Alpha Ketoglutarate Assay Kit (Fluorometric)

Alpha Ketoglutarate Assay Kit (Fluorometric) is a detection kit for the quantification of Alpha Ketoglutarate in serum, plasma (heparin, citrate), cell culture supernatants and tissue homogenates.

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MANUFACTURED BY:

Arigo Biolaboratories Corporation

Address: 9F.-7, No. 12, Taiyuan 2nd St., Zhubei City,

Hsinchu County 302082, Taiwan

Tel: +886-3-6221320

Fax: +886-3-5530266

Email: info@arigobio.com

INTRODUCTION

α-Ketoglutaric acid (2-oxoglutaric acid) is one of two ketone derivatives of glutaric acid. The term "ketoglutaric acid," when not further qualified, almost always refers to the alpha variant. β-Ketoglutaric acid varies only by the position of the ketone functional group, and is much less common.

Its carboxylate, α-ketoglutarate also called 2-oxoglutarate, is an important biological compound. It is the keto acid produced by deamination of glutamate, and is an intermediate in the Krebs cycle. [Provide by Wikipedia: α-ketoglutarate]

PRINCIPLE OF THE ASSAY

This Alpha Ketoglutarate Assay Kit (Fluorometric) is a simple fluorometric assay that measures the amount of Alpha Ketoglutarate (α-KG) present in serum, plasma (heparin, citrate), cell culture supernatants and tissue homogenates. In the assay, 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.

MATERIALS PROVIDED & STORAGE INFORMATION

The kit is shipped on ice. Store the α-KG Substrate Mix and 10X Assay Buffer at 4°C, and other components at-20°C.

Component	Quantity	Storage information
α-KG Standard (100 mM solution)	100 µL	-20°C
α-KG Reaction Converter	500 µL	-20°C
α-KG Enzyme Mix	100 µL	-20°C
α-KG Substrate Mix	750 µL	4°C
Fluorescence Probe	100 µL	-20°C (Protect from light)
HRP (100 U/mL solution in glycerol)	100 µL	-20°C
10X Assay Buffer	25 mL	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Fluorescence microplate reader capable of reading excitation at 530-570 nm and emission at 590-600 nm
- Centrifuge and centrifuge tube (eppendorf)
- 1X PBS
- Deionized or distilled water
- Black flat-bottom 96 well microplate
- Pipettes, pipette tips and multichannel micropipette reservoir

TECHNICAL NOTES AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Reagents are for research use only. Normal precautions for laboratory reagents should be exercised while using the reagents. Please refer to Material Safety Data Sheet for detailed information.
- 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.
- 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.
- Avoid samples containing DTT or β-mercaptoethanol since the Fluorescence Probe is not stable in the presence of thiols (above 10 µM).
- The Fluorescence Probe is unstable at high pH (>8.5).
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- It is highly recommended assaying the Standards and samples in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

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.

Serum: Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 2000 x g for 10 minutes at 4°C. Collect the serum and assay directly. 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 and centrifuge at 2000 x g for 10 minutes at 4°C. Collect the plasma layer and assay directly. Perform plasma dilutions in 1X Assay Buffer. Perform several serial dilutions to ensure values are within the range of the standard curve.

Tissue: 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-80°C. Prepare further dilutions in 1X Assay Buffer.

Cell suspension: 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-80°C. Prepare further dilutions in 1X Assay Buffer.

Note:

- Samples should be assayed immediately or stored at -80°C prior to performing the assay.
- 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.
- 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.
- Avoid samples containing DTT or β-mercaptoethanol since the Fluorescence Probe is not stable in the presence of thiols (above 10 µM).
- The Fluorescence Probe is unstable at high pH (>8.5).

REAGENT PREPARATION

- **1X Assay Buffer:** equilibrate the 10X Assay Buffer to room temperature prior to using. Dilute the 10X Assay Buffer to 1X with distilled water. (E.g., mix 25 mL of 10X Assay Buffer with 225 mL of distilled water for 250 mL total) Mix to homogeneity. Store the 1X Assay Buffer at 4°C 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 αKG Enzyme Mix 1:100 in 1X Assay Buffer. See Table below for examples of Reaction Reagent preparation based on the number of assays employed.

α-KG Reaction Converter (µL)	α-KG Substrate Mix (µL)	Fluorescence probe (µL)	HRP (µL)	1X Assay Buffer (µL)	α-KG Enzyme Mix (µL)	Number of Assay (100 µL/well)
500	500	100	20	8780	100	100
250	250	50	10	4390	50	50
125	125	25	5	2195	25	25

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

α-KG Reaction Converter (µL)	α-KG Substrate (µL)	Fluorescence probe (µL)	HRP (µL)	1X Assay Buffer (µL)	α-KG Enzyme Mix (µL)	Number of Assays (100 µL/well)
0	500	100	20	9280	100	100
0	250	50	10	4640	50	50
0	125	25	5	2320	25	25

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

- **Standards:** 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. (E.g., Add 10 µL of the α-Ketoglutarate Standard stock tube to 990 µL of 1X PBS). Dilute standard as follows.

Standard tube	α-Ketoglutarate (µM)	1X PBS (µL)	α-Ketoglutarate Standard *1 mM (µL)	α-Ketoglutarate Quantity (nmoles/well)*
S1	25	975	25	1.25
S2	12.5	250	250 of S1	0.625
S3	6.25	250	250 of S2	0.313
S4	3.13	250	250 of S3	0.156
S5	1.56	250	250 of S4	0.078
S6	0.78	250	250 of S5	0.039
S7	0.39	250	250 of S6	0.02
S8	0.2	250	250 of S7	0.01
S0	0	250	0	0

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

ASSAY PROCEDURE

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.

	Standard well	Reaction Sample well	Negative control well
Each diluted Standard	50 µL	-	-
Each Sample	-	50 µL	50 µL
Reaction Reagent	100 µL	100 µL	-
Negative Reagent	-	-	100 µL
Tap plate to mix briefly and thoroughly. Incubate for **60-120 minutes** at **37°C** in the dark.			
Read the fluorescence intensity at **λex/em** = **530-570/590-600 nm**.			

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

CALCULATION OF RESULTS

1. Calculate the average absorbance values for every standard, control, and sample. Subtract the average zero standard (S0) value from itself and all standard and sample values. This is the corrected absorbance.
2. α-Ketoglutarate molecular weight is 146.11 g/mol.
3. Subtract the sample absorbance 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 absorbance difference. The absorbance difference is due to the endogenous pyruvate activity.

$$\text{Net } \Delta OD = (OD_{+\ \alpha\text{-KG RC}}) - (OD_{-\ \alpha\text{-KG RC}})$$

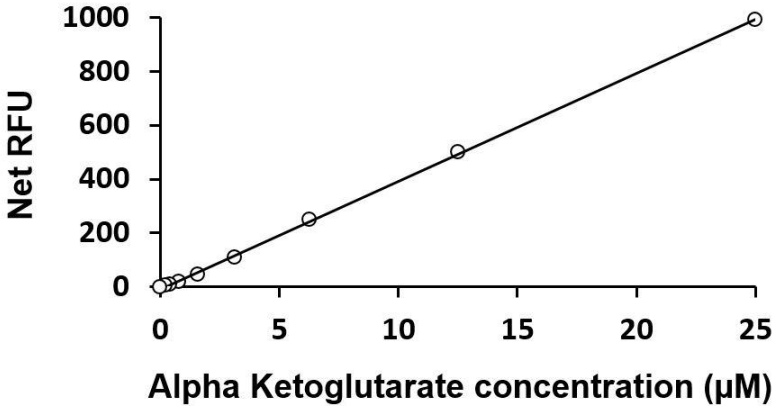
4. Determine the α-Ketoglutarate concentration of the samples with the equation obtained from the linear regression analysis of the standard curve. Substitute the corrected absorbance values for each sample. Only use values within the range of the standard curve. Remember to account for dilution factors.

$$\alpha\text{-Ketoglutarate } (\mu M) = (\text{Sample Corrected Absorbance} / \text{Slope}) \times n$$

5. Plot the corrected absorbance for the standards against the final concentration of the α-Ketoglutarate standards curve. Use the 60-120 minutes reading values, or final time point absorbance values, to plot the α-Ketoglutarate standard curve.

EXAMPLE OF RESULT

The following figures demonstrate typical results with the Alpha Ketoglutarate Assay Kit (Fluorometric). One should use the data below for reference only. This data should not be used to interpret actual results.



QUALITY ASSURANCE

Sensitivity

0.2 µM