ab83431

Alpha Ketoglutarate (alpha KG) Assay Kit (Colorimetric/Fluorometric)

Instructions for Use

For the rapid, sensitive and accurate measurement of alpha Ketoglutarate in various samples.

This product is for research use only and is not intended for diagnostic use.

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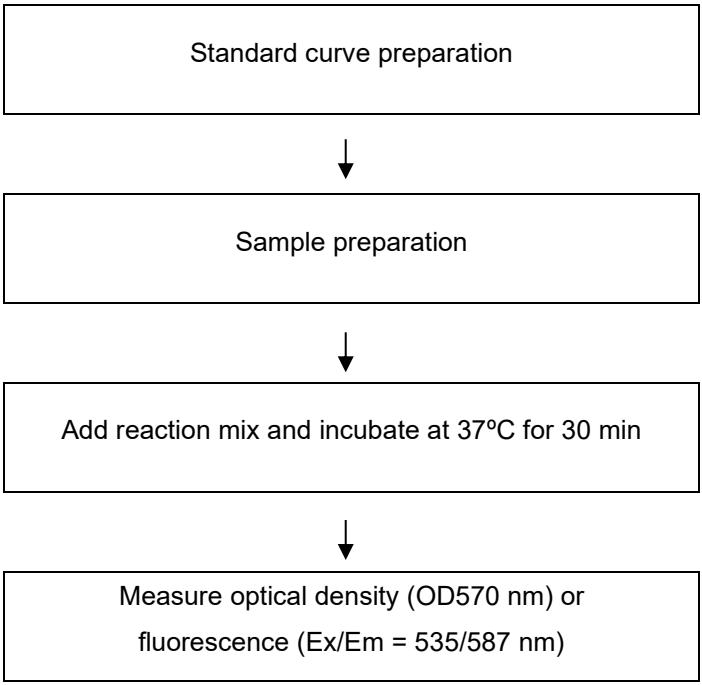
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1. BACKGROUND

Alpha ketoglutarate (alpha KG) Assay Kit (Colorimetric/Fluorometric) (ab83431) provides a simple, sensitive and rapid means for quantifying alpha KG in a variety of samples. In the assay, alpha ketoglutarate is transaminated with the generation of pyruvate which is utilized to convert a nearly colorless probe to both color (λ_{max} = 570 nm) and fluorescence (Ex/Em = 535/587 nm). The alpha ketoglutarate assay kit is useful for detecting alpha ketoglutarate in the range of 0.01- 10 nmoles.

Alpha ketoglutarate (alpha KG) is a key intermediate in the Krebs cycle, coming after isocitrate and before succinyl CoA. Anaplerotic reactions replenish the cycle by synthesizing alpha KG from transamination of glutamate, or through the action of glutamate dehydrogenase. Alpha KG is an important nitrogen transporter. Being a key intermediate, it is one of the organic acids measured in newborns as an indicator of inborn errors of metabolism.

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

If applicable, please refer to the current Safety Data Sheet (SDS) provided with this product for safety, handling, and disposal information. The most up to date and current versions are available on our website https://www.abcam.com/en-us.

4. STORAGE AND STABILITY

Store kit at -20ºC in the dark immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
alpha-KG Assay Buffer	25 mL	-20°C	-20°C
OxiRed™ Probe	0.2 mL	-20°C	-20°C
α-KG Converting Enzyme	1 vial	-20°C	-20°C
Developer Mix A	1 vial	-20°C	-20°C
α-KG Standard	1 vial	-20°C	-20°C

PLEASE NOTE: alpha-KG Assay Buffer was previously labelled as α-KG Assay Buffer and Alpha KG Assay Buffer, and OxiRed™ Probe as OxiRed Probe and Alpha KG OxiRed Probe (in DMSO). Also, Developer Mix A was previously labelled as Development Enzyme Mix I and Alpha KG Development Enzyme Mix (Lyophilized). The composition has not changed.

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- MilliQ water or other type of double distilled water (ddH_2O)
- PBS
- Colorimetric or fluorescent microplate reader – equipped with filter for OD 570 nm or Ex/Em = 535/587 nm (respectively)
- 96 well plate: black plates (clear bottoms) for fluorometric assay; clear plates for colorimetric assay
- Microcentrifuge
- Pipettes and pipette tips
- Heat block or water bath
- Vortex
- Dounce homogenizer or pestle (if using tissue)

If performing deproteinization step, additional reagents are required:

- Perchloric acid (PCA) 4M, ice cold
- Potassium Hydroxide (KOH) 2M

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure complete removal of all solutions and buffers from tubes or plates during wash steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **alpha-KG Assay Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 **OxiRed™ Probe:**

Ready to use as supplied. Warm the DMSO solution at 37 °C until completely thawed (~ 5 minutes), vortex briefly to create a homogenous solution, and spin down briefly to collect any contents trapped in the cap or seal. Keep at room temperature during use and reseal promptly to minimize moisture exposure.
For multiple use aliquot and store at −20 °C, protected from light and moisture. Stable for up to 2 months after thawing (≤3 freeze–thaw cycles).

9.3 **α-KG Converting Enzyme:**

Reconstitute with 220 µL alpha KG Buffer. Pipette up and down to dissolve. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw cycles. Use within two months.

9.4 **Developer Mix A:**

Reconstitute with 220 µL alpha KG Buffer. Pipette up and down to dissolve. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw cycles. Use within two months.

9.5 **α-KG Standard:**

Reconstitute α-KG Standard (10 µmol) in 100 µL of ddH_2O to generate a 100 mM α-KG Standard stock solution. Keep on ice while in use. Aliquot standard so that you have enough to perform the desired number of assays. Store at -20°C.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and must be used within 4 hours.

10.1 **For the colorimetric assay:**

10.1.1 Prepare a 1 nmol/µL α-KG Standard by adding 10 µL of the α-KG Standard to 990 µL of ddH_2O.

10.1.2 Using 1 nmol/µL α-KG Standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (µL)	alpha-KG Assay Buffer (µL)	Final volume standard in well (µL)	End [Alpha KG] in well
1	0	150	50	0 nmol/well
2	6	144	50	2 nmol/well
3	12	138	50	4 nmol/well
4	18	132	50	6 nmol/well
5	24	126	50	8 nmol/well
6	30	120	50	10 nmol/well

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

10.2 **For the flurometric assay:**

10.2.1 Prepare a 1 nmol/µL α-KG Standard by adding 10 µL of the α-KG Standard to 990 µL of ddH_2O.

10.2.2 Prepare 0.1 nmol/µL α-KG Standard by diluting 10 µL of 1 nmol/µL α-KG Standard into 90 µL of ddH_2O.

10.2.3 Using 0.1 nmol/µL α-KG Standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (µL)	alpha-KG Assay Buffer (µL)	Final volume standard in well (µL)	End [Alpha KG] in well
1	0	150	50	0 nmol/well
2	6	144	50	0.2 nmol/well
3	12	138	50	0.4 nmol/well
4	18	132	50	0.6 nmol/well
5	24	126	50	0.8 nmol/well
6	30	120	50	1.0 nmol/well

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 µL).

NOTE: *If your sample readings fall out the range of your fluorometric standard curve, you might need to adjust the dilutions and create a new standard curve.*

11. SAMPLE PREPARATION

General Sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step as well as the deproteinization step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze cells or tissue in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Cell (adherent or suspension) samples:

11.1.1 Harvest the amount of cells necessary for each assay (initial recommendation = 2 x 10^6 cells).

11.1.2 Wash cells with cold PBS.

11.1.3 Resuspend cells in 500 µL (or ~4 volumes) of ice cold alpha-KG Assay Buffer on ice.

11.1.4 Homogenize cells quickly by pipetting up and down a few times.

11.1.5 Centrifuge sample for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.1.6 Collect supernatant and transfer to a clean tube.

11.1.7 Keep on ice.

11.1.8 Perform deproteinization step as described in section 11.3.

11.2 Tissue samples:

11.2.1 Harvest the amount of tissue necessary for each assay (initial recommendation = 20 mg).

11.2.2 Wash tissue in cold PBS.

11.2.3 Resuspend tissue in 500 – 1,000 µL (or ~4-6 volumes) of alpha-KG Assay Buffer on ice.

11.2.4 Homogenize tissue with a Dounce homogenizer sitting on ice, with 10 – 50 passes.

11.2.5 Centrifuge samples for 2 – 5 minutes at 4°C at top speed using a cold microcentrifuge to remove any insoluble material.

11.2.6 Collect supernatant and transfer to a clean tube.

11.2.7 Keep on ice.

11.2.8 Perform deproteinization step as described in section 11.3.

11.3 **Deproteinization step:**

Prepare samples as specified in step 11.1 or 11.2. You should have a clear protein sample after homogenization and centrifugation. Keep your samples on ice.

11.3.1 Add ice cold PCA to a final concentration of 1 M in the homogenate solution and vortex briefly to mix well. ***NOTE:*** *high protein concentration samples might need more PCA.*

11.3.2 Incubate on ice for 5 minutes.

11.3.3 Centrifuge samples at 13,000 x g for 2 minutes at 4°C in a cold centrifuge and transfer supernatant to a fresh tube.

11.3.4 Precipitate excess PCA by adding ice-cold 2 M KOH that equals 34% of the supernatant to your samples (for instance, 34 µL of 2 M KOH to 100 µL sample) and vortexing briefly. This will neutralize the sample and precipitate excess PCA. There may be some gas (CO_2) evolution so vent the sample tube.

11.3.5 After neutralization, it is very important that pH equals 6.5 – 8 (use pH paper to test 1 µL of sample). If necessary, adjust the pH with 0.1 M KOH.

11.3.6 Centrifuge at 13,000 x g for 15 minutes at 4°C and collect supernatant.

11.3.7 Transfer supernatant to a clean tube, and keep on ice.

Samples are now deproteinized, neutralized and PCA has been removed. The samples are now ready to use in the assay.

Sample Recovery

The deproteinized samples will be diluted from the original concentration.

To calculate the dilution factor of your final sample, simply apply the following formula:

$$\% \text{ original concentration} = \frac{\text{Initial sample volume}}{\text{(initial sample volume + vol PCA + vol KOH)}} \text{ X100}$$

NOTE: *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*

12. ASSAY PROCEDURE and DETECTION

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**

12.1 Set up Reaction wells:

- Standard wells = 50 µL standard dilutions.
- Sample wells = 1 – 50 µL samples (adjust volume to 50 µL/well with alpha-KG Assay Buffer).
- Sample background wells = 1 – 50 µL samples (adjust volume to 50 µL/well with alpha-KG Assay Buffer). ***NOTE:*** *for samples containing pyruvate as it can generate background.*
- Background wells = 50 µL alpha-KG Assay Buffer.

12.2 Reaction Mix:

Prepare Colorimetric Reaction Mix for each reaction:

Component	Colorimetric Reaction Mix (µL)	Background Reaction Mix (µL)
alpha-KG Assay Buffer	44	46
α-KG Converting Enzyme	2	0
Developer Mix A	2	2
OxiRed™ Probe	2	2

Prepare Fluorometric Reaction Mix for each reaction:

Component	Fluorometric Reaction Mix (µL)	Background Reaction Mix (µL)
alpha-KG Assay Buffer	45.6	47.6
α-KG Converting Enzyme	2	0
Developer Mix A	2	2
OxiRed™ Probe*	0.4	0.4

NOTE: **For fluorometric readings, using 0.4 µL/well of the OxiRed™ Probe decreases the background readings, therefore increasing detection sensitivity.*

Mix enough reagents for the number of assays (samples, standards and background control) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

X µL component x (Number samples + standards +1)

12.3 Add 50 µL of Reaction Mix to each alpha ketoglutarate standard or sample.

12.4 Add 50 µL of Background Reaction Mix to each sample background well.

12.5 Incubate at 37ºC for 30 minutes protected from light.

12.6 Measure output on a microplate reader.

- Colorimetric assay: measure OD570 nm.
- Fluorometric assay: measure Ex/Em = 535/587 nm.

13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 Average the duplicate reading for each standard and sample.

13.2 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.

Note: In case high background is observed in the samples and sample background controls are included, subtract the mean sample background control readings (instead of the blank) from the corresponding sample readings.

13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of alpha KG.

13.4 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

13.5 Extrapolate sample readings from the standard curve plotted using the following equation:

$$Ay = \left(\frac{Corrected\ absorbance - (y - intercept)}{Slope}\right)$$

13.6 Concentration of α-ketoglutarate (nmol/µL or µmol/mL or mM) in the test samples is calculated as:

$$Concentration = \left(\frac{Ay}{Sv}\right) * D$$

Where:

Ay = amount of alpha KG in sample well (nmol).

Sv = sample volume (µL) added to the reaction well.

D = Sample dilution factor.

Alpha Ketoglutarate molecular weight: 146.11 g/mol.

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

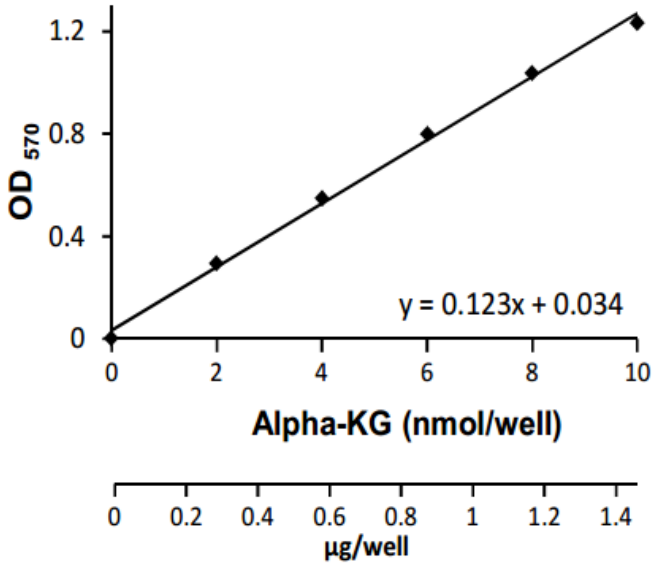


Figure 1. Typical alpha ketoglutarate standard calibration curve using colorimetric reading.

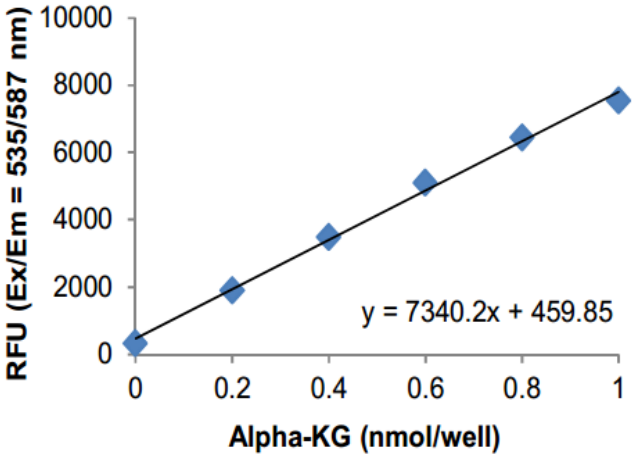


Figure 2: Typical alpha ketoglutarate standard calibration curve using fluorometric reading.

15. QUICK ASSAY PROCEDURE

***NOTE**: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.*

- Prepare α-KG Standard, OxiRed™ Probe and prepare enzyme mix (aliquot if necessary); get equipment ready.
- Prepare appropriate standard curve for your detection method of choice (colorimetric or fluorometric).
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (50 µL), samples (50 µL), sample background wells (50 µL) and background wells (50 µL).
- Prepare either colorimetric or fluorometric Reaction Mix

Component	Colorimetric Reaction Mix (µL)	Background Reaction Mix (µL)
alpha-KG Assay Buffer	**44**	**46**
α-KG Converting Enzyme	**2**	**0**
Developer Mix A	**2**	**2**
OxiRed™ Probe	**2**	**2**

Component	Fluorometric Reaction Mix (µL)	Background Reaction Mix (µL)
alpha-KG Assay Buffer	**45.6**	**47.6**
α-KG Converting Enzyme	**2**	**0**
Developer Mix A	**2**	**2**
OxiRed™ Probe*	**0.4**	**0.4**

- Add 50 µL Reaction Mix or Background Reaction Mix to appropriate wells.
- Incubate plate 37ºC 30 mins.
- Measure plate at OD 570nm for colorimetric assay or Ex/Em= 535/587 nm for fluorometric assay.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. FAQ

Can PCA precipitated supernatant be directly used in this assay?

You need to neutralize the sample before you use it for this assay.

Is the standard curve and the kit itself only accurate in the 0 - 10 nmol/sample range, or can the curve be extrapolated? That is, if the calculated value for a sample says 15 nmol, is that accurate, or do I need to dilute my samples more?

We recommend diluting the sample to fit within the std. curve linear range.

Will low concentrations of NADH in the sample interfere in this assay?

Theoretically, NADH should not interfere. Pyruvate can generate background in the assay. The endogenous pyruvate interference can be subtracted from the sample reading using the parallel background control. Ideally the relative amount of pyruvate < amount of alpha KG in the sample.

18. **INTERFERENCES**

19. **NOTES**

Technical Support

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For all technical or commercial enquiries please go to:
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