**OROBOROS INSTRUMENTS**
high-resolution respirometry

**Protocols**

*Mitochondrial Physiology Network* 08.15: 1-8 (2010)

Version 3: 2010-08-23

# Laboratory Protocol Complex I (NADH:Ubiquinone Oxidoreductase; EC 1.6.5.3) Mitochondrial Membrane Enzyme

Andrey V Kuznetsov, Erich Gnaiger

**OROBOROS INSTRUMENTS Corp.,** high-resolution respirometry
Schöpfstr. 18, A-6020 Innsbruck, Austria
erich.gnaiger@oroboros.at; www.oroboros.at
Medical University of Innsbruck, Department of Transplant Surgery
D. Swarovski Research Laboratory
Innrain 66/6, 6020 Innsbruck, Austria

Section Page

# 1 Background

Complex I (CI) is the segment of the electron transport system (integral enzyme of the inner mitochondrial membrane) responsible for electron transfer from NADH to ubiquinone. CI is sensitive to different pathologies, particularly to oxidative stress, which is involved in ischemia-reperfusion injury, anoxia/ reoxygenation, aging, etc (Kuznetsov et al 2004; Rouslin & Millard 1981; Rouslin & Ranganathan, 1983; Rouslin, 1983). For the assessment of CI activity, among the ubiquinone isoprenologs, it is most convenient to use ubiquinone-1 ($CoQ_1$) as electron acceptor, because of its relative water solubility. Importantly, $CoQ_1$ yields one of the lowest rotenone insensitive rates and a high enzymatic rate. It is, therefore, the best electron acceptors for the CI assay.

## 1.1 Enzymatic reaction catalyzed by CI

NADH + $H^+$ + ubiquinone-1 ($CoQ_1$) → $NAD^+$ + dihydroubiquinone-1 ($CoQH_2$) (1)

## 1.2 Principle of spectrophotometric CI enzyme assay

**Absorbance and enzyme activity**: The optical density, *OD,* of a liquid sample is related to the absorbance, *A,* by the optical path length, *l* [cm],

$$OD = A / l = \varepsilon_B \cdot c_B \quad (2)$$

The unit of *A* is a dimensionless number. The path length is fixed by the dimension of the spectrophotometric cuvette. The molar extinction coefficient of the absorbing substance B, $\varepsilon_B$ [$mM^{-1} \cdot cm^{-1}$], is specific for the compound studied at a particular wavelength, and absorbance increases with molar concentration, $c_B$ [mM], in the final solution contained in the cuvette. The rate of increase or decrease of the absorbance is the slope, $r_A$ = d*A*/d*t*, which is proportional to enzyme activity.

**CI assay**: In the spectrophotometer, the reaction catalyzed by CI (Eq. 1) is associated with oxidation of the absorbing at 340 nm compound NADH to its non-absorbing oxidized form $NAD^+$ (Hatefi & Stiggall, 1978).

### 1.3 Temperature of enzyme assay

When CI activity is used as an indicator of mitochondrial damage, it is not critical to choose a physiological temperature. A constant reference temperature has to be applied for comparability of measurements. Commonly measurements can be performed at room temperature, but possibly more frequently at 30 °C (Hatefi & Stiggall, 1978; Jewess & Devonshire, 1999). Since citrate synthase activity is measured in our laboratory at 30 °C [MiPNet08.14], the same temperature is chosen for CI.

## 2 Reagents and Buffers

### 2.1 Prepare every month new and store at 4 °C

**K-phosphate buffer (0.1 M, pH 7.4):** Add 1.361 g $KH_2PO_4$ to 80 ml a.d., adjust to pH 7.4 with 5 N KOH, and adjust volume to 100 ml.

### 2.2 Prepare and store at -20 °C

Store at –20° C. Store on ice during measurement, freeze it again after the experiment.

**Coenzyme $Q_1$ (ubiquinone-1) (2.5 mM):** Add 1 ml ethanol to a commercial package of 10.0 mg $CoQ_1$, to obtain a c. 39.9 mM solution (stock A; for long-term storage). Dilute 100 µl of stock solution A with 1.498 ml ethanol to obtain a 2.5 mM stock solution B (for use and short-term storage).

**$NaN_3$ (1 M):** Add 1 ml a.d. to 65 mg $NaN_3$.

**Rotenone (0.5 mM):** Add 10 ml ethanol to 1.97 mg rotenone.

### 2.3 Prepare everyday new

**KCN (0.5 M):** Add 0.4 ml a.d. to 13.0 mg KCN.

**NADH (15 mM):** Add 1 ml a.d to 11.5 mg NADH.

## 2.4 Chemicals

| Name | FW | Source No. | Stock Solution | Comments |
|---|---|---|---|---|
| K-phosphate $KH_2PO_4$ | 136.09 | Merck 4873 | M; 1.361 g/100 ml; a.d. | Adjust to pH 7.4 by KOH, to obtain K-phosphate buffer. |
| Ubiquinone-1 (Coenzyme $Q_1$) $C_{14}H_{18}O_4$ | 250.30 | Sigma C7956 | 39.95 mM; 10.0 mg/1 ml Ethanol 2.5 mM; dilute 15.98 times with a.d. | Light sensitive. Store at –20 °C. Avoid contact and inhalation. |
| NADH (Nicotin-amide adenine dinucleotide reduced) Disodium salt, Trihydrate $C_{21}H_{27}N_7Na_2O_{14}P_2 \cdot 3\ H_2O$ | 763.46 | Fluka 43423 | 15 mM; 11.5 mg/1 ml a.d. | Electron donor. Hydroscopic, sensitive to air. Keep at 4 °C. |
| Sodium azide, $NaN_3$ | 65.01 | Sigma S8032 | M; 65.0 mg/1 ml a.d. | Divide into 0.2 ml portions. Store frozen at –20 °C. Very toxic. |
| Potassium cyanide KCN | 65.12 | Sigma 20,781 O | M: 13.0 mg/0.4 ml phosphate buffer (1 M). | Prepare everyday new. The pH of the solution may be very alkaline; therefore, use 1 M phosphate buffer (pH 7.0 at RT). Photosensitive. Hygroscopic. Very toxic. |
| Rotenone, $C_{23}H_{22}O_6$ | 394.40 | Sigma R8875 | 0.5 mM: 1.97 mg/10 ml Ethanol | Store at –20 °C. Light sensitive. Very toxic. |

## 3 Sample preparation

Freeze samples in liquid nitrogen and store frozen at -80 °C or in liquid nitrogen. CI activity of cardiac homogenates is stable during storage (a few hours) on ice. Separation of the enzyme complex from the mitochondrial inner membrane inhibits enzyme activity and leads to changes in its interactions with ubiquinone and inhibitors, which require phospholipids. On the other hand, intact inner mitochondrial membrane is not permeable for substrates (NADH). It is crucial, therefore, to provide an access of substrates (NADH) to the enzyme complex.

Ultrasound treatment (sonification) produces inverted submitochondrial particles, in which respiratory complexes are exposed to the bulk phase. Thus, it is necessary to sonicate samples before measurement (see below).

15 to 20 samples, including standards, can be processed as a batch.

## 3.1 CI standard

**Preparation:** One drawback is that CI is usually not commercially available as a pure enzyme and additionally it has rather low stability. A sonicated mitochondrial suspension of well defined protein or phospholipids concentration (from rat heart or rat liver) could be considered as a standard for CI assay. Stability of this preparation (at –20 °C) has to be checked and is expected to be better at higher concentrations. Hence the appropriate dilution of the stock suspension (or even additional sonication) may be required.

## 3.2 Sample preparation for the assessment of CI in cardiac muscle (or liver) homogenates

Mince frozen tissue samples (40-100 mg) with scissors, place into ice-cold 0.1 M K-phosphate buffer, pH 7.4 at 25 °C (buffer is added to yield a concentration of 30 mg tissue per ml). Homogenize for 30 s with Ultra-Turrax homogenizer at maximum speed at 0 °C. Centrifuge homogenates at 70,000x*g* for 30 min (4 °C) for complete sedimentation of membranous fractions. (Supernatants can be used for citrate synthase and LDH measurement). Resuspend the resultant pellet in the same volume of ice-cold phosphate buffer, sonicate with Bandelin Sonopuls HD 200 ultrasonicator (MS 73 probe tip; Berlin, Germany) 2 times for 20 s on ice. Keep the resultant suspension on ice during the enzyme assay.

## 3.3 Isolated mitochondria

Due to high CI activity of isolated mitochondria, the frozen mitochondrial stock suspension (-80 °C; usually ca. 50 mg of mitochondrial protein per ml) is diluted (1:10). Immediately after thawing, therefore, add 20 µl of mitochondrial suspension to 180 µl of 0.1 M Tris-HCl buffer, pH 7.0 (RT). During measurement, store on ice. Freeze stock suspension again. 25 µl mitochondrial suspension (5 mg/ml) is used for each spectrophotometric measurement. Sonicate the diluted suspension like a tissue homogenate.

## 3.4 Suspended cells

For typical cells (HUVEC, lymphocytes) at 1-2·$10^6$ cells/ml, take replicates of 110- 200 µl samples into

Eppendorf tubes, freeze in liquid nitrogen, and store at –80 °C until measurement. Sonicate cell suspensions before measurement like a tissue homogenate or mitochondria.

# 4 Measurement: Spectrophotometer Beckman DU 640

## 4.1 Switch on the spectrophotometer

Switch on the spectrophotometer ca. 10 min before measurement.
Power up diagnostic **--> *quit***
Routine measurement **--> *kinetics/time***
**--> *method name: A:\Complex1.*** There is the possibility to change options; simply click on the desired option.
**--> *exit***

## 4.2 Blank-measurement

**--> *vis on*** [visible lamp, light will be switched on immediately, the sign becomes red]. ***uv on*** [mercury lamp, light will be switched with delay, the sign becomes also red].
Add 1 ml phosphate buffer into quartz cuvette. Do not use plastic cuvettes, since these yield lower activities compared to quartz.
Insert cuvette into spectrophotometer.
**--> *blank*** [value of blank will be saved automatically, blue sign „reading blank"].

## 4.3 Preparation of incubation medium

To obtain a total volume, $V_{cuvette}$, of 1000 µl in the cuvette (including the volume of sample, $V_{sample}$, normally 25 µl for heart homogenates) add into glass tubes:

1. (940 µl - $V_{sample}$) phosphate buffer
2. 20 µl 15 mM NADH (final concentration ca. 0.3 mM)
3. 40 µl Coenzyme $Q_1$ stock B (final concentration ca. 0.1 mM)
4. 2 µl KCN (final concentration ca. 1 mM)
5. 2 µl $NaN_3$ (final concentration ca. 2 mM)

Mix carefully.
Pre-incubate medium in thermostat at 31 °C (5-10 min) for measurements at 30 °C.

## 4.4 Measurement of changes in absorbance

.

Transfer incubation medium into pre-thermostated spectrophotometric quartz cuvette.
Immediately put the cuvette into the cell holder of spectrophotometer.
Add the sample, $V_{sample}$, i.e. 100-200 µl cell suspension, 20 µl heart homogenates (50 µl liver homogenates) or 10-20 µl mitochondrial suspension, into prepared quartz cuvettes.
Mix carefully with plastic stick, wash stick with a.d.
***--> read sample, --> start.***
The linear decrease of absorbance is measured over ca. 100 s (normally this is proportional up to 0.15-0.2 units of absorbance per minute).
Another possible way for mixing is to add sample directly into pre incubated thermostat medium, mix with Vortex and immediately transfer into the quartz cuvette pre-thermostated (5 min, 30 °C) in the spectrophotometer cuvette holder.
After ca. 100 s (middle of time range) add 2 µl 0.5 mM rotenone (final concentration 1 µM) to recorder (measurement time 100 s) rotenone insensitive activity of the preparation. Normally it should be less than 15 % of original rate for isolated mitochondria (typically 2-5 % for heart and 30-50 % for liver homogenates), which will be later subtracted to obtain rotenone sensitive CI activity.
***--> rates*** [squares show data points].
Change *A* scale if necessary [click on upper or lower y-axis value], or use ***zoom.***
***--> trace*** [click on trace and define your starting and final times, cursor is placed on your defined starting or final points], change initial and final time points to measure the rate within linear part of the curve.
Write into the protocol the measured rate of absorbance change, $r_A = dA/dt$ (see Section 5).
***--> exit***
***--> save clear -***
There is a possibility to save the data ***file name*** [choose file name] ***- OK - OK***
Use ***save*** in exceptional cases, due to very limited memory of Beckman's computer.
For non-saving mode make square empty (click with mouse).
Device is ready for measurement of next sample.

# 5 Data Analysis: Calculation of Specific CI activity

## 5.1 Absorbance, concentration and rate of reaction

The rate of concentration change of the absorbing compound B in the cuvette, $dc_B/dt$, is calculated from the rate of the absorption change (Eq. 2),

$$dc_B/dt = \frac{dA/dt}{l \cdot \varepsilon_B} = \frac{r_A}{l \cdot \varepsilon_B} \quad (3)$$

The reaction flux per unit volume, $J_V$, in the cuvette is,

$$J_V = dc_B/dt \cdot \nu_B^{-1} \quad (4)$$

where $\nu_B$ is the stoichiometric number of compound B (Gnaiger, 1993), which is equal to 1 in the reaction (1).

## 5.2 Specific enzyme activity: reaction rate per unit sample

The specific enzyme activity is proportional to the experimental reaction flux (Eq. 4) and inversely proportional to the dilution factor, $V_{sample}/V_{cuvette}$ and to the mass concentration, $\rho$ [mg·cm$^{-3}$] or cell density [$10^6$·cm$^{-3}$] in the sample, $V_{sample}$. The specific enzyme activity, $v$, is the velocity of the enzyme-catalyzed step per unit of sample, measured under experimental incubation conditions with saturating substrate. Combining Eq.(3) and (4),

$$\text{Specific activity:} \qquad v = \frac{r_A}{l \cdot \varepsilon_B \cdot \nu_B} \cdot \frac{V_{cuvette}}{V_{sample} \cdot \rho} \quad (5)$$

| | |
|---|---|
| $v$ | Specific activity of the enzyme is expressed per mg protein or per million cells [IU/mg protein or IU/$10^6$ cells], depending on $\rho$. Enzyme activity is frequently expressed in international units, IU [µmol/min]. 1 IU of CI oxidizes 1 µmol of NADH per min. Note that the minute is used here as the unit of time (although the second is the preferred *SI* base unit; Gnaiger, 1993). |
| $r_A = dA/dt$ | Rate of absorbance change [min$^{-1}$] (Eq. 3). |
| $l$ | Optical path length (= 1 cm). |
| $\varepsilon_B$ | Extinction coefficient of B (NADH) at 340 nm and pH 7.4 (= 6.22 mM$^{-1}$·cm$^{-1}$). |
| $\nu_B$ | Stoichiometric number of B (NADH) in the reaction (Eq. 1) (= 1). |
| $V_{cuvette}$ | Volume of solution in the cuvette (= 1000 µl). |
| $V_{sample}$ | Volume of samle added to cuvette (100 µl, 25 µl, 5 µl). |

$\rho$ Mass concentration or density of biological material in the sample, $V_{sample}$ (fresh weight of tissue, protein concentration: $mg \cdot cm^{-3}$; cell density: $10^6 \cdot cm^{-3}$).

## 6 References

Gnaiger E. (1993) Nonequilibrium thermodynamics of energy transformations. *Pure & Applied Chem.* 65: 1983-2002.

Hatefi Y, Stiggall DL (1978) Preparation and properties of NADH: cytochrome c oxidoreductase (complex I--III). *Methods Enzymol.* 53: 5-10.

Jewess PJ, Devonshire AL (1999) Kinetic microplate-based assays for inhibitors of mitochondrial NADH:ubiquinone oxidoreductase (complex I) and succinate:cytochrome c oxidoreductase. *Anal. Biochem.* 272: 56-63.

Rouslin W (1983) Mitochondrial complexes I, II, III, IV, and V in myocardial ischemia and autolysis. *Am. J. Physiol* 244: H743-H748.

Rouslin W, Millard RW (1981) Mitochondrial inner membrane enzyme defects in porcine myocardial ischemia. *Am. J. Physiol*. 240: H308-H313.

Rouslin W, Ranganathan S (1983) Impaired function of mitochondrial electron transfer complex I in canine myocardial ischemia: loss of flavin mononucleotide. *J. Mol. Cell Cardiol.* 15: 537-542.

### Protocols

MiPNet08.14 Citrate synthase: laboratory protocol.