

Mouse Acetylcholinesterase Fluorescent Activity Kit

CATALOG NO: IRAAKT2540

LOT NO: SAMPLE

INTENDED USE

The Acetylcholinesterase Activity kit is designed to quantitatively measure acetylcholinesterase (AChE) activity in a variety of samples.

BACKGROUND

Acetylcholinesterases (AChE) appear critical to both development and function of the nervous system. The use of AChE inhibitors as therapeutic agents and pesticides has spurred detailed investigations of cholinesterases since their identification by Dale¹. Acetylcholine (ACh) is an essential neurotransmitter in the central and peripheral nervous systems. In the brain multiple areas exist where cholinergic neurons are concentrated². Nicotinic and muscarinic ACh receptors are recognized as binding and effector proteins to mediate chemical neurotransmission at neurons, ganglia, heart and smooth muscle fibers and glands. This traditional view of AChE acting solely as neurotransmitter has to be revised based on the findings published both early and late in the last century, demonstrating the non-neuronal cholinergic system.



Acetylcholinesterase is encoded by the single AChE gene; and the structural diversity in the gene products arises from alternative mRNA splicing and post translational associations of catalytic and structural subunits. The major form of acetylcholinesterase found in brain, muscle, and other tissues is the hydrophilic species, which forms disulfide-linked oligomers with collagenous, lipid-containing structural subunits. The other alternatively-spliced form, expressed primarily in the erythroid tissues, is structurally different at the C-terminal end and contains a cleavable peptide with a GPI anchor. It associates with membrane receptors through phosphoinositide moieties added post-translationally³. Impairment of cholinergic neurotransmission is well-established in Alzheimer's disease, but there is controversy about its relevance at the early stages of the disease as well as in mild cognitive impairment. In vivo positron emission tomography imaging of cortical AChE activity as a marker of cholinergic function that is expressed by cholinergic axons and neurons has demonstrated a reduction of this enzyme activity in manifest Alzheimer's patients⁴. Other intentional or environmental methods of impairment is with organophosphates and carbamates with anticholinergic properties which are used as insecticides worldwide or as warfare agents. Thousands of cases of acute poisoning have been reported⁵. This acute toxicity inhibits AChE at nerve terminals where inhibition causes accumulation of ACh. This, in turn, induces overstimulation of nicotinic and muscarinic receptors in the central and peripheral nervous systems and the consequent signs and symptoms⁶.

1. Dale, HH. "The action of certain esters and ethers of choline, and their relation to mescaline." *J. Pharmacol. Exp. Ther.* 1914; 6:147-190.
2. Perry E, Walker M, Grace J, Perry R. "Acetylcholine in mind: a neurotransmitter correlate of consciousness?" *Trends Neurosci.* 1999; 22:273-280.
3. "Gene Result." NCBI HomePage. Web. 14 Oct. 2009.
<<http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=ShowDetailView&TermToSearch=43>>
4. Herholz, K., "Acetylcholine esterase activity in mild cognitive impairment and Alzheimer's disease." *Eur J Nucl Med Mol Imaging* 2008; 35(Suppl 1):S25-S29.
5. Moretto, A and Lotti, M., "Toxicity of pesticides." In: Stacey, N.H. Ed., *Occupational Toxicology*. Taylor and Francis, London, pp. 177-204.
6. Lotti, M. "Treatment of acute organophosphate poisoning." *Med. J. Aust.* 1991; 154: 51-55

ASSAY PRINCIPLE

The Acetylcholinesterase Activity kit is designed to quantitatively measure acetylcholinesterase (AChE) activity in a variety of samples. Please read the complete kit insert before performing this assay. A human AChE standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. The kit utilizes a proprietary non-fluorescent molecule that covalently binds to the thiol product of the reaction between the AChE Substrate and AChE in the standards or samples, yielding a fluorescent product read at 510 nm in a fluorescent plate reader with excitation at 390 nm.

The kit is suitable for measuring AChE activity in appropriately diluted serum, plasma and RBC ghosts from a number of species. It will also measure AChE in extracted tissue samples and cell lysates. Because the readout of AChE activity is purely chemical, there are few interferants that will affect the readings obtained.

REACTION OVERVIEW

1. Sample or standard added to well.
2. The reaction is initiated with the addition of the Reaction Mix containing AChE
4. Substrate and reagent.
3. Incubate for 20 minutes and read fluorescent signal. Calculate AChE activity from standard curve.
4. Alternatively samples can be read kinetically. Follow steps 1 and 2 above.
5. Add Reaction Mix and read signal at 510 nm over time. Compare rates for samples and standards to determine sample AChE activity.

SUPPLIED COMPONENTS

- **Black 96 Well Plates**
- **Acetylcholinesterase Standard 225 µL:** Acetylcholinesterase (AChE) at 1,000 mU/mL in a special stabilizing solution. CAUTION: This material is isolated from human erythrocytes. Treat as potentially infectious.
- **Detection Reagent 2 vials:** thiol detection substrate stored in a desiccator. Reconstitute with dry DMSO.
- **Dry DMSO 14 mL:** Dry Dimethyl sulfoxide solvent over molecular sieves. May be stored at room temperature.
- **Assay Buffer Concentrate 28 mL:** A 10x concentrated Tris buffer containing detergents and stabilizers.
- **AChE Substrate 2 vials:** Acetylthiocholine iodide freeze dried with stabilizers.

STORAGE INSTRUCTIONS

- All components of this kit should be stored at 4°C until the expiration date of the kit.
- DMSO, when stored at 4°C, will freeze. Can be stored tightly capped at room temperature.

OTHER MATERIALS REQUIRED

- Distilled or deionized water.
- Repeater pipet with disposable tips capable of dispensing 50 µL.
- Fluorescence 96 well plate reader capable of reading fluorescent emission at 510 nm, with excitation at 390 nm. Contact your plate reader manufacturer for correct filter sets. Set plate parameters for a 96-well Corning Costar 3915 plate.
- Software for converting raw relative fluorescent unit (FLU) readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

PRECAUTIONS

- As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.
- Dimethyl sulfoxide is a powerful aprotic organic solvent that has been shown to enhance the rate of skin absorption of skin-permeable substances. Wear protective gloves when using the solvent especially when it contains dissolved chemicals.
- The Acetylcholinesterase Standard is derived from human blood. It has been extensively tested for viral contamination, but all human blood products should be treated as potentially infectious and adequate precautions taken.
- **Detection Reagent should be stored at 4°C in the desiccator. Allow to warm to room temperature prior to opening. The reagent will react with strong nucleophiles. Buffers containing the preservatives sodium azide will react with the substrate.**

SAMPLE TYPES

- This assay has been validated for serum, EDTA and heparin plasma, and solubilized RBC ghosts from a variety of species. Samples containing visible particulate should be centrifuged prior to using.

SAMPLE PREPARATION

Serum & Plasma

Store separated serum or plasma on ice until assaying or freeze in aliquots for later use. Samples must be diluted in Assay Buffer prior to running in the kit. Any samples with AChE activity outside the standard curve range should be diluted further with Assay Buffer to obtain readings within the standard curve. Serum and plasma typically have to be diluted $\geq 1:300$ to read in the assay.

Erythrocytes (RBCs)

Blood is collected in the presence of heparin or EDTA. The sample is then centrifuged and the plasma and white cell layer are removed from the RBC layer. The RBCs are suspended and gently washed twice with three volumes of isotonic saline (0.9%), separating the cells by centrifugation at $600 \times g$ for 10 minutes and discarding the saline after each step. To lyse the RBCs, four volumes of cold deionized water are added to the RBCs. The cells are then vortexed and incubated for 10 minutes at 4°C , or allowed to undergo a freeze/thaw. Samples are centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatant discarded.

Further wash the membrane pellet with two or three volumes of isotonic saline, with centrifugation in between, until it is only slightly pink. The smaller dark red pellet on the bottom is non-lysed RBCs and should be avoided. Solubilize the white membrane ghost pellet with Triton X-100. Final assay dilution of the solubilized RBC ghost sample must be sufficient that the assay sample contains $\leq 0.01\%$ Triton X-100.

Use all samples within 2 hours of dilution.

REAGENT PREPARATION

Allow the kit reagents to come to room temperature for 30 minutes. We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine AChE activity. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.

Assay Buffer

Prepare the Assay Buffer by diluting one part of the 10x Assay Buffer Concentrate with nine parts deionized water for a 1:10 dilution. It is stable for up to 3 months when stored at 4°C.

Detection Reagent

Remove a vial of Reagent from the desiccator and add 700 µL of the provided DMSO to the vial. Vortex thoroughly. Store any unused reconstituted Detection Reagent at 4°C in the desiccator and use within 2 weeks.

Acetylcholinesterase Substrate

Add 700 µL of the provided DMSO to the AChE Substrate vial and vortex thoroughly. This is a 10x concentrate of the substrate. Store any unused reconstituted AChE Substrate at room temperature and use within 2 weeks.

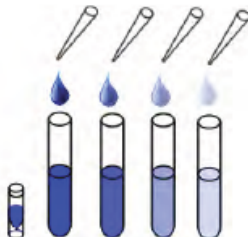
Reaction Mix Dilution Table

	1/2 Plate	Full Plate
10X AChE Substrate Concentrate	300 µL	550 µL
10X ThioStar® Concentrate	300 µL	550 µL
DMSO	2.4 mL	4.4 mL

Standard Preparation

AChE Standards are prepared by labeling five test tubes as #1 through #5. Briefly spin vial of standard in a microcentrifuge to ensure contents are at bottom of vial. Pipet 450 μL of Assay Buffer into tube #1 and 250 μL into tubes #2 to #5. Carefully add 50 μL of the AChE Standard to tube #1 and vortex completely. Take 250 μL of the AChE solution in tube #1 and add it to tube #2 and vortex completely. Repeat these serial dilutions for tubes #3 through #5. The activity of AChE in tubes 1 through 5 will be 100, 50, 25, 12.5, and 6.25 mU/mL.

Use all Standards within 2 hours of preparation.



	Std 1	Std 2	Std 3	Std 4	Std 5
Assay Buffer Volume (μL)	450	250	250	250	250
Addition	Stock	Std 1	Std 2	Std 3	Std 4
Volume of Addition (μL)	50	250	250	250	250
Final Conc. (mU/mL)	100	50	25	12.5	6.25

ASSAY PROTOCOL

1. Use the plate layout sheet on the back page of the insert to aid in proper sample and standard identification. Set plate parameters for a 96-well Corning Costar 3915 plate.
2. Pipet 100 μL of samples or standards into duplicate wells in the plate.
3. Pipet 100 μL of Assay Buffer into duplicate wells as a Zero standard.
4. Add 50 μL of the prepared Reaction Mix to each of the wells using a repeater pipet.
5. Gently tap the sides of the plate to ensure adequate mixing of the reagents.
6. Incubate at room temperature for 20 minutes.
7. Read the fluorescent emission at 510 nm with excitation at 370-410 nm. Please contact your plate reader manufacturer for suitable filter sets.

CALCULATION OF RESULTS

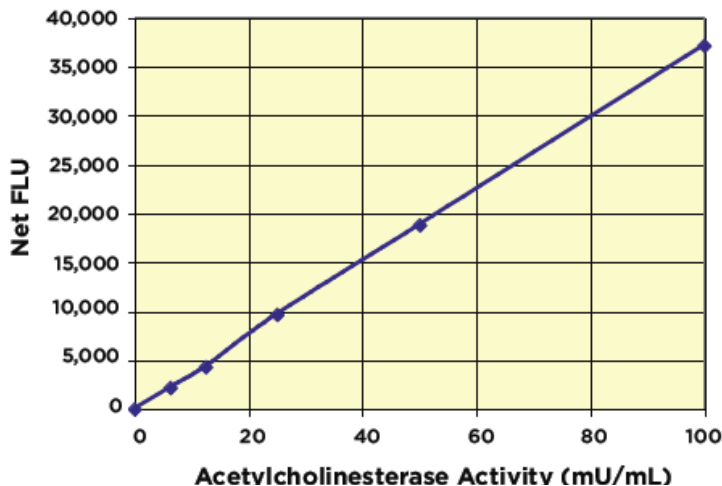
Average the duplicate FLU readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean FLUs for the zero standard. The sample activity obtained should be multiplied by the dilution factor to obtain neat sample values.

TYPICAL DATA

Sample	Mean FLU	Net FLU	AChE Activity (mU/mL)
Standard 1	39,677	37,116	100
Standard 2	21,336	18,775	50
Standard 3	12,194	9,633	25
Standard 4	6,845	4,284	12.5
Standard 5	4,714	2,153	6.25
Zero	2,561	0	0
Sample 1	5,108	2,547	7.4
Sample 2	18,287	15,726	41.6

Always run your own standard curve for calculation of results.
Do not use this data.

Typical Standard Curve



VALIDATION DATA

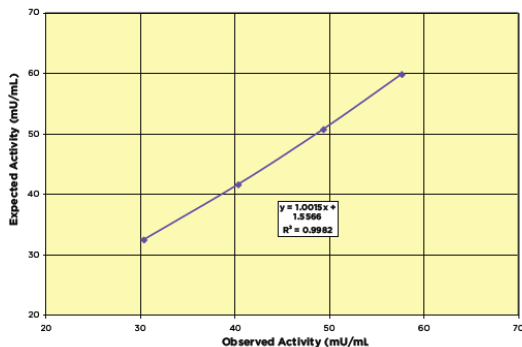
Sensitivity and Limit of Detection

- Sensitivity was calculated by comparing the FLUs for twenty wells run for each of the zero and standard #5. The detection limit was determined at two (2) standard deviations from the zero along the standard curve.
- **Sensitivity was determined as 0.218 mU/mL.**
- The Limit of Detection was determined in a similar manner by comparing the FLUs for twenty wells run for each of the zero and a low activity plasma sample.
- **The Limit of Detection was determined as 0.321 mU/mL.**

LINEARITY

Linearity was determined by taking two serum samples, one high sample diluted 1:600 and one low sample diluted 1:1,000, and mixing in the ratios given below. The measured activities were compared to the expected values based on the ratios used.

Low Sample	High Sample	Observed Activity (mU/mL)	Expected Activity (mU/mL)	% Recovery
100%	0%	23.3	---	---
80%	20%	30.4	32.4	93.8
60%	40%	40.4	41.5	97.3
40%	60%	49.4	50.7	97.5
20%	80%	57.7	59.8	96.5
0%	100%	68.9	---	---
			Mean Recovery	96.3%



Intra Assay Precision

Three mammalian samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated AChE activities were:

Sample	AChE Activity. (mU/mL)	%CV
1	63.9	8.9
2	41.6	9.1
3	11.5	6.7

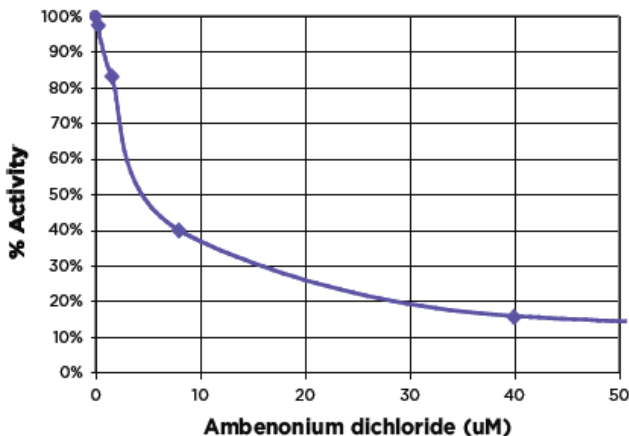
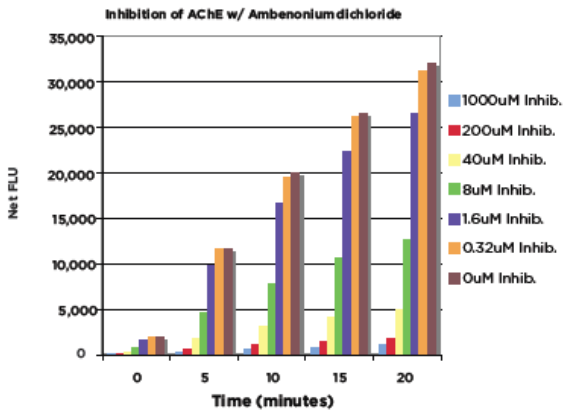
Inter Assay Precision

Three mammalian samples were diluted with Assay Buffer and run in duplicates in thirteen assays run over multiple days by four operators. The mean and precision of the calculated AChE activities were:

Sample	AChE Activity. (mU/mL)	%CV
1	59.1	7.9
2	39.9	8.5
3	11.3	11.1

INHIBITION STUDIES

The human AChE standard was incubated with varying concentrations of a reversible inhibitor of AChE activity, Ambenonium dichloride, from 1,000 down to 0.32 μM for 17 hours at room temperature in the kit Assay Buffer. The activity in the incubated samples was then determined in the normal manner by adding 100 μL of the samples and kinetically reading the activity over 20 minutes.



SAMPLE VALUES

A variety of serum and plasma samples were tested in the assay, including chicken, mouse, rat, dog, monkey, pig and human samples. Values averaged 19,188 mU/mL. RBC ghost samples ranged from 6,216 to 18,552 mU/mL with an average of 13,158 mU/mL.

CROSS REACTIVITY

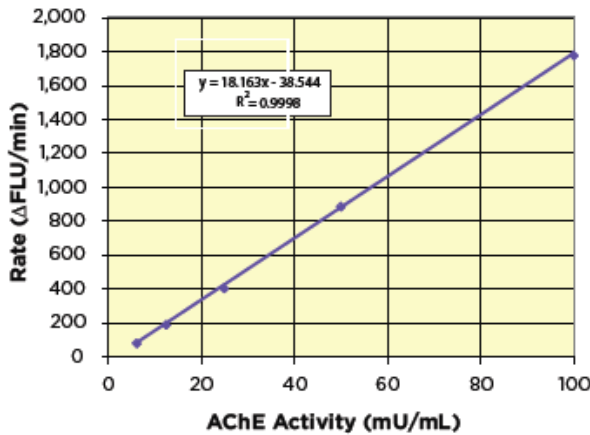
A sample of native human butyrylcholinesterase at 20 mU/mL was tested in the assay. It read 2.45 times higher than the same concentration of AChE tested at the same time.

INTERFERENTS

A variety of solvents were tested as possible interfering substances in the assay. 5% ethanol in the well increased the activity recorded by 6.4%, whereas 10% ethanol in the well decreased activity by almost 43%. 5% DMSO or DMF in the well decreased activity by 12.6% and 19.65% respectively. 10% methanol in the well decreased activity by 6.3%. We expect solvent levels at 1% of well volume to have little or no effect on the measured activity. A solvent only control should be run by the end user when appropriate.

END POINT VERSUS KINETIC ACTIVITY

Human RBC ghosts were read in a standard end point assay and in a kinetic assay. The value obtained from the end point assay was 12,577 mU/mL and in the kinetic assay, it read 12,453 mU/mL.



ASSAY TEMPLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												