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Product Information

Acetoacetate Colorimetric Assay Kit

Catalog Number **MAK199**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Acetoacetate (AcAc) is a β-ketoacid formed in the mitochondria by the condensation of two molecules of acetyl-CoA. AcAc is either enzymatically reduced to 3-hydroxybutyrate or decarboxylated to produce acetone. Ketone bodies (AcAc, 3-HB, and acetone) are used as alternate source of energy in the absence of glucose. Excess ketone bodies are observed in type I diabetes, alcoholism, or starvation.[1,2]

The Acetoacetate Colorimetric Assay kit provides a simple and sensitive procedure for measuring AcAc in various samples (ranging from 20–100 nmole/well). AcAc concentration is determined by generating a colorimetric (550 nm) product proportional to the amount of AcAc present. The kit does not detect 3-hydroxybutyrate and is specific for acetoacetate.

Components

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

Acetoacetate Assay Buffer Catalog Number MAK199A	15 mL
Acetoacetate Standard Catalog Number MAK199B	1 vl
Acetoacetate Substrate Catalog Number MAK199C	1 mL

Reagents and Equipment Required but Not Provided

- 96-well flat-bottom plate – It is recommended to use clear plates for colorimetric assays.
  Note: Cell culture or tissue culture treated plates are **not** recommended.
- Spectrophotometric multiwell plate reader
- 10 kDa Molecular Weight Cut-Off (MWCO) Spin Filters (optional for protein-containing samples), such as CLS431478 or UFC5010.

Precautions and Disclaimer

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.

Acetoacetate Assay Buffer – Allow buffer to come to room temperature before use. A small amount of precipitate might be present. Vortex the bottle to dissolve the precipitate before use.

Acetoacetate Standard – Reconstitute with 100 µL of water to generate a 100 mM Acetoacetate Standard Solution. Mix well by pipetting. Aliquot and store at –20 °C. Use within 2 months of reconstitution. Keep on ice while in use.

Acetoacetate Substrate – Aliquot and store at 2–8 °C, protected from light. Keep at room temperature protected from light during use.

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.

AcAc Standards for Colorimetric Detection

Dilute 10 µL of the 100 mM (100 nmole/µL) AcAc Standard Solution with 90 µL of water to prepare a 10 mM (10 nmole/µL) AcAc Standard Solution. Add 0, 2, 4, 6, 8, and 10 µL of the 10 mM AcAc Standard Solution into a 96 well plate to generate 0 (blank), 20, 40, 60, 80, and 100 nmole/well standards. Add water to each well to bring the volume to 110 µL.

Sample Preparation

Notes: Sample preparation must be rapid to prevent the degradation of the AcAc. Use of fresh samples is recommended. Alternatively, the samples may be stored at –80 °C for 4 weeks.

Enzymes in samples may consume AcAc. To deproteinize, use 10kDa MWCO spin filters.

To correct for background in samples, include a Sample Blank by omitting the AcAc Substrate. The Sample Blank readings can then be subtracted from the sample readings.

For blood samples, either heparin or EDTA anticoagulant tubes may be used.

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

Add 10–100 µL of samples into duplicate wells of a 96-well plate. Bring the samples to a final volume of 110 µL with water.

Spiking duplicate sample wells with a known amount of AcAc Standard Solution is recommended for the accurate determination of AcAc in samples that might contain endogenous compounds that interfere with the reaction.

Assay Reaction

1. Set up the Reaction Mixes according to the scheme in Table 1. 90 µL of Reaction Mix is required for each reaction (well).

Table 1.
Reaction Mixes

Reagent	**Standards and Samples**	**Sample Blank**
Acetoacetate Assay Buffer	80 µL	90 µL
Acetoacetate Substrate	10 µL	–

2. Add 90 µL of the appropriate Reaction Mix to each of the wells. Mix well using a horizontal shaker or by pipetting. Incubate the plate at plate for 10–15 minutes at 25 °C or for 80–110 minutes at 4 °C. Protect the plate from light during the incubation.

3. Measure absorbance of the samples at 550 nm with a microplate reader. It is recommended to take readings in kinetic mode until the absorbance for the 100 nmole AcAc Standard is maximized.

   Note: The stability of final product is improved and absorbance readings are higher when the plate is incubated at lower temperature.

Results

Calculations

The background for either a spiked or unspiked assay is the value obtained for the 0 (blank) AcAc Standard. Correct for the background by subtracting the blank standard value from that of the standards and samples. Background values can be significant and must be subtracted from all readings. Use the corrected values (A_{550}) obtained from the appropriate AcAc Standards to plot a standard curve.

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

Concentration of AcAc

$$C = S_a/S_v$$

where:

S_a = Amount of AcAc in unknown sample well (nmole) from standard curve

S_v = Sample volume (μL) added into the well

C = Concentration of AcAc in sample (nmole/μL)

Sample Calculation

Amount of AcAc (S_a) = 25.84 nmole (from standard curve)

Sample volume (S_v) = 10 μL

Concentration of AcAc in sample:

$$25.84 \text{ nmole}/10\ \mu\text{L} = 2.584 \text{ nmole}/\mu\text{L}$$

Concentration of AcAc in spiked samples

For spiked samples, calculate the amount of AcAc in the sample wells after correcting for the Sample Blank and background.

$$C = \frac{S_p \times A_s}{(A_{sp} - A_s) \times S_v}$$

where:

S_p = Known amount of AcAc Standard spiked in well (nmole)

A_s = Corrected sample reading (A_{550}) (unspiked well)

A_{sp} = Corrected sample + spike reading (A_{550})

S_v = Sample volume (μL) added into the well

C = Concentration of AcAc in sample (nmole/μL)

Sample Calculation

Amount of AcAc Standard spike (S_p) = 30 nmole

Sample volume (S_v) = 10 μL

Corrected sample reading (A_s) = 0.599 (A_{550})

Corrected spike + sample reading (A_{sp}) = 0.702 (A_{550})

Concentration of AcAc in sample:

$$\text{nmole}/\mu\text{L} = \frac{30 \text{ nmole spike} \times 0.599}{[(0.702 - 0.599) \times 10\ \mu\text{L}]} = 17.4$$

References

1. Stadtman, E.R. et al., The mechanism of acetoacetate synthesis. *J. Biol. Chem.*, **191**, 377–382 (1951).
2. Segal, H.L., and Menon, G.K.K., Acetoacetate Formation from Acetoacetyl Coenzyme A in Rat Liver Mitochondria: Effect of Endocrine State and Nature of the System. *J. Biol. Chem.*, **236**, 2872–2878 (1961).

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 improperly stored reagents	Check the storage requirements 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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