MA-0141

α-Ketoglutarate Assay

(100 wells, Colorimetric, OD 570 nm or Fluorometric, Ex/Em = 535/587 nm, Store at -20°C)

Introduction:

α-Ketoglutarate is a key intermediate in multiple pathways, playing essential roles in energy production, amino acid metabolism, and cellular homeostasis. In the TCA cycle, α-ketoglutarate is converted into succinyl-CoA, producing ATP. α-Ketoglutarate is a precursor for the synthesis of several amino acids, including glutamate and glutamine which are critical for neurotransmission, immune function, and cellular signaling. α-Ketoglutarate also acts as a signaling molecule, influencing gene expression and processes such as oxidative stress response and protein synthesis. AkrivisBio's α-Ketoglutarate Assay is a simple, sensitive way to measure this important molecule in a wide range of sample types, with a useful measurement range from 10 nmoles down to below 10 pmoles per well.

Assay Principle:

1 – Alanine aminotransferase transfers the amino group from alanine to α-ketoglutarate forming pyruvate and glutamate
2 – Pyruvate is oxidized by pyruvate oxidase, forming hydrogen peroxide.
3 – Hydrogen peroxide is utilized by peroxidase to oxidize its substrate ADHP to resorufin with intense color and fluorescence.

Assay Components:

Assay Buffer	25 ml	WM	MA-0141-A
ADHP Solution	0.2 ml	Red	MA-0141-B
Alanine aminotransferase	lyoph	Purple	MA-0141-C
Pyruvate oxidase/Peroxidase	lyoph	Green	MA-0141-D
α-Ketoglutarate Standard	lyoph	Yellow	MA-0141-E

Storage and Handling:

Store unopened kit at -20°C. Bring assay buffer and ADHP solution to room temperature before use. Centrifuge all vials briefly prior to opening.
Assay Buffer: Ready to use as supplied.
ADHP Solution: Ready to use as supplied. Light sensitive. Store at -20°C.
Alanine aminotransferase, Pyruvate oxidase/Peroxidase: Reconstitute with 220 µl Assay Buffer each. If the assay is to be used repeatedly over a period of time, aliquot the enzymes into convenient portions and store at -20°C to avoid repeated freeze/thaw cycles.
α-Ketoglutarate Standard: Dissolve in 100 µl DI H_2O, giving a 40 mM Standard solution. Keep on ice while in use. Store at -20°C.

Assay Protocol:

1. Standard Curve:

Absorbance-Based Assay: Dilute the 40 mM Standard to 0.4 mM by transferring 10 µl to 990 µl of DI H_2O. Transfer 0 – 5 – 10 – 15 – 20 - 25 µl to a series of wells in a 96-well plate. Adjust all well volumes to 50 µl with Assay Buffer to giving 0, 2, 4, 6, 8, 10 nmol of Standard.

Fluorescence-based Assay: Dilute the standards as for the absorbance-based assay then further dilute another 10-fold by transferring 10 µl of the 0.4 mM solution to 90 µl of DI H_2O. Transfer 0 – 5 – 10 – 15 – 20 - 25 µl to a series of wells in a 96-well plate and adjust all wells to 50 µl giving 0, 200, 400, 600, 800 and 1000 pmoles of Standard.

2. Sample Preparation: Place a portion of the Assay Buffer on ice for 30 minutes before proceeding. Homogenize tissue (10 mg) or cells (10^6) in 100 µl of ice-cold Assay Buffer. Deproteinize samples either using protein precipitation or 10 kDa centrifugal filters. Transfer 5-50 µl in duplicate to wells of a 96-well plate and adjust all well volumes to 50 µl with Assay Buffer.
Note: It is important that all samples are within the range of the standard curve. If any are outside this range, dilute appropriately and rerun.

3. Initiate Reaction: Each well requires 50 µl of Reaction Mix. Prepare sufficient Reaction Mix for the number of wells to be run, containing:

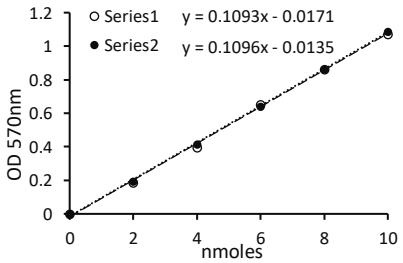
	Absorbance-Based		Fluorescence-Based	
	Sample	**Bkgd. Control**	**Sample**	**Bkgd. Control**
Assay Buffer	44 µl	46 µl	45.6 µl	47.6 µl
Alanine aminotransferase	2 µl	-----	2 µl	-----
Pyruvate oxidase/Peroxidase	2 µl	2 µl	2 µl	2 µl
ADHP Solution	2 µl	2 µl	0.4 µl	0.4 µl

Add 50 µl of the Reaction Mix to each well containing the α-KG Standard, samples or background control*.
Note: Pyruvate in samples causes background. If pyruvate is suspected, prepare background control omitting alanine aminotransferase.

4. Measurement: Monitor wells in kinetic mode at 37°C, with either absorbance at 570 nm or fluorescence at Ex/Em = 535/587 nm.

5. Typical results:

nmol Standard	Standard Raw Values OD		Background Corrected Values OD	
0	0.0835	0.0824	0	0
2	0.2718	0.2787	0.1883	0.1963
4	0.4808	0.4989	0.3973	0.4165
6	0.7376	0.7253	0.6541	0.6429
8	0.9451	0.9452	0.8616	0.8628
10	1.1584	1.172	1.0749	1.0896



6. Calculations: Subtract the value of the zero standard from all other standards. Plot the standard curve and determine its slope. This slope defines the system sensitivity. If background control wells have been run, subtract the background control from its paired test sample value. Divide the background corrected sample values by the slope of the standard curve to convert absorbance or fluorescence to nmoles or pmoles per well. To convert these values to ketoglutarate in the original samples:

A. Divide the nmoles (pmoles) per well by the volume of sample added to the well = nmoles (pmoles)/ µl of sample
B. Multiply the nmoles (pmoles)/ µl of sample by the total volume of the deproteinized samples = total nmoles (pmoles) per sample.
C. Divide the total nmoles (pmoles) per sample by the mg tissue, # of cells or other sample characterization = nmoles (pmoles/mg (or # of cells, etc.)

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