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Mitochondrial Enzyme Activities Protocol

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Protocol status: Working
We use this protocol and it's working

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Abstract

Mitochondrial enzymatic activity assays developed in the Picard lab at Departments of Psychiatry and Neurology, Robert N Butler Columbia Aging Center, Columbia University Irving Medical Center, New York, NY, USA

Homogenization buffer

1

Product	[final]	For 800 ml
EDTA (MW: 292.24) Sigma # E9884	1 mM	0.2338 g
Triethanolamine (MW: 185.65) Sigma # T1502-100g	50 mM	7.4260 g
ddH2O		QSP 700 mL

Homogenization procedure for Mouse Tissue:

2
- Weigh tissue samples by transferring into pre-weighed 1.5ml Eppendorf tube
- Subtract empty tube weight from weight of tube containing sample to get sample weight
- Add 180µl of homogenization buffer per mg of tissue (1mg:180µl, weight:volume)
- Add 2 Tungsten beads (Qiagen cat# 69997) / tube

3
- Move tubes to pre-chilled Tissue Lyser (Qiagen cat # 85300) rack and run at 30 cycles/sec for 1 min
- Put rack back on ice and set a timer for 5 min
- Repeat homogenization in Tissue Lyser for 1min
- Quick spin in pre-cooled microcentrifuge to remove liquid from cap of tubes
- Place samples in -80 ^{O}C freezer until frozen
- Once frozen, thaw samples in room temperature water bath. Move racks around slowly in water bath to ensure circulation of water
- Repeat freeze-thaw cycle 2X. Final thaw of samples is performed on the day assays are run
- Vortex samples for 2 seconds to ensure homogeneity, transfer samples to 96-well block

CITRATE SYNTHASE (CS) ENZYMATIC ACTIVITY ASSAY

4 ***Assay Principle:***

Citrate synthase (CS) enzymatic activity is determined by the change in absorbance of DTNB (Ellman's reagent) measured at 412 nm. Thiol groups (R-SH) reach with DTNB, cleaving the disulfide bond to generate 2 NTB^{-} molecules, which ionizes to an NTB^{2-} dianion in water at neutral and alkaline pH. This NTB^{2-} ion has a yellow color and absorbs light at 412 nm. The reaction requires oxaloacetate to

proceed and generate thiol groups. The enzymatic activity of CS is determined by quantifying the rate of conversion of DTNB into the yellow NTB^{2-}. The molar extinction coefficient of DTNB is 13.6 L x mol^{-1} x cm^{-1}.

5 **Buffers and Reagents**

Buffer Tris 200 mM

Product	[final]	For 800 ml
Tris (MW: 121.14) (Sigma # T6066)	200 mM	19.3824 g
dH2O		(750 ml) QSP 800 ml

Adjust pH at 7.4 @ room temperature with 10M HCl (~ 5ml), store at 4^{O}C. Aliquot into 40 ml. Store at -30

Acetyl CoA 10 mM

Product	[final]	For 28 ml
Acetyl CoA trilithium salt (MW: 809.57) (Sigma # A2181)	10 mM	0.2267 g
ddH2O		28 ml

Make aliquots and store at -80^{O}C indefinitely. (6.2ml per 50mg bottle). Aliquot into 1.4 ml.

DTNB (Ellman's reagent) 10 mM

Product	[final]	For 28 ml
5,5'-dithiobis-(2-nitrobenzoic acid) DTNB (MW: 396.35) (Sigma # D8130)	10 mM	0.1109 g
Ethanol (ETOH)		28 ml

Make aliquots and store at -80^{O}C indefinitely. Aliquot into 1.4ml.

Oxaloacetic acid 2 mM

Product	[final]	For 32 ml
Oxaloacetate (MW: 132.07) (Sigma #O4126)	2 mM	0.0085 g
ddH2O		(25 ml) QSP 30 ml

Adjust pH to ~7.3 to 7.4 with KOH – this is difficult, be gentle. Make aliquots and store at -80^{O}C. Aliquot into 1.6 ml.

Triton X-100 10%

Product	[final]	For 14 ml
Triton X-100 (Sigma #T8532)	10% v/v	1.4 ml
ddH2O		14 ml

Preparation of assay buffer.

for ~1ml of buffer	*for 66 ml of buffer (50 samples)*

0.45 ml dH2O	29.7 ml
0.5 ml Tris Buffer	***33 ml***
20 ml Acetyl CoA, [final] = 200mM	***1.32 ml***
20 ml DTNB, [final] = 200mM	***1.32 ml***
10 ml Triton X-100 10%	***0.66 ml***

6 ***Spectrophotometer settings:***

- Assays are recorded on a Spectramax M2 (Spectramax Pro 6, Molecular Devices)
- The assay is run at 30 ^{o}C
- Read absorbance at 412 nm
- Set spectrophotometer to read only half of the plate at a time (to maximize reading time)

7 ***Running the assay:***

· In a 96-well plate, pipet **10 ml** of Oxaloacetate ([final] = 100mM).
· Pipette **10ml** of sample homogenate.
· Add **200ml** of CS assay mix to each well.
· Quickly put plate in spectrophotometer and read.
· Record slopes for each sample
· The negative control is the assay mix + samples without oxaloacetate; the slope should be ~0.
· Obtain a measure of each sample in duplicates

The enzymatic activity is determined with the molar extinction coefficient of DTNB = 13.6 mM^{-1}

COMPLEX II (SUCCINATE-UBIQUINONE OXIDOREDUCTASE) ENZYMATIC ACTIVITY ASSAY

8 ***Principle of assay:***

Succinate dehydrogenase (SDH, also succinate-ubiquinone reductase or Complex II of the electron transport system – EC 1.3.99.1) activity is determined by the change in absorbance of DCIP measured at 600nm. Oxidation of succinate and decylubiquinone by complex II reduces DCIP, which decreases its absorbance at 600nm. Rotenone and KCN are used to inhibit electron flow through complexes I and IV, respectively.
Addition of malonate (an inhibitor of SDH) at the end of the assay allows to measure DCIP reduction independently from SDH. SDH enzymatic activity is then computed from the

difference between the two slopes. The molar extinction coefficient of DCIP is 16.3 L x mol^{-1} x cm^{-1}.

Buffers and Reagents

Buffer KPi-EDTA (KPi same as for COX assay + EDTA)

Product	[final]	For 2.5 L
K2HPO4 (MW: 174.18)	50 mM	21.775 g
dH2O		QSP 2.5 L

Product	[final]	(From COX buffer)
KH2PO4 (MW: 136.09)	50 mM	--
dH2O		--

- Take 2.5 L of K_2HPO_4 and acidify by adding KH_2PO_4 (approx 50-100ml) until pH 7.8 to give ❶
- Take 2.5 L of ❶ and add 1.461 g EDTA (FW: 292.24) for a [final] of 2 mM, pH will go to 7.4
- Heat for 10 min on hotplate set to 200°C to dissolve EDTA, buffer temperature was at 40°C
- Aliquot into 125 ml. Store at -30

2,6-dichloroindophenol – DCIP (20mM)

Product	[final]	For 8.4ml
DCIP (MW: 290.08) Sigma # D1878	20 mM	0.0487 g
dH2O		QSP 8.4 mL

Aliquot into 420ul, Store at -30

Sodium-malonate (500mM)

Product	[final]	For 7 ml
Na-Malonate (MW: 166.04)	500 mM	0.5812g

Sigma# M1875-25g		
KPi-EDTA assay buffer		QSP 7 mL

Aliquot 7ml into 350 ul; Store at -30

Succinate-Tris (500mM)

Product	[final]	For 28 ml
Sodium succinate dibasic hexahydrate (MW: 270.14) Sigma # S2378	500 mM	3.7820 g
Make in Tris Base (MW: 121.14)	2 M	QSP 28ml

Tris Base 2M is made by adding 9.688g of Tris Base (Fisher #152-1, FW:121.1) in 40ml ddH_2O. Then take 25ml of this, and add succinate. Then QSP to 28ml in graduated cylinder. Aliquot into 1.4 ml, Store at -30

Decylubiquinone (20mM)

Product	[final]	For 14 ml
Decylubiquinone (MW: 322.4) Sigma # D7911	20 mM	0.0903 g
DMSO		QSP 14 mL

For a **25mg bottle**, add 3.877ml of DMSO for a [final] 20 mM. Aliquot into 700 ul; Store at -30

Potassium cyanide – KCN (50mM)

Product	[final]	For 14 ml
KCN (MW: 65.12) Sigma # 207810	50 mM	0.0456g
DMSO		QSP 14 mL

Aliquot 14ml into 700ul; Store at -30

Rotenone (500 µM)

Product	[final]	For 17 ml

Rotenone (MW: 394.42) Sigma # 8875	0.5mM (500 µM)	0.0034 g
DMSO		QSP 17 mL

Note: Dilute 9.8mg in 1ml of DMSO for 25 mM stock. Then 1:50 dilution (0.4ml 25mM in 19.6ml) in
DMSO to get 500uM.
Aliquot into 850 ul; Store at -30

5-Adenosine Triphosphate ATP (50 mM)

Product	[final]	For 6 ml
ATP (MW: 551.15) Sigma # A2383	50 mM	0.1654 g
ddH2O		QSP 6 mL

Aliquot 6ml into 300 ul; Store at -30

Antimycin A (400 µM)

Product	[final]	For 3 ml
Antimycin A (MW: 548.46) Sigma # A8674	0.4 mM (400 µM)	0.0007 g
DMSO		QSP 3 mL

Aliquot 3ml into 150 ul. Freeze 3 x 3ml aliquots.

Bovine Serum Albumin – BSA (50mg/ml)

Product	[final]	For 200 ml
BSA, essentially fatty acid free 99% Sigma # A3059 (or A6003, A3733)	50 mg/ml	10.0000 g
ddH2O		QSP 200 mL

Aliquot into 10 ml.

Preparation of buffer and samples:

For 1ml of buffer	For 66ml buffer (50 samples)
934 µl KPi-EDTA buffer	61.644
20 µl BSA [final] = 1 mg/ml	1.320
8 µl Rotenone [final] = 4 µM	0.528
20 µl Succinate [final]= 10 mM	1.320
5 µl KCN [final] = 0.25 mM	0.330
5 µl Decylubiquinone [final] = 100 µM	0.330
5 µl DCIP [final] = 100 µM	0.330
4 µl ATP [final] = 0.2 mM	0.264
1 µl Antimycin A [final] = 0.4 µM	0.066

Take **33ml** of the total 66ml, and add 330ul of Malonate for **Malonate^{+}**, and 330ul of KPi-EDTA for **Malonate^{-}**.

9 ***Spectrophotometer settings:***

- Assays are recorded on a Spectramax M2 (Spectramax Pro 6, Molecular Devices)
- The assay is done at 30 ^{o}C
- Read absorbance at 600 nm

- Set spectrophotometer to read only half of the plate at a time to maximize reading time (read every 17 sec)

Running the assay:

· Set the spectrophotometer to read absorbance at **600nm**, in Kinetics mode
· Pipette **10ml** of homogenate in each well.
· Add **200ml** of SDH assay mix to each well.
· Read plate for **30 min**.

· The negative control is the assay mix with
Malonate, an inhibitor of Complex II
· Obtain a measure of each sample in duplicates
· The enzymatic activity is determined with the molar extinction coefficient of DCIP = 16.3 L x mol^{-1} x cm^{-1}.

COMPLEX I (NADH-UBIQUINONE OXIDOREDUCTASE, EC 1.6.99.3) ENZYMATIC ACTIVITY ASSAY

10 ***Principle of assay :***
The activity of Complex I of the electron transport system (also NADH-Ubiquinone Oxidoreductase EC 1.3.99.3) is determined by the change in absorbance of DCIP, which accept electrons from CI.
Oxidation of NADH and decylubiquinone by the enzyme leads to a decrease in the absorbance of DCIP as it is reduced. Antimycin A and KCN are used to inhibit electron flow through complexes III and IV, respectively. Addition of Rotenone and Piericidin A (inhibitors of Complex I) allows measurement of non-specific NADH oxidation. Specific Complex I enzymatic activity is then computed from the difference between the two slopes. The molar extinction coefficient of DCIP is 16.3 L x mol^{-1} x cm^{-1}.
The molar extinction coefficient of NADH is 6.22 L x mol^{-1} x cm^{-1}.
This assay was modified based on the method of Janssen et al. Clin Chemistr 2007; 53(4):729-734. [BSA] was increased from 1 to 3.5mg/ml, and DCIP 1000uM was added.

Buffers and Reagents
NADH
(80mM)

Product	[finale]	For3.5 mL
NADH (MW: 709.4) Sigma N8129	80 mM	0.1987 g
KPi buffer (from COX assay)		QSP **3.5** mL

Aliquot 3.5ml into 175 ul; Freeze 3.5ml.

Piericidin A (200mM)

Product	[finale]	For 3.609 mL
Piericidin A (MW: 415.57) Sigma P4368-100mg	0.2mM (200 **µ**M)	0.0003 g
DMSO		--

Piericidin A comes as 100ug in 100ul solution. Take three vial (3), and add 1.103ml of DMSO to each for a final of 200uM. Combine the three for a final of 3.609ml. Piericidin A is 4X more potent than Rotenone, [final] will be 1/4.

Aliquot into 175ul

Products already prepared under the heading of other assays, and aliquoted in double amounts.

- 250 ml of Buffer KPi (50mM)
- BSA (50mg/ml)
- Decylubiquinone (20mM)
- KCN (20 mM)
- Antimycin A (400 µM)
- Rotenone (200 µM)

Preparation of buffer and samples:

For 1ml	66ml buffer (50 samples)
912 µl KPi-EDTA buffer	60.16
70 µl BSA [final] = 3.5 mg/ml	4.620

5 µl KCN [final] = 0.25 mM	0.330
5 µl DCIP [final] = 100 µM	0.330
2.5 µl NADH [final] = 200 µM	0.165
5 µl Decylubiquinone [final] = 100 µM	0.330
1 µl Antimycin A [final] = 0.4 µM	0.066

Take **33ml** of the total 66ml, and add 264ul of Rot (500uM) and 165ul of Pier A (200uM) for **Rot/PierA^{+}**, and 429ul of DMSO for **No-inhibitor**.
Be careful not to introduce inhibitors in the non-inhibited mix.

Spectrophotometer settings:

- Assays are recorded on a Spectramax M2 (Spectramax Pro 6, Molecular Devices)
- The assay is run at 37^{o}C
- Read absorbance at 600 nm

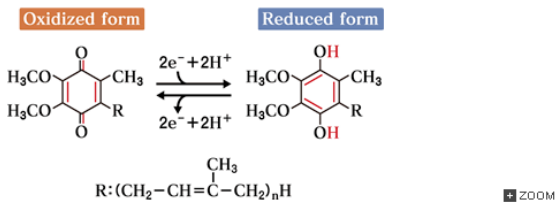
Running the assay:

· Set the spectrophotometer to **37^{o}C** to read absorbance at **600nm**,
in Kinetics mode.
· Pipette **10ml** of homogenate in each well
· Add **200ml of assay buffer** to each well.
· Quickly put plate in spectramax and read.
· The adequate negative control is the assay mix with Rotenone and Piericidin A (Complex I inhibitors)
· Obtain a measure of each sample in duplicates
· The enzymatic activity is determined with the molar extinction coefficient of DCIP = 16.3 L x mol^{-1} x cm^{-1}.

COMPLEX IV (COX – CYTOCHROME C OXIDASE) ENZYMATIC ACTIVITY ASSAY

11 ***Assay Principle:***

Cytochrome c oxidase (COX, Complex IV of the electron transport system) enzymatic activity is determined by the decrease in absorbance of cytochrome c (cyt c) induced through its oxidation by COX. For the assay to work, it is essential to provide purified reduced cyt c. This is accomplished by preparing cyt c with dithionite, and then by purifying it from dithionite with filter tubes. Enzymatic activity is obtained by quantifying the rate of conversion from reduced to oxidized cyt c. The molar extinction coefficient of reduced cyt c is 29.5 L x mol^{-1} x cm^{-1}.



12 **Buffers and Reagents**

Buffer KPi 50mM

Product	[final]	For 700 ml
K2HPO4 (FW : 174.18)	50 mM	6.096 g
dH2O		QSP 700 mL

Product	[final]	For 700 ml
KH2PO4 (FW : 136.09)	50 mM	4.76 g

dH2O		QSP 700 mL

- Take 700 ml of K_2HPO_4 and acidify by adding KH_2PO_4 (about 100-200 ml) until pH 7.5.
- KEEP KH_2PO_4 to acidify SDH buffer

Aliquot into 35 ml for both COX and Complex I assays. Store at -30

n-Dodecylmaltoside 10% w/v

Product	[final]	For 10 ml
n-Dodecylmaltoside (1 g) Calbiochem # 324355 (Sigma #D4641)	10% weight/volume	1.0 g
dH2O		QSP 10 mL

Add 10ml to 1g bottle and rock slowly because this product emulsifies easily. Aliquot into 700ul. Store at -30

13 **Buffers and Reagents**

Buffer KPi 50mM

Product	[final]	For 700 ml
K2HPO4 (FW : 174.18)	50 mM	6.096 g
dH2O		QSP 700 mL

Product	[final]	For 700 ml
KH2PO4 (FW : 136.09)	50 mM	4.76 g
dH2O		QSP 700 mL

- Take 700 ml of K_2HPO_4 and acidify by adding KH_2PO_4 (about 100-200 ml) until pH 7.5.
- KEEP KH_2PO_4 to acidify SDH buffer

  Aliquot into 35 ml for both COX and Complex I assays. Store at -30

n-Dodecylmaltoside 10% w/v

Product	[final]	For 10 ml
n-Dodecylmaltoside (1 g) Calbiochem # 324355 (Sigma #D4641)	10% weight/volume	1.0 g
dH2O		QSP 10 mL

Add 10ml to 1g bottle and rock slowly because this product emulsifies easily. Aliquot into 700ul. Store at -30

14 **Preparation of buffer and samples:**

For 1ml of buffer:	***For 66ml of buffer:***
0.49 ml dH2O	***31.9 ml***
0.5 ml KPi Buffer	***33 ml***
10 ml Dodecylmaltoside 10% ([final] = 0.1%)	***0.66 ml***
(variable amount) ml reduced cyt c [final cyt c] = 120mM	(variable amount) ml

Spectrophotometer settings:

- Assays are recorded on a Spectramax M2 (Spectramax Pro 6, Molecular Devices)
- The assay is run at 30^{o}C
- Read absorbance at 550 nm

Running the assay:

- Set the spectrophotometer to read absorbance at **550nm**, in Kinetics mode
- Pipette **10ml** of homogenate in each well.
- Add **200ml** of COX assay mix to each well.
- Quickly put plate in spectrophotometer and read for 10min.
- The negative control is the assay mix without tissue homogenate; the slope should be 0.
- Obtain a measure of each sample in duplicates.
- The enzymatic activity is determined with the molar extinction coefficient of Cyt c = 29.5 L x mol^{-1} x cm^{-1}.

PREPARING REDUCED CYTOCHROME C FOR COX ASSAY

15 **PREPARING REDUCED CYTOCHROME C FOR COX ASSAY**

Buffers and Reagents (Potassium Phosphate – KPi 50mM)

Product	[finale]	Pour 700 ml
K2HPO4 (FW : 174.18)	50 mM	6.096 g
ddH2O		QSP 700 mL

Product	[finale]	Pour 700 ml
KH2PO4 (FW : 136.09)	50 mM	4.76 g
ddH2O		QSP 700 mL

Take 250 ml of K_2HPO_4 and acidify by adding KH_2PO_4 (about 75ml) until pH 7.5

For the final COX assay mix, 1 ml of reaction buffer requires 2-6ml of the final concentrated cyt c. When measuring the reaction in a 96-well plate, each well will contain 200ml; so you need 0.5-1.2ml of concentrated cyt c per well. For example, if you are running 50 different samples measured in triplicates, you will need about 360ml of cyt c: [1.2ml x (50 samples x 6 measures) = 360ml]. Make sure there is enough in the freezer before you start.

NOTE: the final usable volume for the assay will be about 20-25% of that prepared from the beginning. For example, preparing 20 ml will yield about 9-10 ml of concentrated reduced cyt c.

16 **Dilution and reduction**

- To prepare 20ml (initial) of cyt c, take 100ml of KPi buffer (50mM) and adjust pH at 7.0

- The extra 80 ml are for the washing steps later in the protocol

- Add 20ml of KPi buffer 50mM to a 5 g bottle of cytochrome c (Sigma C7752-5g) (250mg/ml)

- Transfer to a 50ml Falcon tube and put on ice in the dark

· Add 150 (300mg was added) mg of sodium bisulfite (dithionite) (7.5 mg/ml) (Sigma 157953, MW: 174.11)

· Incubate for 5 minutes on ice and protect from light

· Keep at 4^{o}C from that point on

Purification

· Put 500 ml of the mix (KPi buffer + cyt c + dithionite) in 1.5 ml size ultracentrifugation filter tubes 10K MWCO (Amicon Ultra-0.5 mL 10kD Centrifugal Filters – Millipore UFV2BGC10[[1]]) [*Note: 3kD will also work*]

o Vivaspin 2, # VS0291 (3000MWKO)

· Centrifuge for 10 minutes at 10 000g for 1.5mltubes. The goal is to drain about 75% of the added volume. The sodium dithionite leaves through the filter with the buffer but cyt c is too big to go
through and remains in the tube. [*Note: 3kD columns will need to be spun down faster and for much longer*]

· Re-fill the filter tube with ice cold buffer (KPi 50mM) to re-suspend and rinse the cyt c.

· Repeat centrifugation and rinse steps for a total of 4 washes and about 40 minutes of centrifugation.

· After the last centrifugation, invert filters in new tube bottoms provided in the kit. Spin these tubes at 1000g for one minute to retrieve the concentrated, purified cyt c. For a starting volume in each tube of 600ml, you should extract 100-200ml at the end.

· Pool all collected product in one tube so that you have an homogenous mix. Quickly vortex.

· Estimate the volume and leave on ice and protected from light.

Measuring the concentration and purity of reduced cytochrome c

· Prepare two tubes for quantification

o **1ml** of purified cyt c to 0.5 ml of KPi buffer (1:500)

o **2ml** of purified cyt c to 0.5 ml of KPi buffer (1:250)

o **4ml** of purified cyt c to 0.5 ml of KPi buffer (1:125)

· Use the spectrophotometer in *Absorbance/Spectrum* mode to quantify optical density

.

Set “Blank” wells in plate layout (to correct for background) with only KPi buffer

.

Load 200ul of each sample in duplicates

.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank										
B	1:500	1:500										
C	1:250	1:250										
D	1:125	1:125										
E												
F												
G												
H												

E.g.:

· Run a *Spectrum* between 500 and 600nm (5nm intervals) to obtain the absorbance of cytochrome c at different wavelength

· **The purity** of reduced cytochrome c is calculated by the ratio of $OD_{\mathbf{550}}$(reduced cyt c) et $OD_{\mathbf{565}}$
(oxidized cyt c). Oxidized cytochrome c will not contribute to the reaction rate. The ratio OD_{550}/OD_{565} should be > 6-10.

· **The concentration** of reduced cytochrome c is determined by OD_{550} x 500[**[1]**] / ($29.5^{[3]}$ L x mol^{-1} x cm^{-1}[4]). Use the concentrated cyt c at a [final] of 100mM for the COX assay. Determine the amount to add per ml of buffer to arrive at **120mM of reduced cyt c**. This should be between 2-10ml of cyt c per ml of buffer.