3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

α-Ketoglutarate Dehydrogenase Activity Colorimetric Assay Kit

Catalog Number MAK189
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

α-Ketoglutarate Dehydrogenase (α-KGDH, EC 1.2.4.2) forms the E1 subunit of the mitochondrial 2-oxoglutarate dehydrogenase complex that catalyzes the conversion of 2-oxoglutarate to succinyl-CoA and CO_2 during the Krebs cycle. Decreased α-KGDH complex activity has been associated with oxidative stress and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases.[1,2]

The α-Ketoglutarate Dehydrogenase Activity Colorimetric Assay Kit provides a simple procedure for measuring α-ketoglutarate dehydrogenase activity in a variety of tissues, cells, and isolated mitochondria. α-KGDH activity is determined by measuring a colorimetric product with absorbance at 450 nm (A_{450}) proportional to the enzymatic activity present. One unit of α-ketoglutarate dehydrogenase is the amount of enzyme that generates 1.0 μmole of NADH per minute at pH 7.5 at 37 °C.

Components

The kit is sufficient for 100 assays in 96 well plates.

KGDH Assay Buffer Catalog Number MAK189A	25 mL
KGDH Substrate Catalog Number MAK189B	1 vl
KGDH Developer Catalog Number MAK189C	1 vl
NADH Standard Catalog Number MAK189D	1 vl
KGDH Positive Control Catalog Number MAK189E	50 μL

Reagents and Equipment Required but Not Provided

- 96 well flat-bottom plate – It is recommended to use clear plates for colorimetric assays.
- Spectrophotometric multiwell plate reader
- Saturated ammonium sulfate (~4.1 M, optional for samples containing small interfering molecues)
- Mitochondria Isolation Kit (optional for mitochondria samples, Catalog Number MITOISO1 for tissue, MITOISO2 for cells, MITOISO3 for yeast, or equivalent)

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

KGDH Assay Buffer – Store at –20 °C or 2–8 °C. Allow buffer to come to room temperature before use.

KGDH Substrate – Reconstitute with 220 μL of water. Mix well by pipetting. Aliquot and store at –20 °C. Keep on ice during use. Use within 2 months.

KGDH Developer – Reconstitute with 220 μL of water. Mix well by pipetting. Aliquot and store at –20 °C. Use within 2 months.

NADH Standard – Reconstitute with 400 µL of water to generate a 1.25 mM (1.25 nmole/µL) NADH Standard Solution. Aliquot and store at –20 °C. Keep on ice during use. Use within 2 months.

KGDH Positive Control – Reconstitute with 100 µL of KGDH Assay Buffer. Mix well by pipetting. Aliquot and store at –20 °C. Keep on ice during use. Use within 2 months.

Storage/Stability
The kit is shipped on wet ice and storage at –20 °C, protected from light, is recommended.

Procedure
All samples and standards should be run in duplicate.

NADH Standards for Colorimetric Detection
Add 0, 2, 4, 6, 8, and 10 µL of the 1.25 mM NADH Standard Solution into a 96 well plate, generating 0 (blank), 2.5, 5.0, 7.5, 10, and 12.5 nmole/well standards. Add KGDH Assay Buffer to each well to bring the volume to 50 µL.

Sample Preparation
Tissue samples (10 mg) or cells (1×10^6) can be homogenized in 100 µL of ice cold KGDH Assay Buffer. Keep on ice for 10 minutes. Centrifuge the samples at 10,000 × *g* for 5 minutes to remove insoluble material. Transfer supernatant to a fresh tube.

When analyzing KGDH activity in mitochondria, it is recommended to isolate the mitochondria from fresh tissue or cells.

Add 5–50 µL of the samples into duplicate wells. Bring samples to a final volume of 50 µL using KGDH Assay Buffer.

Notes: For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

Small molecules in some tissues such as liver may interfere with the assay. To remove small molecules, it is suggested to use an ammonium sulfate precipitation method. Pipette 50–100 mL of lysate into a fresh tube, add 2× volume of saturated ammonium sulfate (~4.1 M at room temperature) and keep on ice for 20 minutes. Centrifuge at 10,000 × *g* for 5 minutes, remove and discard the supernatant, and resuspend the pellet to the original volume with KGDH Assay Buffer.

For samples exhibiting significant background, include a Sample Blank for each sample by omitting the KGDH Substrate. The Sample Blank readings can then be subtracted from the sample readings.

For a positive control (optional), add 2–10 µL of the KGDH Positive Control solution to the desired wells. Adjust the final volume to 50 µL with the KGDH Assay Buffer.

Assay Reaction
1. Set up Reaction Mixes according to the scheme in Table 1. 50 µL of Reaction Mix is required for each reaction (well).

Table 1.
Reaction Mixes

Reagent	Standards, Controls, and Samples	Sample Blank
KGDH Assay Buffer	46 µL	48 µL
KGDH Developer	2 µL	2 µL
KGDH Substrate	2 µL	–

2. Add 50 µL of the appropriate Reaction Mix to each of the wells. Mix well using a horizontal shaker or by pipetting.

3. Measure the absorbance (A_{450}) in a microplate reader in kinetic mode for 10–60 minutes at 37 °C. Protect the plate from light during the incubation. It is recommended to take absorbance readings every minute.
   Note: Incubation time depends on the activity of KGDH in the samples.

4. Continue taking measurements until the value of the most active sample is greater than the value of the highest standard (12.5 nmole/well). At this time the most active sample is near or exceeds the end of the linear range of the standard curve.

Note: The NADH Standards can be read at the end of the incubation time.

Results

Calculations

Plot the absorbance (A_{450}) for each well versus time.

Choose two time points (T1 and T2) in the linear range of the plot and determine the A_{450} at each time (ABS1 and ABS2).

Note: It is essential that ABS1 and ABS2 fall within the linear range of the standard curve.

Correct for the background by subtracting the measurement obtained for the 0 (blank) NADH standard from that of the standards, controls, and samples. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate NADH Standards to plot a standard curve.

Note: A new standard curve must be set up each time the assay is run.

Calculate the change in absorbance from T1 to T2 for the samples.

$$\Delta ABS = ABS2 - ABS1$$

Subtract the Sample Blank ΔABS value from the Sample ΔABS reading to obtain the corrected measurement. Using the corrected measurement, determine the amount of NADH (nmole/well) generated by the KGDH assay between T1 and T2 (S_a).

α-Ketoglutarate Dehydrogenase (KGDH) activity

$$\text{KGDH Activity} = \frac{S_a}{(\text{Reaction Time}) \times S_v}$$

where:

S_a = Amount of NADH (nmole) generated in unknown sample well between T1 and T2 from standard curve

Reaction Time = T2 – T1 (minutes)

S_v = sample volume (mL) added to well

KGDH activity is reported as nmole/min/mL = milliunit/mL.

One unit of α-ketoglutarate dehydrogenase is the amount of enzyme that generates 1.0 μmole of NADH per minute at pH 7.5 at 37 °C.

Sample Calculation:

Amount of NADH (S_a) = 5.84 nmole (from standard curve)

(T1) = 3 minutes

(T2) = 32 minutes

Sample volume (S_v) = 0.050 mL

KGDH activity in sample well:

$$\text{nmole/min/mL (milliunits/mL)} = \frac{5.84 \text{ nmole/well}}{(32 \text{ min} - 3 \text{ min}) \times 0.050 \text{ mL /well}} = 4.03$$

References

1. Gibson, G.E. et al., The alpha-ketoglutarate-dehydrogenase complex: a mediator between mitochondria and oxidative stress in neurodegeneration. Mol. Neurobiol., **31(1-3)**, 43-63 (2005).
2. Gibson, G.E., et al., The alpha-ketoglutarate dehydrogenase complex in neurodegeneration. Neurochem. Int., **36(2)**, 97-112 (2000).

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	For colorimetric assays, use clear plates
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until 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	Check the expiration date and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Reaction Mixes before each use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the Reaction Mixes
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare Reaction Mixes whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

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