Citrate Assay Kit

Citrate Assay Kit can be used to measure Citrate in serum, plasma, urine, Tissue/cell lysate and cell culture supernatants.

Catalog number: ARG81388

Package: 96 wells

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INTRODUCTION

Citric acid (Citric) is a weak organic acid that has the chemical formula C6H8O7. It occurs naturally in citrus fruits. In biochemistry, Citrate is an intermediate in the TCA cycle (aka TriCarboxylic Acid cycle, or Krebs cycle, Szent-Györgyi), a central metabolic pathway for animals, plants, and bacteria. Citrate synthase catalyzes the condensation of oxaloacetate with acetyl CoA to form citrate. Citrate then acts as the substrate for aconitase and is converted into aconitic acid. The cycle ends with regeneration of oxaloacetate. This series of chemical reactions is the source of two-thirds of the food-derived energy in higher organisms. Hans Adolf Krebs received the 1953 Nobel Prize in Physiology or Medicine for the discovery.

Citrate can be transported out of the mitochondria and into the cytoplasm, then broken down into acetyl-CoA for fatty acid synthesis, and into oxaloacetate. High concentrations of cytosolic citrate can inhibit phosphofructokinase, the catalyst of a rate-limiting step of glycolysis. This effect is advantageous: high concentrations of citrate indicate that there is a large supply of biosynthetic precursor molecules, so there is no need for phosphofructokinase to continue to send molecules of its substrate, fructose 6-phosphate, into glycolysis. Citrate acts by augmenting the inhibitory effect of high concentrations of ATP, another sign that there is no need to carry out glycolysis. [Wikipedia: Citric acid]

PRINCIPLE OF THE ASSAY

This Citrate Activity Assay Kit provides a simple, and rapid procedure for measuring citrate concentration in serum, plasma, urine and cell culture supernatants samples. In this assay, citrate is converted to oxaloacetate and finally oxaloacetate is converted to pyruvate. The pyruvate is then oxidized with the conversion of the dye in the kit into a colored and fluorescent form. The color intensity at 570 nm or fluorescence intensity at λ ex/em = 530/585 nm is directly proportional to the citrate concentration in the sample. The concentration of citrate in the sample is then determined by comparing the signals of samples to the standard.

MATERIALS PROVIDED & STORAGE INFORMATION

Component	Quantity	Storage information
Citrate Developer	10 ml (Ready to use)	-20°C
Citrate Standard (10mM)	500 µl	-20°C
Citrate lyase Enzyme	1 vial (lyophilized)	-20°C
Oxaloacetate decarboxylase Enzyme	120 µl	-20°C
Dye Reagent	120 µl	-20°C

The kit is shipped on ice. Store all components at -20°C in dark. Shelf life of six months after receipt.

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 570 nm. Or fluorescence Microplate Reader capable of measuring fluorescence at λ ex/em = 530/585 nm.
- Flat bottomed 96-well microplate or Black flat bottomed 96-well microplate
- Pipettes and pipette tips
- Deionized or distilled water.

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Prior to assay, concentrations of protein, inhibitor, substrate and incubation time may need to be established for a given sample.
- Briefly spin down the reagents before use.
- It is recommended that the standards and samples be assayed in at least duplicates.
- Change pipette tips between the addition of different reagent or samples.
- All reagents should be warmed to room temperature before use.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g at 2-8°C. Collect the serum, serum should be deproteinated using a 10 kDa spin filter (e.g. Amicon Ultra-0.5). Alternatively, the untreated serum can measured directly if an internal standard is used. Aliquot and store samples at-80°C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g at 2-8°C within 30 minutes of collection. Plasma samples should be deproteinated using a 10 kDa spin filter (e.g. Amicon Ultra-0.5). Alternatively, the untreated plasma can measured directly if an internal standard is used. Aliquot and store samples at-80°C. Avoid repeated freeze-thaw cycles.

Urine- Collect the urine by micturating directly into a sterile container. Remove impurities by centrifugation at 10,000 x g for 1 min. Collect the supernatants and assay immediately or aliquot and store samples at ≤-80°C. Urine samples should be diluted at least 5-fold and an internal standard should be used.

Tissue or cell samples- Homogenized 2 x10^6 of Tissue or cells in 100 µL PBS. Then centrifuge at 14,000 rpm for 5 min. Use clear supernatant for assay.

Cell Culture Supernatants- Media with high pyruvate concentrations should be avoid. (e.g. DMEM, L-15, F12, etc.).

Remove particulates by centrifugation for 10 min at 1500 x g at 4°C and aliquot

& store samples at ≤-80°C. Avoid repeated freeze-thaw cycles.

Note: Samples should be clear and free of particles or precipitates. Avoid using haemolytic, icteric or lipaemic samples.

REAGENT PREPARATION

- **Citrate lyase Enzyme:** Dissolve the Citrate lyase Enzyme in 120 µl of Citrate Developer. Pipette up and down to mix well, and make sure the enzyme is fully dissolved completely before assay. The reconstituted Citrate lyase Enzyme is stable for 4 weeks when it stored at-20°C. Before each use of the Citrate lyase Enzyme, pipette up and down or brief mix to assure the enzyme is resuspended.
- **Standard:**

  For Colorimetric Procedure:

  - Dilute 10 mM Citrate Standard solutions with deionized water to 400 µM, 240 µM and 120 µM as following table, and use deionized water serves as zero standard (0 µM). If assaying culture media with phenol red, dilute the Citrate Standard in culture media instate of deionized water.

  The example of the dilution of standards

Standard No.	Standard Conc. µM	Deionized water (culture media) (µl)	Standard (µl)
S1	400	240	10 µl of 10 mM Stock
S2	240	40	60 µl of S1
S3	120	70	30 µl of S1
S0	0	100	0

  -Internal standard: Mixing 50 µL of 10 mM Standard stock and 450 µL deionized water to yield 500 µL of 1000 µM citrate Internal standard. 5 µL of 1000 µM citrate is used for each internal standard test.

For fluorimetric Procedure:

- For fluorimetric assays, the linear detection range is 0.5 to 40 µM citrate. Dilute the standards prepared in Colorimetric Procedure 1:10 with deionized water (or culture media) to yield standard concentration as 40 µM, 24 µM and 12 µM.
- 5 µL of 100 µM citrate is used for the internal standard.

- **Working Reagent for Standards and Samples:**

  For each reaction combine the following (*Prepare before use)*:

  85 µL of Citrate Developer

  1 µL of Citrate lyase Enzyme

  1 µL of Oxaloacetate decarboxylase Enzyme

  1 µL Dye Reagent.

  Transfer 80 µl of Working Reagent to each sample and standard wells.

- **Sample Blank Working Reagent:**

  For each reaction combine the following (*Prepare before use)*:

  86 µL of Citrate Developer

  (without Citrate lyase Enzyme)

  1 µL of Oxaloacetate decarboxylase Enzyme

  1 µL Dye Reagent.

  Transfer 80 µl of Working Reagent to each Sample Blank wells.

- **Citrate Developer:** Citrate Developer is ready to use, mix it well by vigorous shaking before use.

ASSAY PROCEDURE

All materials should be equilibrated to room temperature (RT) before use, each vial should be mixed thoroughly without foaming and briefly centrifuge tubes prior to use. Each sample requires a sample blank.

For Colorimetric Procedure:

1.1 *If the internal standard does not include in the assay. (For Tissue/cell samples and Amicon Ultra column filtered serum/plasma samples)*

- Add 20 µl of each sample in two separate wells in flat bottomed 96 well plate. One is for sample assay the other one well is for sample blank.

1.2 *If the internal standard is used in the assay. (For un-filtered serum/plasma samples and urine)*

- Add 20 µl of each sample in three separate wells in flat bottomed 96 well plate.

Well 1: sample alone for sample assay:

Add 20 µL of sample and 5 µL of dH2O.

Well 2: sample blank:

Add 20 µL of sample and 5 µL of dH2O.

Well 3: sample plus internal standard:

Add 20 µL of sample and 5 µL of 1000 µM citrate (internal standard).

Note: the S0-S3 standards are not necessary when internal standard is used in the assay. Skip step 2 as below.

2. Add 20 µL of each standard (S0-S3) to separate wells in a 96 well plate. *(Note: If the internal standard is used in the assay the regular standard (S0-*

S3) are not necessary for the assay)

3. Add 80 µL of the Working Reagent for Standards and Samples to each Standard (or internal standard) and Sample wells.
4. Add 80 µL of the Sample Blank Working Reagent to each sample blank wells.
5. Gently tap the plate to ensure thorough mixing. Incubate for 15 min at room temperature in dark.
6. Read the OD with a microplate reader at 570 nm immediately.

For Fluorimetric Procedure (black 96 well plate is used):

1.1 If the internal standard does not include in the assay. (For Tissue/cell samples and Amicon Ultra column filtered serum/plasma samples)

- Add 20 µl of each sample in two separate wells in black 96 well plate. One is for sample assay the other one well is for sample blank.

1.2 If the internal standard is used in the assay. (For un-filtered serum/plasma samples and urine)

- Add 20 µl of each sample in three separate wells in black 96 well plate.

Well 1: sample alone for sample assay:

Add 20 µL of sample and 5 µL of dH2O.

Well 2: sample blank:

Add 20 µL of sample and 5 µL of dH2O.

Well 3: sample plus internal standard:

Add 20 µL of sample and 5 µL of 100 µM citrate (internal standard).

Note: the S0-S3 standards are not necessary when internal standard

is used in the assay. Skip step 2 as below.

2. Add 20 µL of each standard (S0-S3) to separate wells in the black 96 well plate. *(Note: If the internal standard is used in the assay the regular standard (S0-S3) are not necessary for the assay)*
3. Add 80 µL of the Working Reagent for Standards and Samples to each Standard (or internal standard) and Sample wells.
4. Add 80 µL of the Sample Blank Working Reagent to each sample blank wells.
5. Gently tap the plate to ensure thorough mixing. Incubate for 15 min at room temperature in dark.
6. Read fluorescence intensity at λ ex = 530 nm and λ em = 585 nm immediately.

Summary:

A. The internal standard does not include in the assay. (For Tissue/cell samples and Amicon Ultra column filtered serum/plasma samples)

	Assayed sample	Sample blank	Standard S0-S3
Sample	20 µl	20 µl	
Standards			20 µl
Working Reagent for Standards and Samples	80 µl		80 µl
Sample Blank Working Reagent		80 µl	
Mix well and incubate for 15 min at RT in dark.			
Read the OD with a microplate reader at 570 nm immediately. (Or read fluorescence intensity at λ ex/em = 530 / 585 nm immediately.)			

Note: The concentration of Standard S1-S3 are different between Colorimetric

Procedure (400 µM, 240 µM, 120 µM) and Fluorimetric Procedure (40 µM, 24 µM, 12 µM). Please refer the detail at REAGENT PREPARATION section.

B. If the internal standard is used in the assay. (For un-filtered serum/plasma samples and urine)

	Assayed sample	Sample blank	Internal Standard
Sample	20 µl	20 µl	20 µl
Standard			5 µl
dH2O	5 µl	5 µl	
Working Reagent for Standards and Samples	80 µl		80 µl
Sample Blank Working Reagent		80 µl	
Mix well and incubate for 15 min at RT in dark.			
Read the OD with a microplate reader at 570nm immediately. (Or read fluorescence intensity at λ ex/em = 530 / 585 nm immediately.)			

Note: The concentration of Standard: 1000 µM citrate for Colorimetric Procedure, 100 µM citrate for Fluorimetric Procedure. Please refer the detail at REAGENT PREPARATION section.

CALCULATION OF RESULTS

1. If S0-S3 are assayed in the kit. Subtract the blank value (S0) from the standard values and plot the ΔOD or ΔF against standard concentrations. Determine the slope and calculate the citrate concentration of the Samples as follows:

   [Citrate] (µM)=

   $N \times [(R_{SAMPLE} - R_{BLANK}) / \text{Slope } (\mu M^{-1})]$

2. If an internal standard was used, the sample citrate concentration is computed as follows:

   [Citrate] (µM)=

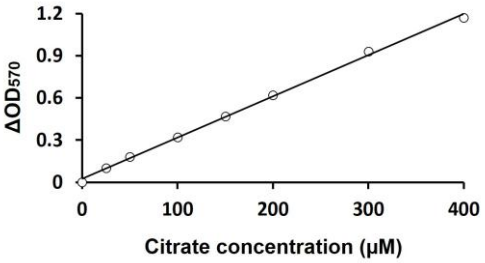
   $N \times [(R_{Sample} - R_{Blank}) / (R_{Standard} - R_{Sample})] \times (\text{Standard Conc.} / 4]$

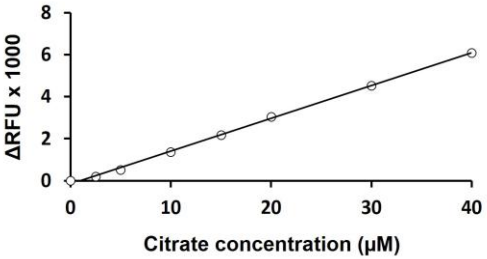
3. The R_{Sample} , R_{Blank} and $R_{Standard}$ are OD or fluorescence readings of the Sample, Sample Blank and the Sample plus Internal Standard respectively . N is the sample dilution factor. Standard Conc. is the concentration of internal standard (1000 µM citrate for Colorimetric Procedure, 100 µM citrate for Fluorimetric Procedure).

   Notes: The volume of the internal standard is 4× lower than the sample volume; thus, the internal standard concentration should be divided by 4.

4. If the calculated citrate concentration is >400 µM for the colorimetric assay, or >40 µM for the fluorimetric assay, dilute sample in deionized water and repeat assay. Multiply result by the dilution factor N.
5. Conversions: 100 µM citrate equals 19.1 mg/L, 0.0019% or 19.1 ppm.

EXAMPLE OF ASSAY





QUALITY ASSURANCE

Sensitivity

Linear detection range:

Colorimetric assays: 4 to 400 µM

Fluorimetric assays: 0.5 to 40 µM

The minimum detectable dose (MDD) of Citrate was:

Colorimetric assays: 4 µM

Fluorimetric assays: 0.5 µM