GenWay Biotech, Inc.

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For research use only

α-Ketoglutarate Assay Kit

(Catalog #GWB-AXR321; 100 assays; Store Kit at -20°C)

I. Introduction:

α-Ketoglutarate (α-KG) is a key intermediate in the Krebs cycle, coming after isocitrate and before succinyl CoA. Anaplerotic reactions replenish the cycle by synthesizing α-KG from transamination of glutamate, or through the action of glutamate dehydrogenase. α-KG is an importantnitrogen transporter. Being a key intermediate, it is one of the organic acids measured in newborns as an indicator of inborn errors of metabolism. GenWay's α-Ketoglutarate Assay Kit provides a simple, sensitive and rapid means for quantifying α-KG in a variety of samples. In the assay, α-ketoglutarate is transaminated with the generation of pyruvate which is utilized to convert a nearly colorless probe to both color (λ_{max} = 570nm) and fluorescence (Ex/Em = 535/587 nm).The α-Ketoglutarate Assay Kit is useful for detecting α-ketoglutarate in the range of 0.01 to 10 nmoles.

II. Kit Contents:

Components		Cap Code	Part Number
α-KG Assay Buffer	25 ml	WM	GWB-AXR321-1
α-KG OxiRed Probe (in DMSO)	0.2 ml	Red	GWB-AXR321-2A
α-KG Converting Enzyme (lyophilized)	1 vial	Purple	GWB-AXR321-4
α-KG Development Enzyme Mix (lyophilized)	1 vial	Green	GWB-AXR321-5
α-KG Standard (10 µmol, lyophilized)	1 vial	Yellow	GWB-AXR321-6

III. Storage and Handling:

Store kit at –20˚C, protect from light. Warm α-KGAssay Buffer to room temperature before use. Briefly centrifuge all small vials prior to opening.

IV. Reagent Preparation and Storage Conditions:

α-KGProbe: Ready to use as supplied. Warm to room temperature before using to melt frozen DMSO. Protect from light and moisture. Stable for 2 months at -20°C.

α-KGConverting Enzyme, α-KGEnzyme Mix: Dissolve with 220 µl α-KG Buffer separately. Pipette up and down to dissolve. Aliquot into vials with sufficient amount for each experiment and store at –20˚C. Avoid repeated freeze/thaw cycles. Use within two months.

α-KG Standard: Dissolve in 100 µl dH_2O to generate 100 mM (100nmol/µl) α-KG Standard solution. Keep cold while in use. Store at -20°C.

V. Assay Protocol:

1. Standard Curve Preparations:

Dilute the α-KGStandard to 1 nmol/µl by adding 10µl of the Standard to 990 µl of dH_2O, mix well. Add 0, 2, 4, 6, 8, 10 µl into a series of standards wells on a 96-well plate. Adjust volume to 50 µl/well with Assay Buffer to generate 0, 2, 4, 6, 8, 10 nmol/well of the Standard.

Fluorometric Assay: Dilute further dilute by adding 10 µl to 90 µl of dH2O. Add 0, 2, 4, 6, 8, 10 µl into a series of standards well on a 96-well plate. Adjust the volume to 50 µl/well to generate 0, 0.2, 0.4, 0.6, 0.8, 1.0 nmol/well.

2. Sample Preparation:

Tissue (20 mg) or cells (2 x 10^6) are rapidly homogenized with 100 µl of ice cold PBS or other buffer (pH 6.5 - 8). Enzymes in samples may interfere with the assay. We suggest deproteinizing samples using a perchloric acid/KOH protocol or 10 kDa molecular weight cut off spin columns. Add1 - 50 µl samples into duplicate wells of a 96-well plate and bring volume to 50 µl with Assay Buffer.We suggest testing several doses of your samples to ensure readings are within the linear range.

3. Mix enough reagent for the number of samples and standards to be performed: For each well, prepare a total 50 µl Reaction Mix containing:

	Sample	Bkgd. Control*
α-KG Assay Buffer	44µl	46µl
α-KG Converting Enzyme	2 µl	-----
α-KGEnzyme Mix	2 µl	2 µl
α-KG probe	2 µl	2 µl

Add 50 µl of the Reaction Mix to each well containing the α-KG Standard, samples or background control*.

***Note1:** Pyruvate generates background. If pyruvate is suspected in your sample, you can do the background control omitting the converting enzyme. The background control can be subtracted from the α-KG reading.

Note2 (optional): for fluorometric assay use 0.4 µl Pyruvate Probe and 45.6 µl (47.6 µl for Bkgd Control) Pyruvate assay buffer to reduce background

4. Incubate for 30 min at 37°C, protect from light.

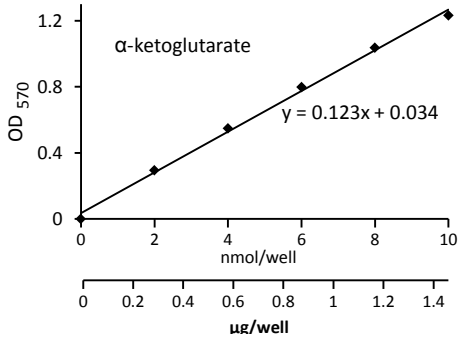
5. Measure OD at 570 nm or fluorescence using Ex/Em = 535/587 nm.

6. Calculation: Correct background by subtracting the value of the 0 α-KGblank from all readings. Plot the standard curve. Apply the corrected sample readings to the standard curve to get α-KGamount in the sample wells.The α-KGconcentrations in the test samples:

C = Ay/Sv (nmol/µl; or µmol/ml; or mM)

Where: Ay is the amount of α-KG (nmol) in your sample from the standard curve.
Sv is the sample volume (µl) added to the sample well.

α-Ketoglutarate molecular weight: 146.11



α-Ketoglutaratestandard curve generated using this kit protocol.

FOR RESEARCH USE ONLY! Not to be used on humans.

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GENERAL TROUBLESHOOTING GUIDE:

Problems	Cause	Solution
Assay not working	• Use of ice-cold assay buffer	• Assay buffer must be at room temperature
	• Omission of a step in the protocol	• Refer and follow the data sheet precisely
	• Plate read at incorrect wavelength	• Check the wavelength in the data sheet and the filter settings of the instrument
	• Use of a different 96-well plate	• Fluorescence: Black plates (clear bottoms) ; Luminescence: White plates ; Colorimeters: Clear plates
Samples with erratic readings	• Use of an incompatible sample type	• Refer data sheet for details about incompatible samples
	• Samples prepared in a different buffer	• Use the assay buffer provided in the kit or refer data sheet for instructions
	• Samples were not deproteinized (if indicated in datasheet)	• Use the 10 kDa spin cut-off filter or PCA precipitation as indicated
	• Cell/ tissue samples were not completely homogenized	• Use Dounce homogenizer (increase the number of strokes); observe for lysis under microscope
	• Samples used after multiple free-thaw cycles	• Aliquot and freeze samples if needed to use multiple times
	• Presence of interfering substance in the sample	• Troubleshoot if needed, deproteinize samples
	• Use of old or inappropriately stored samples	• Use fresh samples or store at correct temperatures till use
Lower/ Higher readings in Samples and Standards	• Improperly thawed components	• Thaw all components completely and mix gently before use
	• Use of expired kit or improperly stored reagents	• Always check the expiry date and store the components appropriately
	• Allowing the reagents to sit for extended times on ice	• Always thaw and prepare fresh reaction mix before use
	• Incorrect incubation times or temperatures	• Refer datasheet & verify correct incubation times and temperatures
	• Incorrect volumes used	• Use calibrated pipettes and aliquot correctly
Readings do not follow a linear pattern for Standard curve	• Use of partially thawed components	• Thaw and resuspend all components before preparing the reaction mix
	• Pipetting errors in the standard	• Avoid pipetting small volumes
	• Pipetting errors in the reaction mix	• Prepare a master reaction mix whenever possible
	• Air bubbles formed in well	• Pipette gently against the wall of the tubes
	• Standard stock is at an incorrect concentration	• Always refer the dilutions in the data sheet
	• Calculation errors	• Recheck calculations after referring the data sheet
	• Substituting reagents from older kits/ lots	• Use fresh components from the same kit
Unanticipated results	• Measured at incorrect wavelength	• Check the equipment and the filter setting
	• Samples contain interfering substances	• Troubleshoot if it interferes with the kit
	• Use of incompatible sample type	• Refer data sheet to check if sample is compatible with the kit or optimization is needed
	• Sample readings above/below the linear range	• Concentrate/ Dilute sample so as to be in the linear range
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		