistical analysis (protocol harmonization).
+G	$PGMOct_E$	FCR_G = 1-PMOct/PGMOct, reveals an additive effect of convergent electron flux through NADH (CI-linked), with a possible contribution by partially activating CII-linked respiration.
+S	$PGMSOct_E$	FCF_{CII} = 1-CI&FAO/CI&II&FAO. Additive effect of CI&II. It may be important if the uncoupler concentration titrated in the PM substrate state is also sufficient for this substrate state.
+Rot	S_E	FCF_{CI} = 1-CII/CI&II&FAO. Rot inhibits CI and FAO simultaneously. Additive effect of CI&II&FAO. In some cases it takes very long, until a steady state is reached after inhibition by Rot. Addition of Gp before Rot would not allow a valid estimation of CII-linked capacity (compare RP2).
+Gp	SGp_E	CGpDH capacity is not measured in the SUIT reference assay. FCF_{CGpDH} = 1-CII/CII&GpDH. This late addition of Gp is a compromise for evaluation of CGpDH capacity. Malonic acid does not effectively inhibit CII at S_{50} (competitive inhibition). Little is known about the diagnostic value of this CGpDH-flux control factor. Gp is expensive.
+Ama	ROX	Inhibition may take very long, particularly in human muscle fibres (Pesta et al 2011; Lemieux et al 2011). This may make ROX correction questionable, particularly if ROX is high in comparison with the initial LEAK state.
+AsTm	CIV_E	$Tm_{0.5}$ is not saturating CIV, and thus represents a compromise, to prevent a too high chemical O_2 background.
+Azd	$cAsTm_{ROX}$	Cyanide is avoided due to the presence of P, but very high Azd concentrations are required. The oxygen dependence of the chemical O_2 is evaluated by a reoxygenation soon after titration of Azd, and is automatically performed by using the DatLab background calibration function (Slope).

2. SUIT_RP2: FAO-OXPHOS

D + mt + Oct + M_{tit} + P + c + (NADH) + G + S + U + Gp + Rot + Ama + AsTm + Azd

		FAO	CI&FAO	CI&FAO	CI&II&FAO	CI&II&FAO&GpDH	CII&GpDH	CIV
E					PGMSOct	PGMSOctGp	SGp	AsTm
P		OctM	PMOct	PGMOct	PGMSOct			
L								
	D	Oct+M	P	G	S	Gp	Rot	Ama+AsTm+Azd

RP2 spotlights:

- Depletion of endogenous substrates with D (State 2).
- FAO_P compared to $CI\&FAO_P$.
- Measurement of maximum ETS capacity, obtained in state $PGMSOctGp_E$.
- Harmonization between protocols RP1 and RP2 in states (1) $PGMSOct_E$, (2) SGp_E, (3) CIV_E.
- Harmonization with many previous protocols up to (+S).
- *P/E* at high ETS capacity compared to RP1.

Limitations:

- CII_E is not obtained (but see RP1).
- The full substrate combination, $PGMSOctGp_P$ and $PGMSOctGp_E$, is not covered, and thus the maximum apparent excess *E-P* capacity factor, j_{ExP} = 1-*P/E*, may be missed.

Substrate type: CI-CII-FAO-CGpDH-CIV

Substrate state category:
FAO + CI&FAO + CI&II&FAO + CI&II&FAO&GpDH + CII&GpDH + CIV

SUIT protocol name:
D(ROX)+Oct(P)+M+P+c+G+S(PE)+Gp+Rot+Ama+AsTm+Azd

RP2-Pfi

Add	State	Comment
+D		ADP is added to the medium before the mt-preparation (mt).
+mt	D_{ROX}	D accelerates the depletion of endogenous substrates (State 2; Chance, Williams 1955; ROX; Gnaiger 2014).
+Oct	Oct_{ROX}	Oct alone does not establish an ETS (and OXPHOS) competent substrate state in many mt-types, since M is required to form oxaloacetate and prevent accumulation of acetyl-Co A by the citrate synthase reaction.
	Oct_P	Stimulation of OXPHOS by Oct alone in the presence of D indicates an obscure mechanism of anaplerosis.
+M	$OctM_P$	M is titrated stepwise: M.05; M.1; M2. Note that M alone can support OXPHOS if mt-malic enzyme is active, and thus FAO may be overestimated.

+P	$PMOct_P$	M_2 is required to reduce flux through CII (minimize inhibition by malonate), such that CI-linked OXPHOS capacity can be estimated without high scope of compensation by CII-linked respiration. GM_P includes a higher share of CII-linked respiration in comparison with PM_P. FCF_{CI} = 1-FAO/CI, important information on training status or cardiac failure (Pesta et al 2011; Lemieux et al, 2011).
+c		See RP1.
(NADH)		See RP1.
+G	$PGMOct_P$	See RP1.
+S	$PGMSOct_P$	See RP1. The state CI&II&FAO$_P$ is identical in RP1 and RP2, and may thus link the two protocols in the SUIT reference assay for statistical analysis (protocol harmonization).
+U	$PGMSOct_E$	CCCP is titrated in the CI&II state with high ETS capacity, to evaluate limitation of OXPHOS by the phosphorylation system. The apparent excess *E-P* capacity factor (*E-P* coupling control factor), j_{ExP} = (*E-P*)/*E* = 1-*P*/*E*, is not measured in the state of maximum ETS capacity, if Gp exerts an additional stimulation (RP1). If Gp stimulates ETS further, the *E-P* coupling control factor is underestimated in the absence of Gp.
+Gp	$PGMSOctGp_E$	RP2 focuses on maximum *E*. FCF_{CGpDH} = 1-CI&II&FAO/CI&II&FAO&GpDH. This CGpDH-flux control factor evaluates additivity at high ETS capacity, which can be compared with additivity on the basis of CII_E (RP1).
+Rot	SGp_E	This state is not a generally valid estimate of CII_E (compare RP1).
Further steps		See RP1.

3. Test experiments

Test experiments: Test experiments are required to finalize the RP2 for specific applications.

G	10 mM may not be saturating, and higher concentrations should be checked in a test experiment.
Gp	Different sources of GP are tested. Gp (type) is expensive.

3.1. SUIT RP1

D	D_5 ($D_{7.5}$ in Pfi) is tested to be saturating CI&II&FAO&CGpDH$_P$. 7.5 mM may not be saturating in all cases, and higher concentrations of ADP should be checked. Depletion of endogenous substrates with D is not possible, to obtain CI$_L$ without inhibitors. Check with Omy after (+c).
Oct	Oct.5 (0.5 mM) might be generally applicable, but in preliminary experiments a higher concentration (1 mM) should be evaluated to check for saturation of flux.
+S	Step titration from S_{10} to S_{50} to test if S_{10} is saturating CI&II- and CII-linked respiratory capacity. If fluxes with $S_{50} > S_{10}$ in CI&II$_E$, then S_{50} is added immediatle in the OXPHOS state. If $S_{50} < S_{10}$, then it is tested if $S_{50} > S_{10}$ in CII$_E$, in which case S_{50} is only added in CII$_E$. If S_{10} is saturating in all states, S_{50} may be tested only occasionally, to exclude a shift in the succinate kinetics (in pathologies, ageing, etc).

3.2. SUIT RP2

Oct	$Oct_{0.5}$ is tested to be saturating in OXPHOS and not inhibiting or uncoupling (titration of high Oct after M.05 or M.1).
M	$M_{0.1}$ is tested to be saturating FAO in OXPHOS without activating CI-linked respiration beyond FAO capacity (HEK: mtME). M should be titrated stepwise (M.05; M.1; M2) in the presence of D, to compare the malate kinetics of FAO$_P$ and CI$_P$.

4. Technical details

Temperature: 37 °C.
Data recording interval: 2 s.
Effective chamber volume: 2 ml
Stirrer speed: 750 rpm.

DatLab file:	The default name of the DatLab file contains the date, Power-O2k number and serial experimental number for each day. 2016-01-17 P1-02.DLD
O2k:	Enter Power-O2k number: P1, P2, P3, P4, ..
Experiment:	Experimental code, as in DatLab [F3].
Event:	Set an 'Event' in DatLab at the time of titration. Use the abbreviated event name, and add information in the comment.

MiR05+CtlCr:

Ctl is present in all cells, hence addition of Ctl is considered physiological, even if reoxygenations are not required with H_2O_2.

Cr is present in many vertebrate cells, and thus should be added generally. With Cr, lower ADP concentrations are saturating for OXPHOS. It may be argued that it should be replaced in invertebrates (*Drosophila, C. rabditis*).

MiR / O2: Mitochondrial respiration medium, 2 ml in the O2k-chamber, plus 100 µl in the capillary of the stopper (more accurately: 88 µl without meniscus). Increase the oxygen concentration to ~450 µM. Close the chamber.

mt mt-preparation: Imt, Pfi, Pc.

D If there is time available (20 min), this period may yield a single point for the instrumental high-O2k background. D may be added just before titrating mt or before opening the chamber for addition of Pfi.

Pfi / O2: During addition of Pfi, the O_2 concentration drops and should be increased immediately to ~450 µM before closing the O2k-chamber.

U: 'Slope smoothing' may be reduced, e.g. to 25 (=25 data points used for calculation of the slope), to evaluate very quickly the stimulation of respiration and the need for additional titration steps of CCCP. If only FCCP (more expensive) is available, this can be used and be fully compared with CCCP titrations (a minimally high CCCP than FCCP concentration may be required for maximum flux).

Cleaning After the experiment clean the O2k-chambers: 3x water, 1x liver homogenate (20 min), 3x water, 3x EtOH 70% (5 min), 1x EtOH 100% (15 min).

O2k-cleaning SOP
» http://bioblast.at/index.php/MiPNet19.03_O2k-cleaning_and_ISS

5. Author contributions, publication versions, references

This communication is a pre-publication prepared by CD and EG. CD, ZS, GL and GK performed test experiments, contributed to the concept and co-wrote the manuscript.

Contribution to the project MitoFit, funded by the Tyrolian Government within the program K-Regio of Standortagentur Tirol.
http://www.mitofit.org/index.php/O2k-MitoFit

Full version with references
» http://wiki.oroboros.at/index.php/MiPNet21.06_SUIT_reference_assay

SUIT reference protocol: RP1-Pfi(pre03)

RP1 spotlight: CI-coupling/substrate control

2016-01-19

PM + mt + D + c + (NADH) + U + Oct + G + S + Rot + Gp + Ama + AsTm + Azd

	CI	CI&FAO	CI&FAO	CI&II&FAO	CII	CII&GpDH	CIV
E	**PM**	**PMOct**	**PGMOct**	**PGMSOct**	**S**	**SGp**	**CIV**
P	**PM**						
L	**PM**						
	PM	Oct	G	S	Rot	Gp	Ama+AsTm+Azd

DatLab file: 2016- **Experimental code:** **Operator:**					**O2k:**	**P__** Chamber	
Event	**Mark name**	**Stock [mM]**	**Final conc. in O2k 2 ml**	**Comment**	**Titration [µl]**	**A**	**B**
MiR			MiR05+CtlCr		2000+100		
O2			~450 µM O_2				
P		2000	5 mM		5		
M		400	2 mM		10		
Pfi							
O2	PM(L)		~450 µM				
D	PM(P)	500	7.5 mM		30		
c	PMc(P)	4	10 µM		5		
NADH	PMcNADH(P)	280	2.8 mM	only if FCF_c >0.1	20		
U	PM(E)	1 CCCP	0.5 – 5 µM		1 µl steps		
Oct	PMOct(E)	100	0.5 mM		10		
G	PGMOct(E)	2000	10 mM		10		
S	PGMSOct(E)	1000	50 mM		100		
Rot	S(E)	1	0.5 µM		1		
Gp	SGp(E)	1000	10 mM		20		
Ama	ROX	5	2.5 µM		1		
O2			~450 µM				
As		800	2 mM		5		
Tm	CIV(E)	200	0.5 mM	~20 min	5		
Azd	ROX	4000	≥100 mM	~10 min	100		
O2	ROX		~450 µM	400 -> 250 µM			

SUIT reference protocol: RP2-Pfi(pre03)

RP2 spotlight: FAO-OXPHOS

2016-01-19

D + mt + Oct + M_{tit} + P + c + (NADH) + G + S + U + Gp + Rot + Ama + AsTm + Azd

		FAO	CI&FAO	CI&FAO	CI&II&FAO	CI&II&FAO&GpDH	CII&GpDH	CIV
E					PGMSOct	PGMSOctGp	SGp	AsTm
P		OctM	PMOct	PGMOct	PGMSOct			
L								
	D	Oct+M	P	G	S	Gp	Rot	Ama+AsTm+Azd

DatLab file: 2016- Experimental code: Operator:					O2k:	P__ Chamber	
Event	**Mark name**	**Stock [mM]**	**Final conc. in O2k 2 ml**	**Comment**	**Titration [µl]**	**A**	**B**
MiR			MiR05+CtlCr		2000+100		
O2			~450 µM				
D		500	7.5 mM		30		
Pfi							
O2	ROX		~450 µM				
Oct	Oct(P)	100	0.5 mM		10		
M.05	OctM.05(P)	50	0.05 mM		2		
M.1	OctM.1(P)	50	0.1 mM		2		
M2	OctM2(P)	400	2 mM		9.5		
P	PMOct(P)	2000	5 mM		5		
c	PMOctc(P)	4	10 µM		5		
NADH	PMOctcNADH(P)	280	2.8 mM	only if FCF_c >0.1	20		
G	PGMOct(P)	2000	10 mM		10		
S	PGMSOct(P)	1000	50 mM		100		
U	PGMSOct(E)	1 CCCP	0.5 – 5 µM		1 µl steps		
Gp	PGMSOctGp(E)	1000	10 mM		20		
Rot	SGp(E)	1	0.5 µM		1		
Ama	ROX	5	2.5 µM		1		
O2			~450 µM				
As		800	2 mM		5		
Tm	CIV(E)	200	0.5 mM	~20 min	5		
Azd	ROX	4000	≥100 mM	~10 min	100		
O2	ROX		~450 µM	400 -> 250 µM			

Supplement

A. General links

Introduction
» http://wiki.oroboros.at/index.php/Gnaiger_2014_MitoPathways

Table of titrations
» http://wiki.oroboros.at/index.php/MiPNet09.12_O2k-Titrations

Definition
» http://www.bioblast.at/index.php/Substrate-uncoupler-inhibitor_titration

Context
» http://www.mitofit.org/index.php/SUIT_protocol_library

Abbreviations
» http://www.bioblast.at/index.php/MitoPedia