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# Laboratory Protocol
# Citrate Synthase
# Mitochondrial Marker Enzyme

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## 1 Background

Citrate synthase (E.c. 4.1.3.7) is a pace-maker enzyme in the Krebs cycle (citric acid cycle). Citrate synthase, CS, has a molecular weight of 51,709 Da, with gene

map locus 12q13.2-q13.3. CS is localized in the mitochondrial matrix, but is nuclear encoded, synthesized on cytoplasmic ribosomes and transported into the mitochondrial matrix. CS, therefore, is commonly used as a quantitative marker enzyme for the content of intact mitochondria (Holloszy et al., 1970; Willimas et al., 1986; Hood et al., 1989), although this role of CS has been questioned in developmental (Drahota et al., 2004) and age-related studies (Marin-Garcia et al., 1998). Proliferation of mitochondria in pathological states is normally associated with an increase in citrate synthase activity per cell, but CS activity in a specific tissue is frequently constant when expressed per mitochondrial protein. Mitochondrial, cellular or tissue respiration, therefore, may be expressed per CS activity for specific applications (Kuznetsov et al., 2002; Renner et al., 2003; Hütter el al., 2004).

## 1.1 Enzymatic reaction catalyzed by citrate synthase

CS catalyzes the reaction of 2 carbon acetyl CoA with 4 carbon oxaloacetate to form 6 carbon citrate, thus regenerating coenzyme A.

Acetyl-CoA + oxalacetate + $H_2O \rightarrow$ citrate + CoA-SH (1)

## 1.2 Principle of spectrophotometric enzyme assay

(Srere, 1969; Bergmeier, 1970)

**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],

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

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 of the absorbance is the slope, $r_A$ = d*A*/d*t*, which is proportional to enzyme activity.

**Citrate synthase assay**: In the spectrophotometer, the rate-limiting reaction catalyzed by CS (Eq. 1) is coupled to the effectively irreversible chemical reaction (Eq. 3),

$$\text{CoA-SH} + \text{DTNB} \rightarrow \text{TNB} + \text{CoA-S-S-TNB} \quad (3)$$

The reaction product TNB (thionitrobenzoic acid) is the absorbing substance B (Eq. 2) with intense absorption at 412 nm. Therefore, the working wavelength is 412 nm. The absorbance increases linearly with time, up to 0.6-0.8 units of absorbance (over 200 s of measurement). The enzyme activity is not affected by up to 1% Triton X-100.

### 1.3 Temperature of enzyme assay

When CS activity is used as a marker, it is not critical to choose a physiological temperature. A constant reference temperature has to be applied for comparability of measurements. Commonly measurements are performed at room temperature, but possibly more frequently at 30 °C (Hütter et al., 2004; Kuznetsov et al., 2002; Renner et al., 2003; Trounce et al., 1989).

## 2 Reagents and buffers

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

**Tris-HCl buffer (1.0 M, pH 8.1):** 2.4228 g Tris/20 ml a.d., adjust to pH 8.1 with 37% HCl (ca. 100 µl/20 ml).

**Triethanolamine-HCl buffer (0.5 M, pH 8.0) + EDTA (5 mM):** 8.06 g triethanolamine/100 ml a.d., adjust pH with 37% HCl, add 186.1 mg EDTA.

**Triton X-100 (10 % solution):** reagent solution is 100%, add 90 ml a.d. to 10 g (ca 10 ml) Triton X-100.

### 2.2 Prepare 12.2 mM acetyl-CoA, store at -20 °C.

25 mg acetyl CoA + 2.5 ml a.d., make aliquots of 250 µl and store at –20° C. Store on ice during measurement, freeze it again after the experiment.

## 2.3 Prepare fresh every day

**Triethanolamine-HCl-buffer (0.1 M):** 1 ml of 0.5 M triethanolamine-HCl-buffer of pH 8.0 + 4 ml a.d.

**Oxalacetate (10 mM):** 6.6 mg oxalacetate + 5 ml of 0.1 M triethanolamine-HCl-buffer of pH 8.0.

**DTNB (1.01 mM):** 2 mg DTNB + 5 ml of 1 M Tris-HCl-buffer of pH 8.1.

## 2.4 Chemicals

| Name | FW | Source No. | Stock Solution | Comments |
|---|---|---|---|---|
| Tris [Tris-(hydroxymethyl)-aminomethan], C4H11NO3 | 121.14 | Merck 8382 | 1.0 M; 2.4228 g/20 ml a.d. | Adjust to pH 8.1 by HCl, to obtain Tris-HCl buffer. |
| Triethanolamine (2,2',2''-nitrilotriethanol), C6H15NO3 | 149.19 | Fluka 90279 | 0.5 M; 8.06 g/100 ml a.d. | pH 8.0, viscous liquid. Harmful. |
| EDTA (ethylenediaminetetra-acetic acid), disodium salt, dihydrate, C10H14N2O8Na2.2H2O | 372.2 | Sigma E-1644 | 5 mM; 186.1 mg/100 ml of 0.5 M triethanolamine-HCl buffer pH 8.0 | Chelator for heavy metals, added to avoid interference with SH-groups. |
| Triton X-100, C34H62O11 | 646.87 | Serva 37238 | 10%; 10 g/100 ml a.d. | Viscous liquid; detergent. Keep at 4 °C. |
| Oxalacetic acid (oxobutanedioic acid), C4H4O5 | 132.1 | Sigma O-4126 | 10 mM; 6.6 mg/5 ml of triethanolamine-HCl-buffer | Irritant. |
| DTNB [5,5`-dithio-bis(2-nitrobenzoic acid); 3-carboxy-4-nitrophenyl disulfide], Ellman's reagent, C14H8N2O8S2 | 396.3 | Sigma D-8130 | 1.01 mM; 2 mg/5 ml of Tris-HCl-buffer | TNB-S-S-TNB (dithionitrobenzoic acid); irritant. |
| Acetyl CoA (acetyl coenzyme A), lithium salt, C23H38N7O17P3SLi | 816.5 | Sigma A-2181 | 12.2 mM; 25 mg/2.5 ml a.d. | Prepared enzymatically, toxic. |
| Citrate synthase, CS | | Sigma C-3260 | 8.6 mg prot./ml | From porcine heart, stored at 4 °C. Harmful. Crystalline suspension in 2.2 M (NH4)2SO4, pH 7. Specific activity: 200 IU/mg protein at 37 °C, varies with Lot Number. |

# 3 Sample preparation

Freeze sample in liquid nitrogen and store frozen at -80 °C. During measurements, store on ice. Citrate synthase activity of cells is stable during storage (a few hours) on ice. Incubate suspension at 30 °C for 5-10 min before measurement.

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

## 3.1 CS Standard

**Preparation:** As a standard, commercial citrate synthase is diluted 1:500 in 0.1 M Tris-HCl buffer, at pH 7.0 (RT). Accurate dilution is critical and is achieved by adding 2 µl of CS standard (using a 10 µl Hamilton syringe) to 998 µl of buffer. Starting with a protein concentration of 8.6 $mg \cdot cm^{-3}$ in the undiluted CS standard, this yields a final protein concentration of 0.0172 $mg \cdot cm^{-3}$ in the sample, of which 5 µl are added to a volume of 995 µl of incubation medium. Use freshly diluted enzyme.

**Application**: The CS standard serves as a check of chemicals and assay conditions, and may even be used for final correction of results. A standard (at least in replicate) is included at each day of measurement, if a large number of samples is processed collectively.

**Different CS standards:** The age of the standard has to be checked critically. If a new CS standard is applied, the Lot Number is noted, together with the specific activity and the protein concentration provided by the supplier. For instance, a general value of 200 IU/mg protein is listed in the Sigma catalogue, whereas a specific activity of 215 IU/mg (8.6 mg/ml) is given for Lot Number 121H9500 (37 °C).

## 3.2 Isolated mitochondria

Due to very high CS activity of isolated mitochondria, the suspension for measurement can be prepared by dilution (1:10) of a frozen stock mitochondrial suspension (-80 °C; usually ca. 50 mg of mitochondrial protein per ml). Immediately after thawing, add 20 µl of mitchondrial suspension to 180 µl of 0.1 M Tris-HCl buffer, at 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.

### 3.3 Suspended cells

For typical cells (HUVEC, lymphocytes) at 1-2·$10^6$ cells/ml, take replicates of 110 µl samples into Eppendorf tubes, freeze in liquid nitrogen, and store until measurement.

## 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:\CS.*** There is possibility to change options, simply click on desired option.
**--> *exit***

### 4.2 Blank-measurement

**--> *vis on*** [visible lamp, light will be switched on immediately, the sign becomes red].
Add (800 µl – $V_{medium}$) a.d. 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 1,000 µl in the cuvette (including the volume of sample, $V_{sample}$), add into glass tubes:

1. (800 µl - $V_{sample}$) a.d.
2. 100 µl 1.01 mM DTNB (final concentration ca. 0.1 mM)
3. 25 µl 10% Triton X-100 (final concentration ca. 0.25%)
4. 50 µl oxalacetate (final concentration ca. 0.5 mM)
5. 25 µl acetyl CoA (final concentration ca. 0.31 mM)

Mix carefully.
Pre-incubate medium in thermostat at 30 °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 µl cell suspension, 25 µl mitochondrial suspension, 5 µl CS standard (see Section 3) 5 µl heart or 20 µl liver homogenates, into prepared quartz cuvettes.
Mix carefully with plastic stick, wash stick with a.d.
***--> read sample, --> start.***
The linear increase of absorbance is measured over ca. 200 s.
Another possible way for mixing is to add sample directly into pre incubated medium, mix with vortex and immediately transfer into quartz cuvette pre-thermostated (5 min, 30 °C) in spectrophotometer cuvette holder.
*Optional:* Add more oxalacetate (30 µl) or DTNB (50 µl), to check if the concentrations were high enough (saturating). No change of slope should be observed after increasing the substrate concentrations.
*Optional*: After ca. 100 s (middle of time range) it is possible to make a second sample addition (the same volume), in this case the activity must be proportional to the amount of the sample added, taking into account the dilution factor. (normally this is proportional up to 0.2 - 0.3 units of absorbance per minute).
***--> 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 CS 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),

$$dc_B / dt = \frac{dA / dt}{l \cdot \varepsilon_B} = \frac{r_A}{l \cdot \varepsilon_B} \quad (4)$$

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

$$J_V = dc_B/dt \cdot \nu_B^{-1} \quad (5)$$

where $\nu_B$ is the stoichiometric number of compound B (Gnaiger, 1993), which is equal to 1 in the coupled reactions (1) and (3).

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

The specific enzyme activity is proportional to the experimental reaction flux (Eq. 5) 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 sample, measured under experimental incubation conditions with saturating substrate. Combining Eq.(4) and (5),

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

$v$ 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 CS forms 1 µmol of citrate 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. 4).

$l$ Optical path length (= 1 cm).

$\varepsilon_B$ Extinction coefficient of B (TNB) at 412 nm and pH 8.1 (= 13.6 mM$^{-1}$·cm$^{-1}$).

$\nu_B$ Stoichiometric number of B (TNB) in the reaction (Eq. 3) (= 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, $V_{sample}$ (protein concentration: $mg \cdot cm^{-3}$; cell density: $10^6 \cdot cm^{-3}$). |

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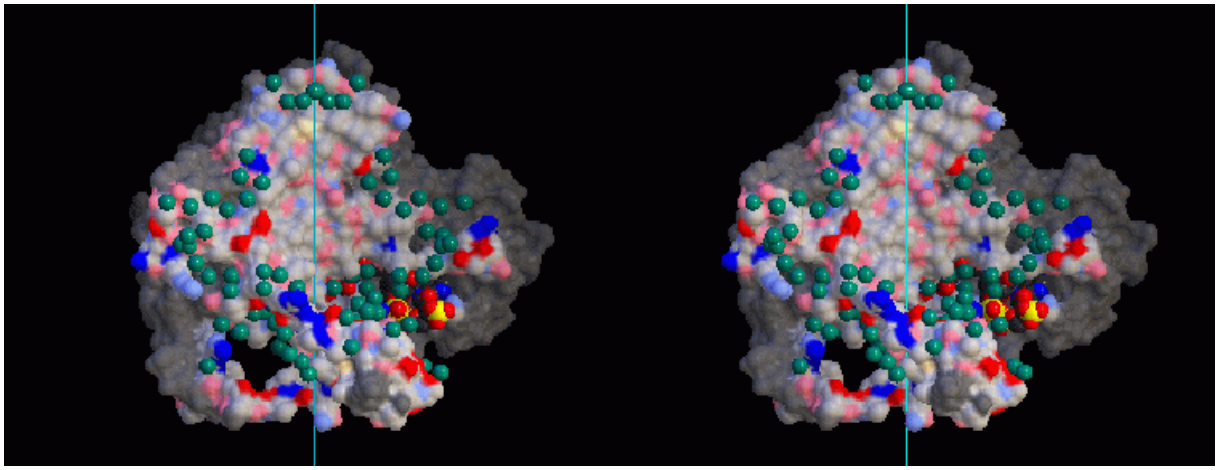
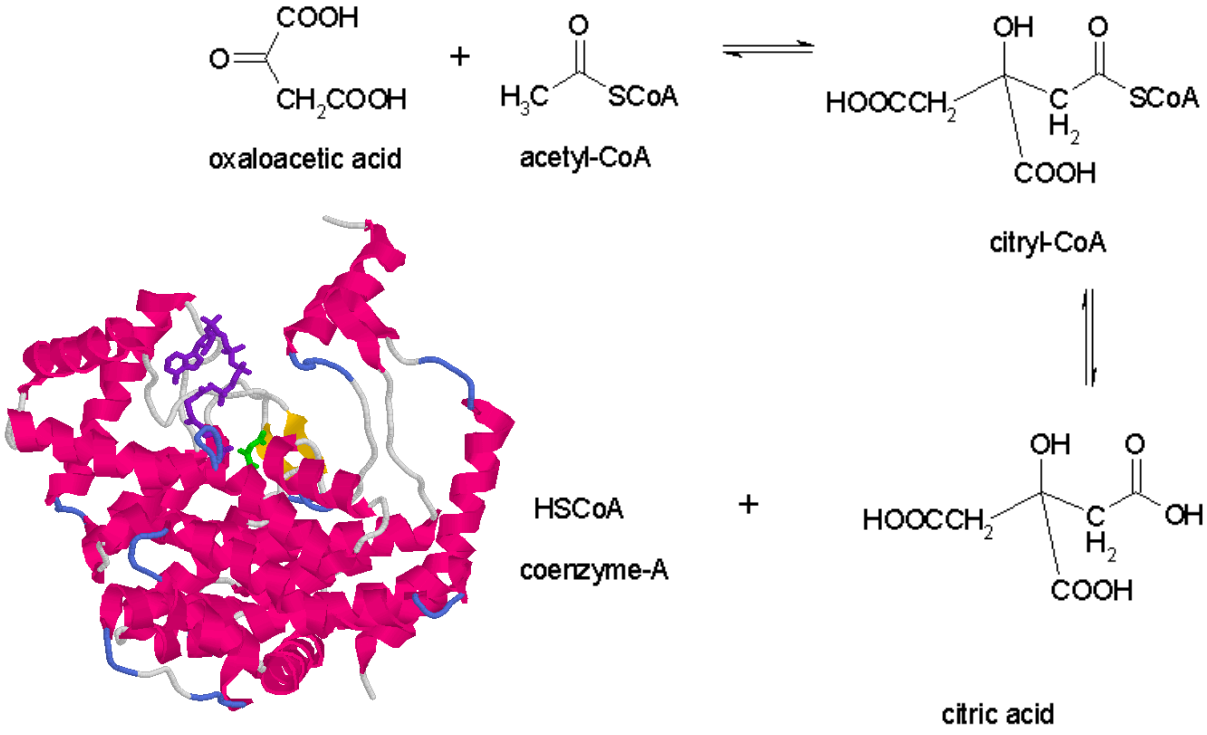
# 7 Appendix



Cross-eyed stereo image of citrate synthase
http://www.scripps.edu/pub/goodsell/interface/interface_images/1csh.html




Citrate synthase complexed with oxaloacetate (green) and the CoASH analog amidocarboxymethyldethia coenzyme A (purple).
http://chemistry.gsu.edu/Glactone/PDB/Proteins/Krebs/1csh.html