Technical Bulletin

Acetylcholine Assay Kit

Catalog Number MAK435

Product Description

Acetylcholine is a neurotransmitter produced in acetylcholinergic neurons. It plays important roles in skeletal muscle movement, regulation of smooth and cardiac muscles, as well as in learning, memory and mood.

The Acetylcholine Assay Kit provides a simple, direct and high-throughput assay for measuring acetylcholine in biological samples. In this assay, acetylcholine is hydrolyzed by acetylcholinesterase to choline which is oxidized by choline oxidase to betaine and H_2O_2. The resulting H_2O_2 reacts with a specific dye to form a pink colored product. The color intensity at 570 nm or fluorescence intensity at λ_{Ex} = 530 nm/ λ_{Em} = 585 nm is directly proportional to the acetylcholine concentration in the sample. The linear detection range for the acetylcholine assay method is 10-200 µM by colorimetric detection and 0.4-10 µM by fluorimetric detection.

The kit is suitable for the quantitative determination of acetylcholine and evaluation of drug effects on acetylcholine metabolism in serum, plasma, urine, saliva, milk, tissue, and cell culture etc.

Components

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

- Assay Buffer 10 mL
  Catalog Number MAK435A
- ACHE Enzyme 120 µL
  Catalog Number MAK435B
- Enzyme Mix 1 vial
  Catalog Number MAK4345C
- Dye Reagent 120 µL
  Catalog Number MAK435D
- Standard (2 mM Acetylcholine) 400 µL
  Catalog Number MAK435E

Reagents and Equipment Required but Not Provided

- Pipetting devices and accessories (e.g., multichannel pipettor)
- Multiwell plate reader
- Clear flat-bottom 96-well plates for colorimetric assay or black flat-bottom 96-well plates for fluorometric assay. Cell culture or tissue culture treated plates are **not** recommended.
- Dounce tissue grinder set
  (Catalog Number D9063 or equivalent)
- Microcentrifuge capable of
  RCF ≥ 14,000 × g
- Hydrochloric Acid (HCl), 6 N
  (Catalog Number 84429 or equivalent)
- Phosphate Buffered Saline (PBS)
  (Catalog Number P3813 or equivalent)
- Sodium Hydroxide (NaOH), 6 N

Precautions and Disclaimer

For Research Use Only. Not for use in diagnostic procedures. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The kit is shipped on wet ice. Store components at -20 °C.

Preparation Instructions

Briefly centrifuge small vials prior to opening. Equilibrate all components to room temperature prior to use and keep on ice during the assay.

Enzyme Mix: Reconstitute vial with 120 µL of Assay Buffer. Reconstituted Enzyme Mix is stable for 1 month when stored at -20 °C.

Note: A yellow precipitate may form after thawing reconstituted Enzyme Mix. If a precipitate forms, pellet it by centrifuging for 2 minutes at 14,000× g and use the clear supernatant.

Procedure

All samples and standards should be run in duplicate.

Sample Preparation

Note: SH-containing reagents (e.g., > 5 µM of β–mercaptoethanol or dithiothreitol) are known to interfere in this assay and should be avoided in sample preparation.

1. Liquid samples such as serum and plasma can be assayed directly.
2. Tissue and cell lysates can be prepared by homogenization in cold 1× PBS and centrifugation (5 minutes at 14,000 × g). Use clear supernatants for assay.
3. Milk samples should be clarified by mixing 600 µL of milk with 100 µL of 6 N HCl. Centrifuge for 5 minutes at 14,000 × g. Transfer 300 µL of the supernatant into a clean tube and neutralize with 50 µL of 6 N NaOH. The neutralized supernatant is ready for assay (dilution factor DF = 1.36).
4. Transfer 20 µL of each sample into separate wells of an appropriate 96-well plate (clear for colorimetric assay, black for fluorometric assay).
5. If a specific Sample is known to contain choline, prepare a parallel Sample Blank well with 20 µL of sample.

Colorimetric Standard Curve Preparation

1. Prepare a 200 µM Acetylcholine Standard by mixing 24 µL of the 2 mM Standard with 216 µL of purified water.
2. Prepare Acetylcholine standards in 1.5 mL microcentrifuge tubes according to Table 2.

Table 2.
Preparation of Colorimetric Acetylcholine Standards

Well	200 µM Acetylcholine Standard	Purified Water	Acetylcholine (µM)
1	100 µL	-	200
2	60 µL	40 µL	120
3	30 µL	70 µL	60
4	-	100 µL	0

3. Mix well and transfer 20 µL of each Standard into separate wells of a clear 96-well plate.

Fluorometric Standard Curve Preparation

1. Prepare a 10 µM Acetylcholine Standard by mixing 5 µL of the 2 mM Standard with 995 µL of purified water.
2. Prepare Acetylcholine standards in 1.5 mL microcentrifuge tubes according to Table 3.

Table 3.
Preparation of Fluorometric Acetylcholine Standards

Well	10 µM Acetylcholine Standard	Purified Water	Acetylcholine (µM)
1	100 µL	-	10
2	60 µL	40 µL	6
3	30 µL	70 µL	3
4	-	100 µL	0

3. Mix well and transfer 20 µL of each Standard into separate wells of a clear 96-well plate.

Working Reagents

Note: This assay is based on an enzyme-catalyzed kinetic reaction. Addition of Working Reagent should be quick and mixing should be brief but thorough.

1. Mix enough reagents for the number of assays to be performed. Prepare Working Reagents according to Table 4.
   a. For each Standard and Sample well, prepare 88 µL of Working Reagent. Mix well.
   b. For choline containing samples where a parallel Sample Blank well was prepared, prepare 87 µL of Blank Working Reagent per Sample Blank well. Mix well.

Table 4.
Preparation of Working Reagents

Reagent	Working Reagent	Blank Working Reagent
Assay Buffer	85 µL	85 µL
ACHE Enzyme	1 µL	-
Enzyme Mix	1 µL	1 µL
Dye Reagent	1 µL	1 µL

Measurement

1. Quickly add 80 µL of Working Reagent to each Standard and Sample well and mix thoroughly.
2. If applicable, quickly add 80 µL of Blank Working Reagent to each Sample Blank well and mix thoroughly.
3. Incubate the plate for 30 minutes at room temperature.
4. Measure the optical density at 570 nm (OD_{570}) or fluorescence (RFU) at λ_{Ex} = 530 nm/λ_{Em} = 585 nm.
5. If the Sample reading is higher than the 200 µM standard reading in the Colorimetric Assay or 10 µM standard reading in the Fluorometric Assay, dilute sample in purified water and repeat the assay.

Results

1. Subtract the 0 Standard reading from all Standard readings.
2. Plot the Corrected OD_{570} or Corrected RFU readings for each Standard against Standard concentrations and calculate the slope of the Standard Curve.

3. Calculate the acetylcholine concentration of Sample:

Acetylcholine (µM) =

$$\frac{R_{Sample} - R_{Blank}}{Slope\ (\mu M^{-1})} \times DF$$

where

R_{Sample} = Optical density (OD_{570}) or fluorescence (RFU) intensity reading of the Sample

R_{Blank} = Optical density (OD_{570}) or fluorescence (RFU) intensity reading of the H_2O Blank (Standard #4) or Sample Blank for samples containing choline

DF = Sample dilution factor (DF = 1 for undiluted Samples)

Conversions: 1 mM acetylcholine equals 14.6 mg/dL, 0.015% or 146 ppm.

Figure 1.
Typical Acetylcholine Colorimetric Standard Curve

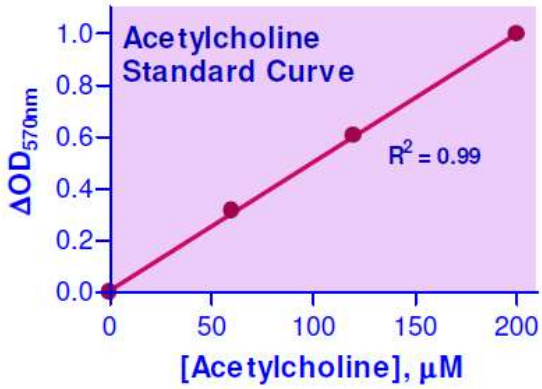
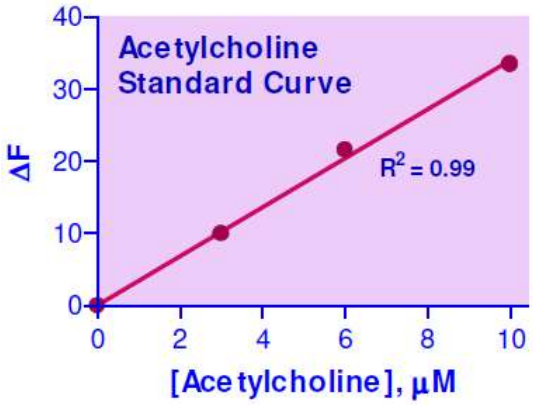


Figure 2.
Typical Acetylcholine Fluorometric Standard Curve



References

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2. Chen, L. et al., Acute exposure to DE-71: Effects on locomotor behavior and developmental neurotoxicity in zebrafish larvae. *Environ. Toxicol. Chem.* **31(10)**:2338-44 (2012).
3. Baitharu, I., et al., *Withania somnifera* root extract ameliorates hypobaric hypoxia induced memory impairment in rats. *J. Ethnopharmacology*, **145(2)**: 431-441. (2013).

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