Citrate Synthase Assay Kit

Cat. #: CB007
(250 tests in 96-well plate)

Description
Citrate synthase (E.c. 4.1.3.7) is a pace-maker enzyme in the Krebs cycle (citric acid cycle or tricarboxylic acid cycle, TCA). 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. CS catalyzes the reaction of two-carbon acetyl CoA with four-carbon oxaloacetate to form six-carbon citrate, thus regenerating coenzyme A.
Acetyl-CoA + oxalacetate + H_2O → citrate + CoA-SH
This colorimetric assay is based on the reaction between 5', 5'-Dithiobis 2-nitrobenzoic acid (DTNB) and CoA-SH to form TNB, which exhibits maximum absorbance at 412 nm:
CoA-SH + DTNB → TNB + CoA-S-S-TNB
The intensity of the absorbance is proportional to the citrate synthase activity. This enzyme is an exclusive marker of the mitochondrial matrix1.

Reagents and buffers
Tris-HCl buffer (40 mM, pH 8.1): Assay buffer.
Tris–HCl buffer (0.1 M, pH 7.0): For Citrate Synthase standard preparation.
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. pH does not change after addition of EDTA.
Triton X-100 (10% solution): Reagent solution is 100%, add 90 ml a.d. to 10 g (ca. 10 ml) Triton X-100. Triton X-100 is viscous and sticky. Weigh on balance in a beaker and dissolve by stirring.

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.

Prepare fresh every day
Triethanolamine-HCl-buffer (0.1 M, pH 8.0): 1 ml of 0.5 M triethanolamine-HCl-buffer of pH 8.0 + 4 ml
Oxalacetate (10 mM, pH 8.0): 6.6 mg oxalacetate + 5 ml of 0.1 M triethanolamine-HCl-buffer of pH 8.0.
DTNB (1.01 mM, pH 8.1): 2 mg DTNB + 5 ml of 1 M Tris-HCl-buffer of pH 8.1.

Sample Preparation
1. Isolated mitochondria
10-25 µl mitochondrial suspension (5 mg/ml) is used for each spectrophotometric measurement.
2. 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.
3. As a standard, citrate synthase is (8.6 mg prot./ml) diluted 1:500 in 0.1 M Tris-HCl buffer, pH 7.0 (RT), this yields a final protein concentration of 0.0172 mg/cm^{-3} in the sample, of which 5 µl are added to a volume of 995 µl of reaction mix (1:200).

Procedure for Citrate Synthase Activity Assay (1mL cuvette)
1. Set the spectrophotometer at 412 nm on a kinetic program:
Duration: 2 minutes
Interval: 10 seconds
2. Warm the assay solutions to room temperature before starting the reaction. Mix until homogenous.
3. Prepare sample reactions according to the reaction scheme (see below)

Assay Buffer (1X)	800-X µL
Acetyl CoA	25 µL
DTNB solution (1X)	100 µL
Triton X-100	25 µL
Mitochondrial protein (5~10 µg)	X µL

4. Mix solution in cuvette.
5. Blank spectrophotometer with reaction mixture.
6. Add 50 µL oxaloacetate solution and mix (cover with parafilm and invert 3-4 times).

7. Immediately read and record decrease in OD for 2 minutes.
8. Calculate ΔA/min by using of the maximum linear rate. ΔA= change in OD reading.
9. Calculate citrate synthase activity of the sample (see calculations).

Procedure for Citrate Synthase Activity Assay (96-well plate)
1. Set the spectrophotometer at 412 nm on a kinetic program:
Duration: 2 minutes
Interval: 10 seconds
2. Warm the assay solutions to room temperature before starting the reaction. Mix until homogenous.
3. Prepare sample reactions according to the reaction scheme (see below)

Assay Buffer (1X)	170-X µL
Acetyl CoA	5 µL
DTNB solution (1X)	20 µL
Triton X-100	5 µL
Mitochondrial protein (1~2 µg)	X µL

4. Add 10 µL oxaloacetate solution and mix.
5. Immediately read and record decrease in OD for 2 minutes.
6. Calculate ΔA/min by using of the maximum linear rate. ΔA= change in OD reading.
7. Calculate citrate synthase activity of the sample (see calculations).

Calculations
Calculate the citrate synthase activity using the following equation:

$$\text{Unit/mg mitochondria} = \frac{\Delta A/\text{min}}{\varepsilon \text{ x L(cm) x mg mitochondria}}$$

ΔA/min = (change in OD reading)/time
ε = 13.6 $mM^{-1}cm^{-1}$ and ε is extinction coefficient of TNB at 412 nm
L (cm) = path length for absorbance

- For 1ml cuvette, path length = 1 cm
- For 96-well plate, path length = 0.625 cm

Unit definition: One unit would make 1.0 µmole DTNB become TNB per minute at pH 7.2 at 25 ºC

Procedure for Measuring Mitochondrial inner Membrane Integrity
Citrate synthase locates in the matrix of the mitochondria. The integrity of the mitochondrial inner membrane is assessed by measuring citrate synthase activity in the presence and absence of the detergent, triton X-100. The ratio between activity without and with triton X-100 presence is a measurement of the integrity of the mitochondrial inner membrane.

Freeze/thaw processes may potentially cause rupture of the membrane of mitochondria. Therefore freshly prepared tissues are recommended, though frozen tissues could still be used for measuring total activity of citrate synthase.

% mitochondria with intact mitochondria inner membrane:

$$\% = \frac{\Delta A/\text{minute(w/ detergent)} - \Delta A/\text{minute (w/o detergent)}}{\Delta A/\text{minute (w/ detergent)}}$$

Storage
1. Store the kit at 2°C to 8°C until first use. The performance of this product is guaranteed for six months from the date of purchase if stored and handled properly.
2. Reconstituted JC-1 staining solution should be aliquoted in small amounts sufficient for one day of experimental work and stored at -20°C, protected from light and moisture (preferably in a desiccator).
3. Avoid multiple freeze-thaw cycles.