Technical Bulletin

Acetyl Coenzyme A Assay Kit

Catalogue Number MAK566

Product Description

Acetyl-CoA (AcCoA) is an essential cofactor and carrier of acyl groups in enzymatic acetyl transfer reactions. It is the starting compound for the citric acid cycle (Kreb's cycle). It is also a key precursor in lipid biosynthesis, and the source of all fatty acid carbons. AcCoA acts as an indicator of fat, sugar, and protein levels and shows up on the nutritional status. In lowered glucose conditions, AcCoA participates in the production of ATP. Increased AcCoA levels are observed in prostate cancer due to elevated fatty acid utilization and thus provides an additional energy source for the tumor cell growth.

In this kit, AcCoA concentration is determined by a coupled enzyme assay, which results in a fluorometric (λex = 340/λem = 460 nm) product, proportional to the Acetyl-CoA present. Typical sensitivities of detection for this kit are 20-1000 pmol of Acetyl CoA. This kit is a highly sensitive assay for determining Acetyl-CoA level in a variety of biological samples.

Components

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

- Assay Buffer 25 mL
  Catalogue Number MAK566A
- Standard Solution 0.1 mL
  Catalogue Number MAK566B
- Substrate Mix 7 mL
  Catalogue Number MAK566C
- Enzyme Mix 0.15 mL
  Catalogue Number MAK566D
- Reaction Initiator Solution 0.07 mL
  Catalogue Number MAK566E
- Quencher 1 vial
  Catalogue Number MAK566F
- Quencher Remover 0.2 mL
  Catalogue Number MAK566G

Reagents and Equipment Required but Not Provided

- 96-well flat-bottom plate.
  - Black plates with clear bottoms for fluorescence assays (Catalogue number CLS3631 or equivalent)
  - Cell culture or tissue culture treated plates are not recommended.
- Plate reader that is capable to read fluorescence at wavelength of λex = 340 nm/λem = 460 nm.
- 3M Potassium bicarbonate solution
- 70% Perchloric acid (Catalog No. 244252)
- Pipettors and Pipettes
- Vortex

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.

Storage/Stability

The product is shipped on dry ice. Store at -20 °C upon receipt, protected from light.

Preparation Instructions

Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. Avoid repeated freeze/thaw cycles.

For MAK566B, MAK566C and MAK566D it is recommended to aliquot after initial thaw.

Assay Buffer (MAK566A)

Allow buffer to come to room temperature before use. Prepare 1 mL of diluted assay buffer for Reaction Initiator Solution preparation by adding 100 µL assay buffer to 900 µL ultrapure water.

Reaction Mixture (MAK566C + MAK566D)

1. Thaw the MAK566C and MAK566D solutions at room temperature prior to use.
2. In a 2 ml microcentrifuge tube, add 875 µl of the Assay Buffer (undiluted), 875 µl of the Substrate Mix MAK566C, 17 µl of Enzyme Mix MAK566D (this amount is sufficient for a calibration curve + 1 sample + 1 blank, in duplicates). If additional tests are needed, adjust the quantities according to this ratio.

Reaction Initiator Solution (MAK565C)

Thaw the Reaction Initiator Solution MAK566E at room temperature. Dilute an aliquot of 10 µl Reaction Initiator Solution by adding 990 µl of diluted Assay buffer.

Standard Solution (MAK566B)

The Standard Solution of 10 mM Acetyl CoA (MAK566B) should be thawed prior to use.

Quencher (MAK566F)

Reconstitute the vial with 1 mL of purified water. Store at 4 °C.

Procedure

All samples and standards should be run in technical duplicates.

Standard Curve Preparation

A new standard curve should be prepared for every assay.

1. The standard solution should be aliquoted at the first opening of the vial in 10 ul portions and then refrozen.
2. First, dilute 10 µL of the 10 mM standard in 990 uL water to create a solution of **0.1 mM**. Mix well by pipetting up and down.
3. For the high range (0–1 nmol), further dilute 100 µL of the 0.1 mM solution in 400 µL water to create a **0.02 mM** Acetyl CoA standard solution.
4. For the low range (0–100 pmol) further dilute 10 µL of the 0.1 mM solution in 490 µL DDW to create a **0.002 mM** Acetyl CoA standard solution.
5. Prepare 6 standards by mixing either the 0.02 or 0.002 mM standard with the assay buffer in the wells of a 96-well plate, according to Table 1:

Table 1.
Preparation of Acetyl Coenzyme A Standards

Std	0.02 or 0.002 mM Std (µL)	Assay Buffer (µL)	Concentration (pmol/well, high/low)
1	0	50	0
2	10	40	200/20
3	20	30	400/40
4	30	20	600/60
5	40	10	800/80
6	50	0	1000/100

Sample Preparation

Tissue Samples:

Samples (20 - 1,000 mg) should be frozen rapidly (liquid N_2 or methanol/dry ice) and pulverized with a pestle and mortar. Deproteinize sample by perchloric acid (PCA) precipitation. Suspend the powdered tissue in 0.6 M Perchloric acid (30 mg powdered tissue/ml) while keeping the sample cold and homogenize or sonicate thoroughly. Centrifuge the samples at 10,000 x g for 10 minutes to remove insoluble material. Neutralize the supernatant with 3M potassium bicarbonate solution, adding in aliquots of 1 µL/10 µL of supernatant while vortexing until bubble evolution ceases (2–5 aliquots). Cool on ice for 5 minutes. Verify pH is in the range of 6–8, using 1 µL of sample. Spin 2 minutes to pellet insoluble potassium bicarbonate.

Cell Culture Samples:

Grow a culture on a suitable dish until at least 80% confluent. Scrape the cells mechanically from the flask and suspend in PBS. After precipitation by centrifugation of the cells and resuspension in 2 ml PBS, count the number of cells present if necessary. Homogenize the cells using a 7 ml Teflon and glass Dounce homogenizer. Centrifuge the samples at 10,000 x g for 10 minutes to remove insoluble material. Deproteinize sample by PCA precipitation: add 0.6M PCA while keeping the sample cold. Centrifuge the samples at 10,000 x g for 10 minutes to remove insoluble material. Neutralize the supernatant with 3M potassium bicarbonate solution, adding in aliquots of 1 µL/10 µL of supernatant while vortexing until bubble evolution ceases (2–5 aliquots). Cool on ice for 5 minutes. Verify pH is in the range of 6–8, using 1 µL of sample. Spin 2 minutes to pellet insoluble potassium bicarbonate.

Notes:

- 70% Perchloric acid (Catalog No. 244252) is 11.6M. To achieve a 600 mM solution, dilute 520 µL to 10 ml with purified water.

- To correct for background created by free Coenzyme A and succinyl-CoA, add 5 µL of Acetyl-CoA Quencher per 100 µL sample and/or standard solution. Incubate at room temperature for 5 minutes. Add 1 µL of Quench Remover, mix well, and incubate for an additional 5 minutes.

- For unknown samples, it is suggested to test several sample volumes to make sure the readings are within the standard curve range. Add 10-50 µL of samples into duplicate wells of a 96 well plate. Bring the sample to a final volume of 50 µL with Acetyl-CoA Assay Buffer.

- Include a blank sample for each unknown sample by omitting the **Reaction Initiator Solution** and using 50 µL of the diluted Assay Buffer instead. This will correct for any endogenous reaction products in the unknown sample.

Assay Reaction

1. According to expected AcCoA concentrations in the sample chose the suitable standards curve (high or low).
2. Add standard or sample to each well and bring to a final volume of 50 µL using Assay Buffer.
3. Add 100 µL of Reaction Mixture to each well using a multichannel pipette and mix by pipetting. Incubate at 30°C for 5 minutes.
4. Add 50 µL of Reaction Initiator Solution to each well with a multichannel pipette. Place in the plate reader and read the results for 60 minutes (at 3-6 min interval), while at 30º C. Fluorometric λex /λem = 340/460 nm. Use extended (high) gain range if possible.

Results

Calculations

The background for the assays is the value obtained for the 0 Acetyl-CoA standard point. Correct for the background by subtracting the 0 standard value from all readings. Input the corrected values of the Acetyl-CoA standards into a spreadsheet to plot a standard curve.

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

If a non-linear behavior is observed, select the highest value from the linear portion of the curve, otherwise select the value at 60 minutes. Subtract the sample blank (a sample without reaction initiator solution) value from the reading to obtain the corrected fluorescence measurement. Calculate the amount of Acetyl-CoA in the

sample according to the standard curve's slope equation.
The final concentration of Acetyl-CoA can be calculated according to the following equation:

$$S_A/S_V \times DF = C$$

S_A = Amount of Acetyl-CoA in unknown sample (pmol) from standard curve
S_v = Sample volume (µL) added into the wells.
DF = dilution factor
C = Concentration of Acetyl-CoA in sample in pmol

Acetyl Coenzyme A molecular weight: 809.6 g/mol.

Sample Calculation

Amount of Acetyl-CoA (Ay) = 520.8 pmol (from standard curve)
Sample volume (Sv) = 50 µL .

Concentration of Acetyl-CoA in sample:

520.8/50 µL = 10.42 pmol/µL = 0.0104 nmol/µL

0.0104 nmol/µL X 809.6 ng/nmol = 8.420 ng/µL

Figure 1.
An example of a low range standard curve.

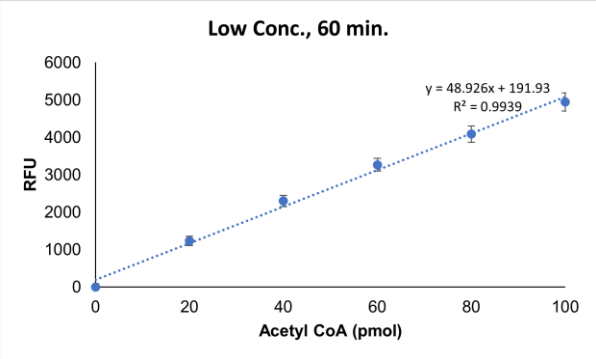


Figure 2.
An example of a high range standard curve.

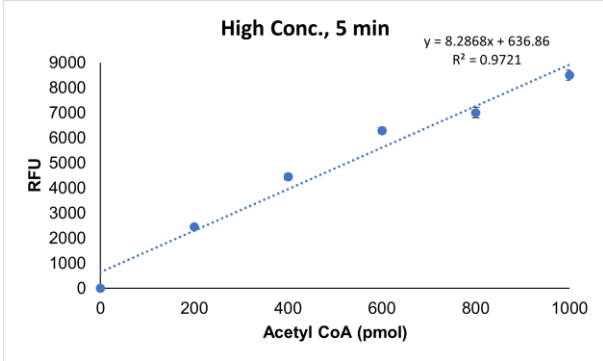


Figure 3.
Analysis of Acetyl Coenzyme A in mouse liver. Frozen mouse liver (1.7 g) was ground with liquid N_2 in a pestle and mortar. A sample (230 mg) was added to 2 mL 1M perchloric acid (PCA). The sample was diluted to about 30 mg tissue/mL and kept on ice for 15 minutes, after which tube was centrifuged at 10,000 x g for 10 min at 4°C. The solution was titrated with 3M potassium bicarbonate until the pH was about 7.0. 1 mL of sample was treated with 50 µL N-Ethylmaleimide solution (NEM, MAK566F) and incubated at 37°C for 10 minutes. Finally, 50 µL MAK566G was added to neutralize the NEM. Both samples (NEM-treated and untreated) were incubated for 60 minutes at 30°C and the fluorescence was read. Analysis was done using the **low** concentration standard curve.

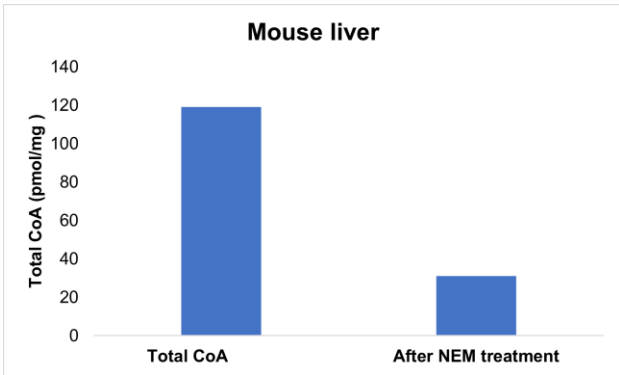
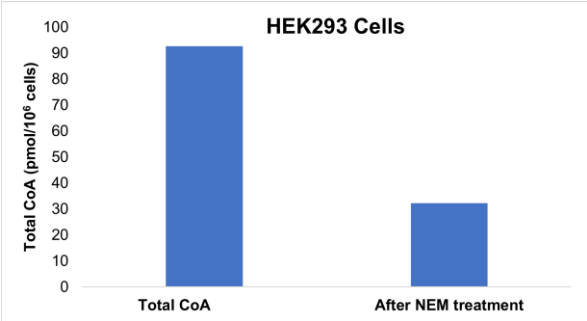


Figure 4.
Analysis of Acetyl Coenzyme A in HEK293 cells. HEK293 cells were grown on a 75 cm^2 dish. The cells were washed with warm PBS and resuspended in 2 mL PBS, 10.5 x 10^6 cells in total. Cells were homogenized with a Teflon/Glass homogenizer, spun down at 10,000 x g and resuspended in 1.9 ml PBS. 100 µL 11M perchloric

acid (PCA) was added to cell suspension and tube was kept in ice for 10 minutes. After centrifugation at 10,000 x g, 1.8 mL supernatant was transferred to a different tube and neutralized with 3M potassium bicarbonate (200 µL/mL) to a pH of 7.5. After centrifugation, 1.88 mL of supernatant was split into 2 equal parts. One sample was treated with 47 µl N-Ethylmaleimide solution (NEM, MAK566F) and incubated at 37ºC for 10 minutes, after which 94 µl MAK566G was added. 47 µl water and 94 µl of MAK566G were added to the second sample. Both samples (NEM-treated and untreated) were incubated for 60 minutes at 30ºC and the fluorescence was read. Analysis was done using the low concentration standard curve.



Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Ice 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 settings of instrument
	Type of 96 well plate used	For fluorescence assays, use black 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
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
	Improperly thawed components	Thaw all components completely and mix gently before use
Lower/higher readings in samples and standards	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 Master Reaction Mix 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
	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
Non-linear standard curve	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare a Master Reaction Mix wheneverpossible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	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
	Samples measured at incorrect wavelength	Check the equipment and filter settings
Unanticipated results	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

Notice

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