Product Manual

α-Ketoglutarate Assay Kit (Fluorometric)

Catalog Number

MET-5132 **100 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

CELL BIOLABS, INC.
Creating Solutions for Life Science Research

Introduction

α-Ketoglutarate (α-KG, or 2-oxoglutarate), is an important molecule with a variety of roles within multiple metabolic pathways. α-KG is produced by the oxidative decarboxylation of isocitrate via isocitrate dehydrogenase and is also the keto acid product of the deamination of glutamate via glutamate dehydrogenase. α-Ketoglutarate exerts its influence in the tricarboxylic acid (TCA) cycle as a rate determining intermediary of cellular energy metabolism. Anaplerotic reactions can refuel the cycle by generating α-Ketoglutarate from glutamate via glutamate dehydrogenase.

α-Ketoglutarate acts as a nitrogen transporter that facilitates amino acid synthesis, while also inhibiting protein degradation in muscles. L-Alanine or L-Aspartate can react with α-ketoglutarate via one of the transaminase enzymes, alanine aminotransferase (ALT) or aspartate aminotransferase (AST), to produce glutamate, which is the basis of nitrogen utilization and elimination. Amino groups from amino acids attach to α-KG where they are transported to the liver and can enter the urea cycle. These transamination and deamination pathways are also important for ammonia detoxification, as well as formation of the neurotransmitters glutamate and *gamma*-aminobutyric acid (GABA).

Other characteristics of α-Ketoglutarate include antioxidant activity by quenching of hydrogen peroxide build up and facilitating its elimination. It also generates ATP via oxidative decarboxylation for energy in intestinal cell processes and redox homeostasis. The molecule can influence hydroxylation of proline to hydroxyproline, thus increasing the formation of collagen and bone matrix formation. Recently, α-KG has been noted to extend cell lifespan and delay age-related diseases (Figure 1).

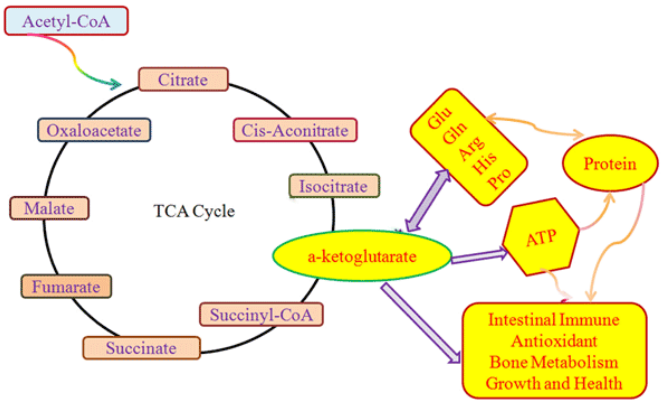

Figure 1: α-Ketoglutarate's influence on many cellular functions.

Cell Biolabs' α-Ketoglutarate Assay Kit is a simple fluorometric assay that measures the activity of α-Ketoglutarate present within plasma, serum, tissue homogenates, or cell suspensions in a 96-well microtiter plate format. Each kit provides sufficient reagents to perform up to 100 assays*, including blanks, standards and samples. Sample α-Ketoglutarate concentrations are determined by comparison with a known α-Ketoglutarate standard. The kit has a detection sensitivity limit of ~0.2 µM α-Ketoglutarate.

Note: Pyruvate present in samples can generate background signal. If pyruvate is present in your testing sample, perform 2 assays per sample. Treat one sample reaction with α-KG Reaction Converter and the other without. α-Ketoglutarate is calculated from the difference in fluorescence readings from the 2 wells.

Assay Principle

Cell Biolabs' α-Ketoglutarate Assay Kit measures α-Ketoglutarate through coupled enzymatic reactions. α-Ketoglutarate is transaminated with the production of pyruvate. Pyruvate is then detected

with the fluorometric probe. Samples and standards are incubated for 60-120 minutes and then read with a standard 96-well fluorometric plate reader (Ex. 530-570 nm/Em. 590-600 nm). Samples are compared to a known concentration of α-Ketoglutarate standard within the 96-well microtiter plate format.

Related Products

1. MET-5013: Lactate Assay (Fluorometric)
2. MET-5023: Glycogen Assay (Fluorometric)
3. MET-5029: Pyruvate Assay Kit (Fluorometric)
4. MET-5055: L-Amino Acid Assay Kit (Fluorometric)
5. MET-5124: Alanine Aminotransferase (ALT) Activity assay Kit (Fluorometric)

Kit Components

Box 1 (shipped on blue ice packs)

1. α-KG Reaction Converter (Part No. 51323C): Two 500 µL tubes
2. α-Ketoglutarate Standard (Part No. 51321C): One 100 µL tube of a 100 mM solution
3. α-KG Enzyme Mix (Part No. 51324C): Two 100 µL amber tubes
4. Fluorescence Probe (100X) (Part No. 260003): Two 100 µL amber tubes
5. HRP (Part No. 234402): One 100 µL tube of a 100 U/mL solution in glycerol

Box 2 (shipped on blue ice packs)

1. 10X Assay Buffer (Part No. 50292A): One 25 mL bottle
2. α-KG Substrate Mix (Part No. 51322B): Two 500 µL tubes

Materials Not Supplied

1. Distilled or deionized water
2. 10 kDa MWCO centrifugal filter (e.g., Amicon Ultra 0.5 mL) (Optional) (for high protein content samples)
3. 1X PBS
4. Standard 96-well fluorescence microtiter plate

Storage

Upon receipt, store the α-KG Substrate Mix and 10X Assay Buffer at 4ºC. Store the remaining components at -20ºC.

Preparation of Reagents

- 1X Assay Buffer: Warm the 10X Assay Buffer to room temperature prior to using. Dilute the Assay Buffer to 1X with deionized water by diluting the 25 mL Buffer with 225 mL deionized water for 250 mL total. Mix to homogeneity. Store the 1X Assay Buffer at 4ºC up to six months.

- Reaction Reagent**:** Prepare the Reaction Reagent by diluting the kit components accordingly (α-KG Reaction Converter 1:20, α-KG Substrate Mix 1:20, Fluorescence Probe 1:100, HRP 1:500, and α-KG Enzyme Mix 1:100 in 1X Assay Buffer). See Table 1A below for examples of Reaction Reagent preparation based on the number of assays employed.

Note: Prepare only enough for immediate use by scaling the above examples proportionally.

Note: Add the α-KG Enzyme Mix last to the Reaction Reagent mixture, vortex the solutions thoroughly between each component addition, and protect the solutions from light until use. For best results, place the Reaction Reagent on ice and use within 30 minutes of preparation. Do not store Reaction Reagent solutions.

α-KG Reaction Converter (µL)	α-KG Substrate Mix (µL)	Fluorescence Probe (100X) (µL)	HRP (µL)	1X Assay Buffer (µL)	α-KG Enzyme Mix (µL)	Number of Assays (150 µL/well)
750	750	150	30	13170	150	**100**
375	375	75	15	6585	75	**50**
188	188	38	8	3290	38	**25**

Table 1A. Preparation of Reaction Reagent.

- Negative Control Reaction Reagent**:** Prepare a Negative Control Reaction Reagent by **omitting** the α-KG Reaction Converter accordingly. Dilute the α-KG Substrate Mix 1:20, Fluorescence Probe 1:100, HRP 1:500, α-KG Enzyme Mix 1:100 in 1X Assay Buffer. See Table 1B below for examples of the Negative Control Reaction Reagent preparation based on the number of assays employed.

Note: Prepare only enough for immediate use by scaling the above examples proportionally.

Note: Add the α-KG Enzyme Mix last to the Negative Control Reaction Reagent mixtures, vortex the solutions thoroughly between each component addition, and protect the solutions from light until use. For best results, place the Negative Control Reaction Reagent on ice and use within 30 minutes of preparation. Do not store Negative Control Reaction Reagent solutions.

α-KG Reaction Converter (µL)	α-KG Substrate Mix (µL)	Fluorescence Probe (100X) (µL)	HRP (µL)	1X Assay Buffer (µL)	α-KG Enzyme Mix (µL)	Number of Assays (150 µL/well)
0	750	150	30	13920	150	**100**
0	375	75	15	6960	75	**50**
0	188	38	8	3478	38	**25**

Table 1B. Preparation of Negative Control Reaction Reagent.

Note: The Fluorescence Probe is light sensitive and must be stored accordingly.

Preparation of Samples

Samples should be assayed immediately or stored at -80ºC prior to performing the assay. Optimal experimental conditions for samples must be determined by the investigator. The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design. A set of serial dilutions is recommended for samples to achieve optimal assay results and minimize possible interfering compounds. Run proper controls as necessary. Always run a standard curve with samples. High protein concentrations may interfere with the assay. In this case, filter the sample with a 10kDa MWCO centrifugal filter before assaying (to reduce the possibility of protein interference and turbidity).

- Tissues: Weigh 500-1000 mg of sample and mince with scissors and a dounce until tissue is thoroughly liquified. Add 2 mL of 1X Assay Buffer or PBS and further sonicate the homogenate for several cycles on ice. Centrifuge 10 minutes at 12,000 x g to remove debris. Recover the supernatant and recentrifuge in a separate tube to clarify it further. Recover supernatant in a fresh eppendorf tube and incubate on ice. Prepare samples for testing and store the remaining supernatant at -80ºC. Prepare further dilutions in 1X Assay Buffer.
- Cell Suspensions: Prepare cells at 1x 10^6 cells/mL and rapidly homogenize the cell pellet with 0.2 mL cold PBS or 1X Assay Buffer. Centrifuge 10 minutes at 12,000 x g to remove debris. Recover supernatant in a fresh eppendorf tube and incubate on ice. Prepare samples for testing and store the remaining supernatant at -80ºC. Prepare further dilutions in 1X Assay Buffer.
- Serum: Collect blood without using an anticoagulant. Allow blood to clot for 30 minutes at room temperature. Centrifuge at 2000 x g and 4ºC for 10 minutes. Remove the serum layer and store on ice. Take care to avoid disturbing the white buffy layer. Aliquot samples for testing and store remaining solution at -80ºC. Perform serum dilutions in 1X Assay Buffer. Perform several serial dilutions to ensure values are within the range of the standard curve.
- Plasma: Collect blood with heparin or citrate (EDTA could cause a quenching effect) and centrifuge at 1000 x g and 4ºC for 10 minutes. Remove the plasma layer and store on ice. Take care to avoid disturbing the white buffy layer. Aliquot samples for testing and store remaining solution at -80ºC. Perform plasma dilutions in 1X Assay Buffer. Perform several serial dilutions to ensure values are within the range of the standard curve.

Notes:

1. *Samples with NADH concentrations above 10 µM and glutathione concentrations above 50 µM will oxidize the probe and could result in erroneous readings. To minimize this interference, it is recommended that superoxide dismutase (SOD) be added to the reaction at a final concentration of 40 U/mL.*
2. *Avoid samples containing DTT or β-mercaptoethanol since the Fluorescence Probe is not stable in the presence of thiols (above 10 µM).*
3. *The Fluorescence Probe is unstable at high pH (>8.5).*

Preparation of α-Ketoglutarate Standard Curve

1. Prepare fresh α-Ketoglutarate standards by **diluting the α-Ketoglutarate Standard stock from 100 mM to 1 mM in 1X PBS for a 1:100 dilution**. (Example: Add 10 µL of the α-Ketoglutarate Standard stock tube to 990 µL of 1X PBS). **Vortex thoroughly**.
2. Continue preparing a dilution series of α-Ketoglutarate in the range of 0-25 µM by diluting the α-Ketoglutarate standard preparation according to Table 2.

Tubes	1 mM α-Ketoglutarate Standard (µL)	1X PBS (µL)	Final α-Ketoglutarate Concentration (µM)	α-Ketoglutarate Quantity (nmoles/well) *
1	25	975	25	1.25
2	250 of Tube #1	250	12.5	0.625
3	250 of Tube #2	250	6.25	0.313
4	250 of Tube #3	250	3.13	0.156
5	250 of Tube #4	250	1.56	0.078
6	250 of Tube #5	250	0.78	0.039
7	250 of Tube #6	250	0.39	0.02
8	250 of Tube #7	250	0.2	0.01
9	0	250	0	0

Table 2. Preparation of α-Ketoglutarate Standards.

*Note: *Based on 50 µL volume/well. Do not store diluted α-Ketoglutarate standard solutions.*

Assay Protocol

Each α-Ketoglutarate standard, controls and samples should be assayed in duplicate or triplicate. A freshly prepared standard curve should be used each time the assay is performed.

Note: Pyruvate present in samples can generate background signal. If pyruvate is present in your testing sample, perform 2 assays per sample. Treat one sample reaction with α-KG Reaction Converter and the other without (1X Assay buffer will be added to the Negative Control Reaction Mix in place of α-KG Reaction Converter). α-Ketoglutarate is calculated from the difference in OD readings from the 2 wells.

1. Add 50 µL of the diluted α-Ketoglutarate standards, controls or samples to each well of a 96-well microtiter plate.
2. Add 100 µL of the prepared Reaction Reagent to the samples and to one half of the paired sample wells, and mix the well contents thoroughly.
3. Add 100 µL of the prepared Negative Control Reaction Reagent to the remaining half of the paired sample wells, and mix the well contents thoroughly.
4. Incubate the wells for 60-120 minutes at 37ºC protected from light.

Note: This assay is continuous (not terminated) and therefore may be measured at multiple time points to follow the reaction kinetics.

5. Read the fluorescence of each microwell used on a fluorescence microplate reader using 530-570 nm excitation filter and 590-600 nm emission filter.

Example of Results

The following figures demonstrate typical α-Ketoglutarate Assay Kit results. One should use the data below for reference only. This data should not be used to interpret or calculate actual sample results.

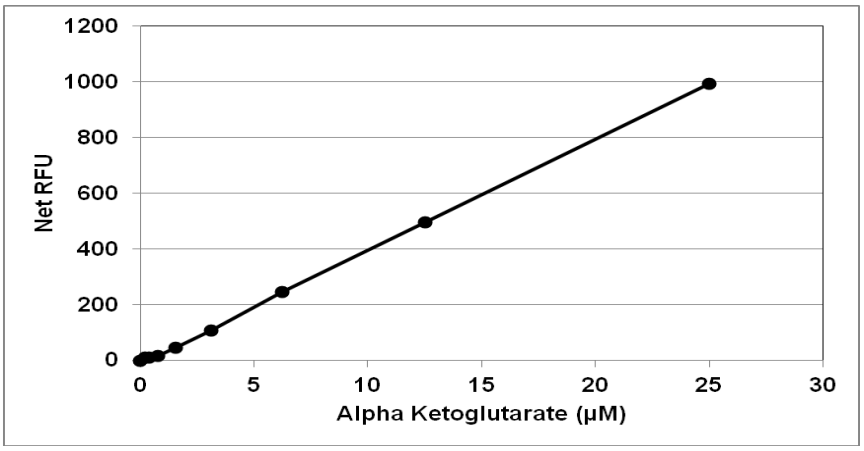

Figure 2: Example α-Ketoglutarate Standard Curve.

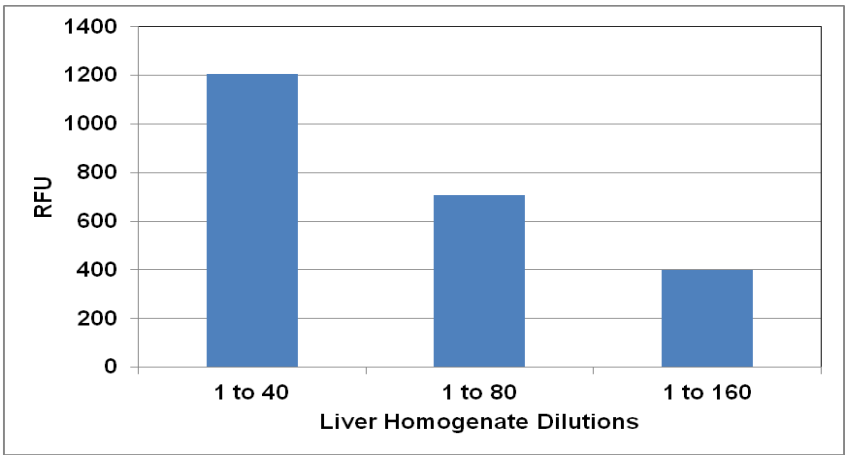

Figure 3: Liver Homogenates. Chicken livers were homogenized and sonicated in cold 1X Assay Buffer. Upon centrifugation, the homogenate was tested according to the assay protocol.

Calculation of Results

1. Calculate the average fluorescence values for every standard, control, and sample. Subtract the average zero standard value from itself and all standard and sample values. This is the corrected fluorescence.
2. Subtract the sample fluorescence well values without α-KG Reaction Converter (-α-KG RC) from the sample well values containing α-KG Reaction Converter (+α-KG RC) to obtain the net fluorescence difference. The fluorescence difference is due to the endogenous pyruvate activity.

$$\textbf{Net } \Delta\textbf{RFU} = (\textbf{RFU}_{+\ \alpha\textbf{-KG RC}}) - (\textbf{RFU}_{-\ \alpha\textbf{-KG RC}})$$

3. Plot the corrected fluorescence for the standards against the final concentration of the α-Ketoglutarate standards from Table 2 to determine the best curve. Use the 60–120-minute reading values, or final time point fluorescence values, to plot the α-Ketoglutarate standard curve. See Figure 2 for an example standard curve.
4. Determine the α-Ketoglutarate concentration of the samples with the equation obtained from the linear regression analysis of the standard curve. Substitute the corrected fluorescence values for each sample. Only use values within the range of the standard curve. Remember to account for dilution factors.

$$\textbf{α-Ketoglutarate (μM)} = \left[\frac{\textbf{Sample Corrected Fluorescence}}{\textbf{Slope}} \right] \textbf{x Sample dilution}$$

Note: α-Ketoglutarate molecular weight is 146.11 g/mol.

References

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3. Hou, Y.Q., et al. (2010) *Front Biosci.* 16: 1186-1196.
4. Tatara M., et al. (2005) *Poult. Sci.* **84(10)**: 1604-1609.
5. Yao, K., et al. (2012) *Amino Acids* **42**: 2491-2500.

Warranty

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