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Product Information

Citrate Synthase Assay Kit

Catalog Number **CS0720**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Citrate synthase is the initial enzyme of the tricarboxylic acid (TCA) cycle. The enzyme catalyzes the reaction between acetyl coenzyme A (acetyl CoA) and oxaloacetic acid to form citric acid. This enzyme is an exclusive marker of the mitochondrial matrix.[1-4]

The Citrate Synthase Assay Kit contains all the required reagents, including a positive control enzyme, for a fast and simple measurement of citrate synthase activity. In addition the kit enables testing of the intactness of the mitochondrial inner membrane.[2]

The kit was tested on NIH 3T3, Jurkat, HepG2, HEK293, HeLa, and A431 cell lines, and on rat liver, kidney, and brain tissues.

Components

The kit is sufficient for 100 reactions (1 ml cuvette) or 480 reactions (96 well plate).

Assay Buffer for Citrate Synthase 5× (Catalog Number B6935)	25 ml
Bicine Buffer (Catalog Number B7060)	10 ml
CelLytic™ M Cell Lysis Reagent (Catalog Number C2978)	10 ml
5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) (Catalog Number D8130)	4 mg
Acetyl Coenzyme A (Acetyl CoA) Trilithium Salt (Catalog Number A2181)	25 mg
Oxaloacetic Acid (Catalog Number O4126)	1 g
Citrate Synthase (Positive control) (Catalog Number C4741)	0.1 ml

Equipment and Reagents Required but Not Provided

- CelLytic MT Cell Lysis Reagent (Catalog Number C3228)
- Protease Inhibitor Cocktail (Catalog Number P8340)
- 1 ml Polystyrene cuvette (Catalog Number C5416)
- 96 well plate, flat bottom (Catalog Number CLS3635)
- Molecular biology grade water (Catalog Number W4502) or ultrapure (17 MΩ·cm or equivalent) water
- Absolute ethanol (Catalog Number E7023 or equivalent)
- Temperature controlled UV/visible spectrophotometer or plate reader

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

It is recommended to use molecular biology grade water or ultrapure (17 MΩ·cm or equivalent) water when preparing the reagents.

1× Assay Buffer for Citrate Synthase - Thaw the Assay Buffer for Citrate Synthase 5× (Catalog Number B6935) and mix until homogenous. Dilute an aliquot of buffer 5-fold with water. The Assay Buffer for Citrate Synthase 5× and the 1× Assay Buffer for Citrate Synthase can be stored at 2–8 °C for up to 6 months.

30 mM Acetyl CoA Solution - Dissolve the contents of the vial with 1 ml of water and mix gently until homogenous. For long-term storage, aliquot and store at –20 °C.

10 mM Oxaloacetate (OAA) Solution – Store the powder at –20 °C. Before each use, dissolve 1.3 mg in 1 ml of 1× Assay Buffer for Citrate Synthase. Mix until homogenous. The 10 mM Oxaloacetate Solution can be stored at –20 °C for up to 1 week.

10 mM DTNB Solution - Dissolve the contents of the vial in 1 ml of absolute ethanol. Mix until homogenous. For long-term storage, store at –20 °C in working aliquots.

Citrate Synthase (positive control) Solution - The activity of the Citrate Synthase control enzyme (Catalog Number C4741) supplied in the kit is 2–16 units/ml. Thaw the enzyme solution and dispense in aliquots. The enzyme can be stored at 2–8 °C for up to one week. For long-term storage, store at –20 °C. For each set of reactions, dilute an aliquot 10-fold with 1× Assay Buffer for Citrate Synthase. For each positive control reaction use a volume as indicated in the appropriate reaction scheme (Table 1 or 2). Use the diluted control solution immediately and do not store any remaining diluted control solution.

Storage/Stability
The product is shipped on dry ice and storage at –20 °C is recommended.

Store CelLytic M Cell Lysis Reagent (Catalog Number C2978) at room temperature. CelLytic M Cell Lysis Reagent may appear cloudy after an extended period of storage. Product performance is unaffected and may be used, as is, without further filtration or clarification.

Procedure
Citrate synthase catalyzes the reaction between acetyl coenzyme A (acetyl CoA) and oxaloacetic acid (OAA) to form citric acid. The acetyl CoA contributes 2 carbons to the 4 carbons of oxaloacetate resulting in citrate with 6 carbons. The hydrolysis of the thioester of acetyl CoA results in the formation of CoA with a thiol group (CoA-SH). The thiol reacts with the DTNB in the reaction mixture to form 5-thio-2-nitrobenzoic acid (TNB). This yellow product (TNB) is observed spectrophotometrically by measuring absorbance at 412 nm.[1-3]

Reaction catalyzed by citrate synthase:

$$\text{Acetyl CoA} + \text{Oxaloacetate} \rightarrow \text{Citrate} + \text{CoA-SH} + H^+ + H_2O$$

Colorimetric reaction:

$$\text{CoA-SH} + \text{DTNB} \rightarrow \text{TNB} + \text{CoA-S-S-TNB}$$

A. Sample Preparation

Citrate synthase activity can be measured in isolated mitochondria preparations or in whole cell extracts.[3]

1. Mitochondria Preparation – For preparation of mitochondria from tissues use the MITOISO1 kit and for mitochondria from cells use the MITOISO2 kit and the procedures in the respective Technical Bulletins.

   Total Citrate Synthase Activity – For the detection of total citrate synthase activity in mitochondria, suspend the prepared mitochondrial pellet in CelLytic M Cell Lysis Reagent (Catalog Number C2978) using ~200 µl per g of tissue or per $2–5 \times 10^7$ cells.

   Inner Mitochondrial Membrane Intactness Assay – The citrate synthase activity is not readily measurable in intact mitochondria owing to the impermeability of the inner membrane to the substrates. Therefore, it is a good marker for measuring mitochondrial inner membrane intactness.[2] When using the kit for measurement of the inner mitochondrial membrane intactness, the sample preparation should be divided into two equal aliquots.

   a. Suspend the mitochondrial pellet in Bicine Buffer (Catalog Number B7060, a buffer containing no detergent) using ~200 µl per g of tissue or per $2–5 \times 10^7$ cells.

   b. Split the suspension into two tubes and centrifuge at 11,000 × *g* for 10 minutes.

   c. Remove the supernatant and resuspend the pellet in the first tube with CelLytic M and the pellet in the second tube with Bicine Buffer using for both ~200 µl per g of tissue or per $2–5 \times 10^7$ cells. Mix well.

   d. The samples in Bicine Buffer and in CelLytic M may now be assayed for protein content.

   The activity observed in the sample suspended in the CelLytic M Reagent represents the total citrate synthase activity present in the mitochondria, since the mitochondria in this sample are ruptured and the substrate in the reaction mixture is readily available to the enzyme. The activity of the citrate synthase observed in the sample suspended in Bicine Buffer represents the activity resulting from broken mitochondria in the isolated mitochondria preparation.

   The activity of the Bicine Buffer sample will be much lower than that of the CelLytic M Reagent sample; therefore, it is advisable to use a larger sample volume for the activity measurement, but no more than 50 µl. The ratio between the activities in the two samples represents the fraction of broken mitochondria.

2. Tissue or Whole Cell Extracts

   For **tissue extracts** use the CelLytic MT Cell Lysis Reagent (Catalog Number C3228) according to the procedure in the Technical Bulletin.

   For **whole cell extracts** use the CelLytic M Cell Lysis Reagent. Protease Inhibitor Cocktail (Catalog Number P8340) may be added to the CelLyticM reagent.

   a. Wash the cells twice with PBS. For cells in suspension, collect the cells by centrifugation and resuspend the cell pellet with CelLytic M (125 µl for $10^6–10^7$ cells). For adherent cells scrape the cells on the plate using the appropriate volume of CelLytic M. The plate size will dictate the volume of reagent required to cover the plate surface. Suggested working volumes are 500–1,000 µl for a 100 mm plate and 200–400 µl for a 35 mm plate.

   b. Incubate the cells for 15 minutes on a shaker.

   c. Collect lysed cells.

   d. Centrifuge the lysed cells for 15 minutes at 12,000–20,000 × *g* to pellet the cellular debris.

   e. Transfer the protein-containing supernatant to a chilled test tube.
   Note: For long term storage it is recommended to store the lysate at –70 °C.

   f. Assay for protein content.

B. Measurement of Citrate Synthase Activity in 1 ml Cuvette

1. Set the spectrophotometer at 412 nm on a kinetic program:

   Lag time: 20 second
   Duration: 1.5 minute
   Interval: 10 second

2. Warm the assay solutions to 25 °C before starting the reaction. Mix until homogenous.

3. Prepare sample reactions according to the reaction scheme (see Table 1). Add all the reaction components except for the 10 mM Oxaloacetate (OAA) Solution. Mix well by gentle vortexing. The reaction mixture turns yellow.

Table 1.
Reaction Scheme for Citrate Synthase Activity Measurement in 1 ml Cuvette

Description	Sample	1× Assay Buffer	30 mM Acetyl CoA Solution	10 mM DTNB Solution	10 mM OAA Solution Last to be added
CelLytic M Sample*	x (2-50 µl)	930 - x µl	10 µl	10 µl	50 µl
Bicine Buffer Sample**	x (2-50 µl)	930 - x µl	10 µl	10 µl	50 µl
Citrate Synthase (positive control) Diluted Solution	x (5-10 µl)	930 - x µl	10 µl	10 µl	50 µl

* CelLytic M Cell Lysis Reagent mitochondria sample for mitochondria intactness measurement or whole cell extract for total activity measurement. The amount of protein in the sample should be at least 2 µg of mitochondrial protein or 10 µg of whole cell extract protein.

** Relevant for mitochondria intactness measurement only. The amount of the protein in the sample should be at least 10 µg.

4. Follow the absorbance of the reaction mixture for 1.5 minutes to measure the baseline reaction, endogenous levels of thiol or deacetylase activity.

5. Add 50 µl of 10 mM OAA Solution to initiate the reaction and mix by inversion.

6. Follow the absorbance of the reaction mixture for 1.5 minutes to measure total activity.

7. Calculate activity in the sample (see Calculations).

Note: If a multicuvette holder is available, the endogenous baseline reaction (step 4, the measurement before the addition of 10 mM OAA Solution) and the sample reaction (step 6, after the addition of 10 mM OAA Solution) can be prepared in separate cuvettes and then read simultaneously. For this purpose, prepare 2 ml of the reaction mixture including the sample. In one cuvette add 50 µl of 1× Assay Buffer for Citrate Synthase and in the second cuvette add 50 µl of 10 mM OAA Solution. Add 950 µl of the reaction mixture (sample, 1× Assay Buffer, 30 mM Acetyl CoA Solution, and 10 mM DTNB Solution) to each cuvette, and read in parallel.

C. Measurement of Citrate Synthase Activity in 96 Well Plate

1. Set the Plate reader at 412 nm on a kinetic program:
   Duration: 1.5 minute
   Interval: 10 seconds

2. Warm the assay solutions to 25 °C before starting the reaction. Mix until homogenous.

3. Prepare sample reactions according to the reaction scheme (see Table 2).
   Note: A recommended alternative is to prepare a reaction mixture containing 1× Assay Buffer, 30 mM Acetyl CoA Solution, 10 mM DTNB Solution, and the sample/ enzyme for triplicates of the same test and then place 190 µl of the reaction mixture in each of the 3 wells.

Table 2.
Reaction Scheme for Citrate Synthase Activity Measurement in 96 Well Plate

well	Description	Sample	1× Assay Buffer	30 mM Acetyl CoA Solution	10 mM DTNB Solution	10 mM OAA Solution Last to be added
1-3	CelLytic M Sample*	x (2-8 µl)	186 – x µl	2 µl	2 µl	10 µl
4-6	Bicine Buffer Sample**	x (2-8 µl)	186 – x µl	2 µl	2 µl	10 µl
7-9	Citrate Synthase (positive control) Diluted Solution	x (1-2 µl)	186 – x µl	2 µl	2 µl	10 µl

* CelLytic M Cell Lysis Reagent mitochondria sample for mitochondria intactness measurement or whole cell extract for total activity measurement. The amount of protein in the sample should be at least 0.4 µg of mitochondrial protein or 8 µg of whole cell extract protein.

** Relevant for mitochondria intactness measurement only. The amount of the protein in the sample should be at least 8 µg.

4. Follow the absorbance of the reaction mixture for 1.5 minutes to measure baseline reaction, endogenous levels of thiol or deacetylase activity.

5. Add 10 µl of 10 mM OAA Solution to each well to initiate the reaction. In order to start the reaction in all the wells simultaneously as possible, use a multichannel pipette. Shake the plate for 10 seconds before reading absorbance (or mix with the multichannel pipette).

6. Follow the absorbance of the reaction mixture for 1.5 minutes to measure total activity.

7. Calculate activity in the sample (see Calculations).

Calculations

Determination of Citrate Synthase Activity

Plot the absorbance (A_{412}) values against time for each reaction. Measure the change in absorbance (ΔA_{412})/minute, in the linear range of the plot, for the endogenous activity. Measure the change in absorbance (ΔA_{412})/minute, in the linear range of the plot, for the total activity (after addition of 10 mM OAA Solution). Calculate the net citrate synthase activity by subtracting the (ΔA_{412})/minute of the endogenous activity from the (ΔA_{412})/minute of the total activity of the sample. Use this value for calculating the citrate synthase activity.

Calculate the citrate synthase activity using the following equation:

Citrate Synthase Activity:

$$\text{units } (\mu\text{mole/ml/min}) = \frac{(\Delta A_{412})/\text{min} \times V(\text{ml}) \times \text{dil}}{\varepsilon^{mM} \times L(\text{cm}) \times V_{enz}(\text{ml})}$$

dil - the dilution factor of the original sample

V(ml) – the reaction volume:

- for assay in 1 ml cuvette = 1 ml
- for assay in 96 well plate = 0.2 ml

V_{enz}(ml) – the volume of the enzyme sample in ml

ε^{mM} (mM^{-1} cm^{-1}) – the extinction coefficient of TNB at 412 nm is 13.6.

L(cm) – pathlength for absorbance measurement:

- for 1 ml cuvette, pathlength = 1 cm
- for 96 well plate (Catalog Number CLS3635), pathlength = 0.552 cm

For other cuvettes/wells the pathlength needs to be determined.

Determination of Mitochondrial Inner Membrane Intactness

Calculate the changes in absorbance, (ΔA_{412})/minute, in the linear range of the plot, for the CelLytic M Cell Lysis Reagent sample and for the Bicine Buffer sample. Determine the citrate synthase activity of each sample. The ratio between the citrate synthase activities in the mitochondria preparation in Bicine Buffer versus the activity in the same mitochondria preparation treated with CelLytic M Cell Lysis Reagent (ruptured mitochondria) gives the percentage of ruptured mitochondria.

$$\% \text{ Ruptured mitochondria} = \frac{\text{Activity in Bicine Buffer} \times 100}{\text{Activity in CelLytic M}}$$

References

1. Srere, P.A., Citrate Synthase. Methods Enzymol., **13**, 3-11 (1969).
2. Graham, J.M., and Rickwood, D. (eds.), Subcellular Fractionation: A Practical Approach, Oxford Univ. Press Inc. (New York, NY: 1997) p.135.
3. Trounce, I.A., et al., Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. Methods Enzymol., **264**, 484-509 (1996).
4. Morgunov, I., and Srere, P.A., Interaction between citrate synthase and malate dehydrogenase. Substrate channeling of oxaloacetate. J. Biol. Chem., **273**, 29540-29544 (1998).

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