Acetylcholine Assay Kit

Acetylcholine Assay Kit measures Acetylcholine in serum, plasma and tissues or cell lysates.

Catalog number: ARG81359

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INTRODUCTION

Acetylcholine (ACh) is an organic chemical that functions in the brain and body of many types of animals, including humans, as a neurotransmitter—a chemical message released by nerve cells to send signals to other cells [neurons, muscle cells, and gland cells]. Parts in the body that use or are affected by acetylcholine are referred to as cholinergic. Substances that interfere with acetylcholine activity are called anticholinergics. Acetylcholine is the neurotransmitter used at the neuromuscular junction—in other words, it is the chemical that motor neurons of the nervous system release in order to activate muscles. This property means that drugs that affect cholinergic systems can have very dangerous effects ranging from paralysis to convulsions. Acetylcholine is also a neurotransmitter in the autonomic nervous system, both as an internal transmitter for the sympathetic nervous system and as the final product released by the parasympathetic nervous system. Acetylcholine is synthesized in certain neurons by the enzyme choline acetyltransferase from the compounds choline and acetyl-CoA. Cholinergic neurons are capable of producing ACh. An example of a central cholinergic area is the nucleus basalis of Meynert in the basal forebrain. The enzyme acetylcholinesterase converts acetylcholine into the inactive metabolites choline and acetate and the half-life and activity are very short. Acetylcholine functions in both the central nervous system (CNS) and the peripheral nervous system (PNS). In the CNS, cholinergic projections from the basal forebrain to the cerebral cortex and hippocampus support the cognitive functions of those target areas. In the PNS, acetylcholine activates muscles and is a major neurotransmitter in the

autonomic nervous system. Therefore, different levels of ACh or modified expression and function of Ach receptors in the nervous system maybe related in several neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington as well as in psychiatric disorders such as schizophrenia. In addition, a shortage of acetylcholine by an Ach autoantibody lead to Myasthenia gravis disease characterized by muscle weakness and fatigue occurs when the body inappropriately produces antibodies against acetylcholine nicotinic receptors, and thus inhibits proper acetylcholine signal transmission. [Provide from Wikipedia]

PRINCIPLE OF THE ASSAY

This Acetylcholine Assay Kit employs a convenient colorimetric method for the detection of acetylcholine from serum, plasma and cell / tissue lysates. Acetylcholine in sample and standards are converted into choline and acetic acid by the enzyme acetylcholinesterase. And then choline is oxidized by choline oxidase to betaine and H2O2. The resulting H2O2 is detected by a Colorimetric Probe and then horseradish peroxidase (HRP) catalyzes the reaction between the probe and hydrogen peroxide to form a pink colored product. The intensity of the color is measured at a wavelength of 540-570 nm. The concentration of Acetylcholine in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
10X Assay Buffer	25 ml*2	4°C
Acetylcholine Standard (10 mM)	50 µl	-20°C
50X Colorimetric Probe	100 µl	-20°C
500X Horseradish peroxidase (HRP) (100 U/ml)	100 µl	-20°C
250X Acetylcholinesterase	10 U	-20°C
200X Choline Oxidase	25 µl	-20°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 540 - 570 nm
- Pipettes and pipette tips
- Multichannel micropipette reservoir
- Deionized or distilled water
- 96 well ELISA strips or 96 well microtiter plate
- Centrifugal filters for plasma or serum samples (e.g. Millipore Amicon Ultra-0.5ml, Ultracel® membrane filters, or Thermo Pierce Concentrators PES membrane filters)
- (optional) Chloroform
- (optional) Methanol
- (optional) Superoxide dismutase

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Ensure complete reconstitution and dilution of reagents prior to use.
- All materials should be equilibrated to room temperature (RT, 22-25°C) few minutes before use.
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- It is highly recommended assaying the control and samples in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

Samples should be assayed immediately or stored at -80ºC prior to performing the assay. Optimal experimental conditions for samples must be determined by the investigator. The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design. A set of serial dilutions is recommended for samples to achieve optimal assay results and minimize possible interfering compounds. Run proper controls as necessary. Always run a standard curve with samples.

Tissues or Cell lysates:

1. Homogenize 250 mg of sample (wet tissue or cell pellet) in 4.5 mL of chloroform/methanol (2:1, v/v). Centrifuge to remove debris.
2. After centrifugation, incubate the homogenate at room temperature for 1

hour on an orbital shaker.

3. Add 1.25 mL of distilled water to induce phase separation. Incubate 10 minutes at room temperature and centrifuge at 1000 x g for 10 minutes.
4. Collect the lower (chloroform) organic phase and re-extract the upper phase with 2 mL of solvent mixture whose composition is CHCl3/MeOH/water (86:14:1, v/v/v).
5. Combine organic phases and dry in a vacuum centrifuge.
6. Dissolve in 200 µL CHCl3/MeOH/water (60:30:4.5, v/v/v) for storage.
7. Before acetylcholine assay, samples must be diluted at least 1:50 to 1:400 with Assay Buffer.

Serum:

1. Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 1000 x g, 15 minutes at 4°C.
2. Collect the serum layer and store it on ice. Take care to avoid disturbing the white buffy layer.
3. Aliquot samples for testing and store samples at -80°C.
4. Prior to assay, filter samples with a 3K-10K centrifugal filter (e.g. Millipore Amicon Ultra-0.5mL, Ultracel® membrane filters, or Thermo Pierce Concentrators PES membrane filters).
5. Perform serum dilutions in 1X Assay Buffer. Serum samples must be diluted at least 1:20 with Assay Buffer for accurate determinations. Acetylcholine levels in normal serum samples may be below the kit detection limit.

Plasma:

1. Collect plasma using heparin or citrate as an anticoagulant. Centrifuge for 1000 x g, 10 minutes at 4°C.
2. Collect the plasma layer and store on ice. Take care to avoid disturbing the white buffy layer.
3. Aliquot samples for testing and store at -80°C.
4. Prior to assay, filter samples with a 3K-10K centrifugal filter (e.g. Millipore Amicon Ultra-0.5mL, Ultracel® membrane filters, or Thermo Pierce Concentrators PES membrane filters).
5. Perform plasma dilutions in 1X Assay Buffer. Plasma samples must be diluted at least 1:100 to 1:200 with Assay Buffer for accurate determinations. Acetylcholine levels in normal plasma samples may be below the kit detection limit.

Notes:

1. Samples with NADH concentrations above 10 µM and glutathione concentrations above 50 µM will oxidize the probe and could result in erroneous readings. To minimize this interference, it is recommended that superoxide dismutase (SOD) be added to the reaction at a final concentration of 40 U/mL.
2. Avoid samples containing DTT or β-mercaptoethanol since the probe is not stable in the presence of thiols (above 10 µM).
3. Choline can generate high background if present in samples. If choline may be present, run a background control without Acetylcholinesterase. Subtract this value from sample reading values.

REAGENT PREPARATION

- 1X Assay Buffer: Warm the 10X Assay Buffer to room temperature prior to using. Dilute the 10X Assay Buffer with deionized water to yield 1X Assay Buffer. (E.g. add 25 ml of 10X Assay Buffer into 225 ml of deionized water). Mix to homogeneity. Store the 1X Assay Buffer at 4°C up to 6 months.
- Acetylcholine Reaction Reagent: Prepare a reaction reagent to test for acetylcholine by diluting the Choline Oxidase 1:200, HRP 1:500, Colorimetric Probe 1:50, and Acetylcholinesterase 1:250 in 1X Assay Buffer. (eg. For 50 assays, combine 12.5 µl of Choline Oxidase, 5 µl of HRP, 50 µl of Colorimetric Probe, and 10 µl of Acetylcholinesterase with 2422.5 µl of 1X Assay Buffer to 2.5 ml total solution). Mix thoroughly and protect the solution from light. For best results, place the Acetylcholine Reaction Reagent on ice and use within 30 minutes of preparation. Do not store the Acetylcholine Reaction Reagent solution.
- Standards: Prepare fresh acetylcholine standards by diluting the 10 mM Acetylcholine Standard stock solution 1:50 in 1X Assay Buffer to yield a Standard 1 concentration of 200 µM. The 1X Assay Buffer serves as zero standard (0 µM), and the rest of the standard serial dilution can be diluted as according to the suggested concentration below: 200 µM, 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.13 µM, 1.57 µM and 0.78 µM. Do not store diluted acetylcholine standard solutions.

Dilute Acetylcholine standard as according to the table below:

Standard	Acetylcholine Conc.	µl of 1X Assay Buffer	µl of standard
S1	200 µM	490	10 of 10 mM Stock
S2	100 µM	250	250 of S1
S3	50 µM	250	250 of S2
S4	25 µM	250	250 of S3
S5	12.5 µM	250	250 of S4
S6	6.25 µM	250	250 of S5
S7	3.13 µM	250	250 of S6
S8	1.57 µM	250	250 of S7
S9	0.78 µM	250	250 of S8
S0	0	250	0

ASSAY PROCEDURE

Prepare and mix all reagents thoroughly before use. Each acetylcholine standard and sample should be assayed in duplicate or triplicate. A freshly prepared standard curve should be used each time the assay is performed.

1. Add **50 µL** of the **diluted acetylcholine standards or samples** to a 96-well microtiter plate.
2. Add **50 µL** of the **prepared Acetylcholine Reaction Reagent** to each standard and sample wells, mix thoroughly.
3. Cover the plate wells to protect the reaction from light. Incubate the plate on an orbital shaker for **60 minutes at room temperature.**
4. Read absorbance of each well on a microplate reader using **540-570 nm**.
5. Calculate the concentration of acetylcholine within samples by comparing the sample absorbance to the acetylcholine standard curve.

CALCULATION OF RESULTS

1. Subtract the mean absorbance value of the blank (S0, Standard #0) from all standard and sample readings. This is the corrected absorbance. Calculate the average absorbance values for each set of standards and samples.
2. Using log-log, semi-log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.
3. Using the mean corrected absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Determine the acetylcholine concentration of the samples with the equation obtained from the linear regression analysis of the standard curve. Substitute the corrected absorbance values for each sample. Remember to account for dilution factors.

   Acetylcholine (µM) =
   (Sample corrected absorbance/ Slope)* Sample dilution

5. Conversion: 1 mM acetylcholine = 14.62 mg/dL or 146 ppm.

EXAMPLE OF RESULTS

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.