α-Ketoglutarate Detection Assay Kit

Item No. 701350

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size	Storage
701351	α-Ketoglutarate Assay Buffer	1 vial/10 ml	-20°C
701352	α-Ketoglutarate Standard	2 vials	-20°C
700002	ADHP Assay Reagent	2 vials	-20°C
701353	α-Ketoglutarate Cofactor Mixture	2 vials	-20°C
701354	α-Ketoglutarate Converting Enzyme	2 vials	-20°C
701355	α-Ketoglutarate Development Enzyme	2 vials	-20°C
700001	DMSO Assay Reagent	1 vial/1 ml	RT
400017	96-Well Solid Plate (black)	1 plate	RT
400012	96-Well Cover Sheet	1 cover	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section, on page 3, and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A microplate reader with the capacity to measure fluorescence using an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm or absorbance at 570 nm
2. An orbital microplate shaker
3. A source of pure water: glass-distilled water is acceptable
4. Microcentrifuge tubes
5. A refrigerated centrifuge
6. Dounce homogenizer for tissue samples
7. 10 kDa spin columns
8. Phosphate-buffered saline (PBS)
9. A clear bottom microtiter plate if performing a colorimetric assay

INTRODUCTION

Background

α-Ketoglutarate (α-KG) is a rate-determining metabolic intermediate in the citric acid cycle.[1,2] It is formed *via* oxidative decarboxylation of isocitrate by isocitrate dehydrogenase (IDH), oxidative deamination of glutamate by glutamate dehydrogenase, or by pyridoxal phosphate-dependent transamination.[2] α-KG is decarboxylated to succinyl-CoA by α-KG dehydrogenase with CoA as a coenzyme, a rate-limiting step in the citric acid cycle. It is a precursor of glutamine and glutamate and an antioxidant with roles in immune homeostasis, aging, protein synthesis, and bone development.[2,3] α-KG levels are increased in the urine of individuals with maple syrup urine disease (MSUD), an inborn error of metabolism characterized by branched-chain α-keto acid dehydrogenase (BCKAD) deficiency that leads to progressive ketoacidosis, failure to thrive, neurological dysfunction, and, potentially, death.[4,5]

About This Assay

Cayman's α-KG Detection Assay provides fluorometric- or colorimetric-based methods for quantifying α-KG in biological samples such as serum, plasma, urine, and tissue. It can also be utilized to determine intracellular and extracellular α-KG concentrations in cell culture samples. In this assay, alanine transaminase (ALT) deaminates α-KG to form pyruvate.[6,7] Pyruvate is then oxidized by pyruvate oxidase to yield acetyl phosphate, hydrogen peroxide (H_2O_2), and carbon dioxide.[8] As H_2O_2 is produced, the cycling mechanism of horseradish peroxidase (HRP) results in the concurrent reduction of H_2O_2 to H_2O and the oxidation of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP) to produce the highly fluorescent compound resorufin.[9] Resorufin fluorescence is analyzed with an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm. Alternatively, the absorbance of resorufin can be measured at 570 nm.

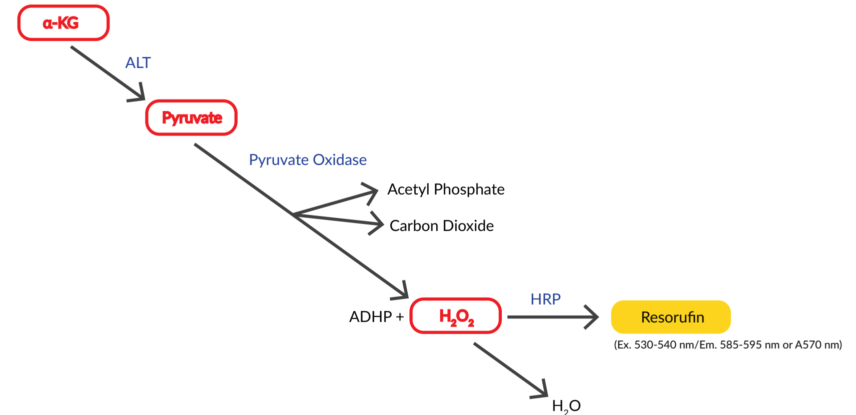

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **α-Ketoglutarate Assay Buffer - (Item No. 701351)**

   Dilute 5 ml of the supplied 10X Assay Buffer with 45 ml of pure water. This 1X Assay Buffer should be used in the assay and for preparing any reagents and samples. Any unused buffer may be stored at -20°C.

2. **ADHP Assay Reagent - (Item No. 700002)**

   The vial contains a lyophilized powder of ADHP. Instructions for preparing the ADHP Assay Reagent as an assay detector are found on page 16. Each vial contains enough reagent to assay 60 wells. Use the prepared assay detector within 15 minutes or increased background will result.

3. **α-Ketoglutarate Cofactor Mixture- (Item No. 701353)**

   The vial contains a lyophilized powder of FAD and thiamine pyrophosphate. Reconstitute the contents of the vial with 600 µl of 1X Assay Buffer. This is enough cofactor to assay 60 wells. Prepare the additional vial as needed.

4. **α-Ketoglutarate Converting Enzyme - (Item No. 701354)**

   The kit contains two vials of lyophilized α-KG Converting Enzyme. One vial contains enough enzyme to run half of a 96-well plate. Reconstitute the contents of the vial with 280 µl of prepared Assay Buffer, place on ice, and use within two hours. Prepare the additional vial as needed.

5. **α-Ketoglutarate Development Enzyme - (Item No. 701355)**

   The kit includes two vials of lyophilized pyruvate oxidase and HRP. One of the vials contains enough enzyme to run half of a 96-well plate. At the time of the assay, reconstitute each vial with 0.8 ml of prepared Assay Buffer, place on ice, and use within two hours. Prepare the additional vial as needed.

6. **α-Ketoglutarate Standard - (Item No. 701352)**

   The vial contains a lyophilized powder of α-KG. Reconstitute the vial with 1 ml of 1X Assay Buffer before use. This 10 mM solution is used to prepare the standard curve. Any unused standard may be stored at -20°C for approximately one week.

7. **DMSO Assay Reagent - (Item No. 700001)**

   The vial contains DMSO, which is ready to use as supplied.

Sample Preparation

The best results can be obtained with samples that are prepared and assayed immediately. This protocol includes recommended starting dilutions. Optimal dilutions should be determined by the end user.

Conditioned Media

1. Harvest and count the target cells necessary to obtain 2 x 10^6 cells.
2. Centrifuge at 5,000 × g for five minutes to pellet cells.
3. The cell pellet is used for intracellular α-KG determination (see below).
4. Assay the extracellular media sample undiluted.

Intracellular α-KG

1. Wash the cell pellet prepared above with 1 ml of PBS.
2. Centrifuge sample at 5,000 × g for 5 minutes to re-pellet the cells.
3. Remove the PBS and discard.
4. To the cell pellet, add 1 ml of ice-cold 1X Assay Buffer, and pipette up and down or vortex repeatedly to aid in cell lysis.
5. Let sample sit on ice for 20 minutes. Cell lysis can be monitored by examining under a microscope with addition of trypan blue. Some cell lines may take longer to lyse.
6. Centrifuge the samples at 10,000 × g at 4°C for five minutes to pellet the cellular debris.
7. Transfer supernatant to new tube and assay undiluted.

Tissue Homogenate

1. Collect tissue and wash with PBS to remove excess red blood cells.
2. Homogenize tissue in approximately 1 ml of ice-cold 1X Assay Buffer using a Dounce homogenizer with tight-fitting pestle (about 20-30 passes). It is recommended to homogenize about 200 mg of tissue per 1 ml of buffer.
3. Centrifuge homogenate at 10,000 × g for 10 minutes at 4°C to pellet cellular and nuclear debris.
4. Transfer supernatant to new tube.
5. Dilute the supernatant at least 1:4 with prepared 1X Assay Buffer before assaying.

Urine

1. Urine should be centrifuged through a 10 kDa molecular weight cut-off filter and assayed undiluted.

Plasma and Serum

1. Plasma and serum samples should be centrifuged through a 10 kDa molecular weight cut-off filter and assayed undiluted.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of the α-KG standard curve and samples to be measured in triplicate is provided in Figure 2. We suggest recording the contents of each well on the template sheet provided (see page 22).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	A	S1	S1	SB1	S9	S9	SB9	S17	S17	SB17
B	B	B	B	S2	S2	SB2	S10	S10	SB10	S18	S18	SB18
C	C	C	C	S3	S3	SB3	S11	S11	SB11	S19	S19	SB19
D	D	D	D	S4	S4	SB4	S12	S12	SB12	S20	S20	SB20
E	E	E	E	S5	S5	SB5	S13	S13	SB13	S21	S21	SB21
F	F	F	F	S6	S6	SB6	S14	S14	SB14	S22	S22	SB22
G	G	G	G	S7	S7	SB7	S15	S15	SB15	S23	S23	SB23
H	H	H	H	S8	S8	SB8	S16	S16	SB16	S24	S24	SB24

A-H = Standard wells
S1-S24 = Sample wells
SB1-SB24 = Sample background wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps to maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 150 µl in all wells.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in duplicate (triplicate preferred) but it is at the user's discretion to do so.
- The assay is performed at room temperature.
- Monitor the fluorescence with an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 or monitor the absorbance at 570 nm. *NOTE: This assay can be read using fluorescence or absorbance. You do not need to prepare both standard curves; choose the one that matches the format you will be using.*

Standard Preparation

Using the 10 mM α-KG Standard prepared in the Reagent Preparation section (see page 9), add 100 µl of the 10 mM solution to 900 µl of the prepared Assay Buffer to get a 1 mM α-KG stock solution.

To prepare the standard curve for use in the assay: Obtain 8 clean test tubes and label them A-H. Add the amount of 1 mM α-KG stock solution and prepared Assay Buffer to each tube as described in Tables 1 and 2, below and on page 15, respectively. The diluted standards will be stable for approximately one hour on ice.

Tube	1 mM α-KG Stock Solution (µl)	1X Assay Buffer (µl)	Final Concentration (µM)	Final Standard Amount (nmol/well)
A	0	1,000	0	0
B	10	990	10	0.1
C	25	975	25	0.25
D	50	950	50	0.5
E	75	925	75	0.75
F	100	900	100	1
G	150	850	150	1.5
H	200	800	200	2

Table 1. Preparation of α-KG standards - fluorometric

Tube	1 mM α-KG Stock Solution (µl)	1X Assay Buffer (µl)	Final Concentration (µM)	Final Standard Amount (nmol/well)
A	0	1,000	0	0
B	50	950	50	0.5
C	100	900	100	1
D	200	800	200	2
E	400	600	400	4
F	600	400	600	6
G	800	200	800	8
H	1,000	0	1,000	10

Table 2. Preparation of α-KG standards - colorimetric

Performing the Assay

NOTE: Use the 96-Well Plate (black) (Item No. 400017) for the fluorometric assay or a 96-well clear bottom plate (not provided) for the colorimetric assay.

1. **Prepare the Enzyme Reaction Mixtures**

   The following protocol will prepare a sufficient amount of enzyme mixture to run half of a 96-well assay plate. If running a full plate, use the additional vial of α-KG Development Enzyme included in the kit and multiply the following volumes by 2. Label two tubes as Sample and one tube as Sample Background.

   Sample Reaction Mixture - Add 400 µl of the prepared α-KG Development Enzyme and 100 µl of the reconstituted α-KG Converting Enzyme.

   Sample Background Reaction Mixture - Add 320 µl of the prepared α-KG Development Enzyme and 80 µl of prepared 1X Assay Buffer.

2. **Prepare the Assay Detector**

   Immediately before running the assay, add 75 µl of DMSO Assay Reagent to the ADHP Assay Reagent and vortex to dissolve the powder. Then add 700 µl of 1X Assay Buffer and vortex well. This prepared assay detector should be used within 15 minutes. Further delay will result in the increased background.

3. **Prepare the Assay Plate**

   Add the other reagents to each well according to Table 3, on page 17. Initiate the reactions by adding 10 µl of the Sample Reaction Mixture to the standard and sample wells and 10 µl of the Sample Background Reaction Mixture to the sample background wells. Cover with a 96-well Cover Sheet (Item No. 400012) and incubate with shaking for 1 hour at room temperature.

	Standard Wells (µl)	Sample Wells (µl)	Sample Background Wells (µl)
1X Assay Buffer	110	110	110
Standard	10	-	-
Sample	-	10	10
Cofactor Mixture	10	10	10
ADHP Assay Detector	10	10	10
Initiate reactions			
Sample Reaction Mix (µl)	10	10	-
Sample Background Reaction Mix	-	-	10

Table 3. Pipetting summary

4. **Read Fluorescence or Absorbance**

   Remove the cover sheet. Using a microplate reader, read at and excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm. Fluorescence should be stable up to 45 minutes. If the colorimetric method is used, read absorbance at 570 nm.

ANALYSIS

Calculations

1. Determine the average fluorescence or absorbance of each standard, sample, and sample background.
2. Subtract the fluorescence or absorbance value of the standard A (no α-KG) from itself and all other Standards, Samples, and Sample Background. This is the corrected signal (CS).
3. Subtract the CS values of the Sample Background wells from the CS values of their respective Sample wells. This corrects for the presence of pyruvate in the sample.
4. Plot the CS values (from step 2 above) of each standard as a function of the final concentration of α-KG from Tables 1 and 2 on pages 14 and 15, respectively. See Figures 3 and 4, on pages 19 and 20, respectively, for typical standard curves.
5. Interpolate the background-subtracted sample CS (from step 3 above) from the standard curve using the equation below to get the final α-KG concentration of the sample.

$$\alpha\text{-KG } (\mu M) = \left[\frac{\text{background-substracted CS - (y-intercept)}}{\text{Slope}} \right] \text{x Sample Dilution}$$

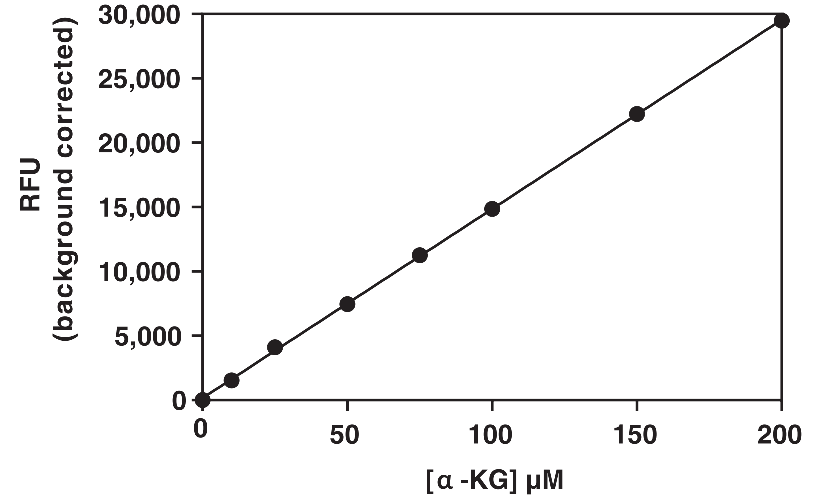

Figure 3. α-KG standard curve - fluorometric

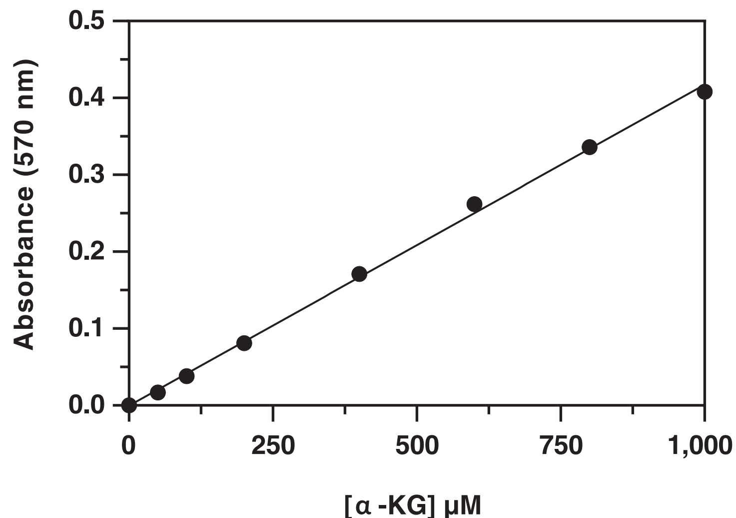

Figure 4. α-KG standard curve - colorimetric

Performance Characteristics

Sensitivity:

The lower limit of detection (LLOD) for the fluorometric assay is 1 µM (0.01 nmol/well) and 13 µM (0.13 nmol/well) for the colorimetric assay.

The lower limit of quantification (LLOQ) for the fluorometric assay is 10 µM (0.1 nmol/well) and 50 µM (0.5 nmol/well) for the colorimetric assay.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/ technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles

References

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	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.

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