Quantikine® ELISA

Human Acetylcholinesterase/ACHE Immunoassay

Catalog Number DACHE0

For the quantitative determination of human Acetylcholinesterase (ACHE) concentrations in cell culture supernates, tissue lysates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Acetylcholinesterase (ACHE), a member of the carboxylesterase family of enzymes, is a serine hydrolase that plays an indispensable role in cholinergic neurotransmission. It rapidly and selectively hydrolyzes the neurotransmitter acetylcholine (ACH) at cholinergic synapses and neuromuscular junctions (1). ACHE is expressed throughout the nervous system and is found in both cholinergic and noncholinergic fibers (2). ACHE is also expressed in many non-neural cells and tissues including muscle, haematopoietic cells, leukocytes, vascular endothelial cells, and osteoblasts, (3, 4).

In the ACHE gene, alternate promoters and alternative splicing produces multiple ACHE isoforms with different N- and C-termini, respectively (1, 4-6). ACHE-Synaptic (ACHE-S) contains a 40 amino acid (aa) C-terminal peptide and can exist in both monomeric and multimeric forms (5-7). The human ACHE-S monomer is synthesized as a 614 aa precursor protein with a predicted molecule weight of approximately 68 kDa. It shares 89% aa sequence identity with the mouse and rat orthologs. ACHE-Erythrocyte (ACHE-E) is a GPI-anchored dimeric protein found in the membranes of erythrocytes. It constitutes the Yt blood group antigen (7-9). ACHE-Read-Through (ACHE-R) is a soluble monomer that is produced by the inclusion of the normally spliced-out intron 4 (6, 7). Its expression has been reported to be elevated during stress (1, 9).

In addition to its classical function of ACH hydrolysis, ACHE has been shown to be involved in many non-cholinergic functions including playing a role in the pathology of Alzheimer's disease (AD). ACHE is found in excess in the aged brain and has been found to co-localize with Aβ deposits (10-12). Considered a chaperone molecule, ACHE has been shown to accelerate the assembly of Aβ peptides into fibrils by complexing with Aβ via its peripheral anionic site (12-16). It also increases the neurotoxicity of aggregated Aβ, augmenting the effects induced by Aβ oligomers and fibrils, such as calcium homeostasis dysregulation and mitochondria dysfunction (12, 14). Additionally, ACHE has been shown to promote neurodegeneration of cholinergic neurons by inducing tau hyperphosphorylation and apoptosis (12, 17). ACHE inhibitors have been used to delay symptoms of AD patients by virtue of their ability to enhance ACH availability, as well as reduce amyloidogenesis and subsequent neurotoxicity (13, 16). ACHE has also been shown to be involved in cell growth, stem cell differentiation, neurite outgrowth, cell adhesion, apoptosis, synaptogenesis, activation of dopaminergic neurons, haematopoiesis, and thrombopoiesis (1, 3, 4, 7, 9, 13, 18-20). It is believed to play a role in tumorigenesis, and low ACHE levels are associated with pesticide toxicity (9, 21). Furthermore, ACHE is involved in the cholinergic anti-inflammatory pathway and is associated with low-grade systemic inflammation in obesity, hypertension, type II diabetes, and AD (22).

The Quantikine Human Acetylcholinesterase/ACHE Immunoassay is a 4.5 hour solid phase ELISA designed to measure ACHE levels in cell culture supernates, tissue lysates, serum, and plasma. It contains CHO cell-expressed recombinant human ACHE and antibodies raised against the recombinant protein. Results obtained for naturally occurring human ACHE showed linear curves that were parallel to the standard curves obtained using the Quantikine Human ACHE Immunoassay standards. These results indicate that this kit can be used to determine relative mass values for natural human ACHE.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human ACHE has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any ACHE present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human ACHE is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of ACHE bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human ACHE Microplate	894869	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human ACHE.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human ACHE Standard	894871	2 vials of recombinant human ACHE in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard for each assay.
Human ACHE Conjugate	894870	21 mL of a polyclonal antibody specific for human ACHE conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-63	895352	12 mL of a buffered protein base with and preservatives.	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:4 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- Test tubes for dilution of standards and samples.
- Human ACHE Controls (optional; R&D Systems, Catalog # QC212).

SUPPLIES REQUIRED FOR TISSUE LYSATE SAMPLES

- RIPA Buffer with protease inhibitors.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Tissue Lysates - Tissue must be lysed prior to assay as directed in the Sample Values section.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Grossly hemolyzed samples are not suitable for use in this assay.*

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

SAMPLE PREPARATION

Serum and plasma samples require a 2-fold dilution due to a matrix effect. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

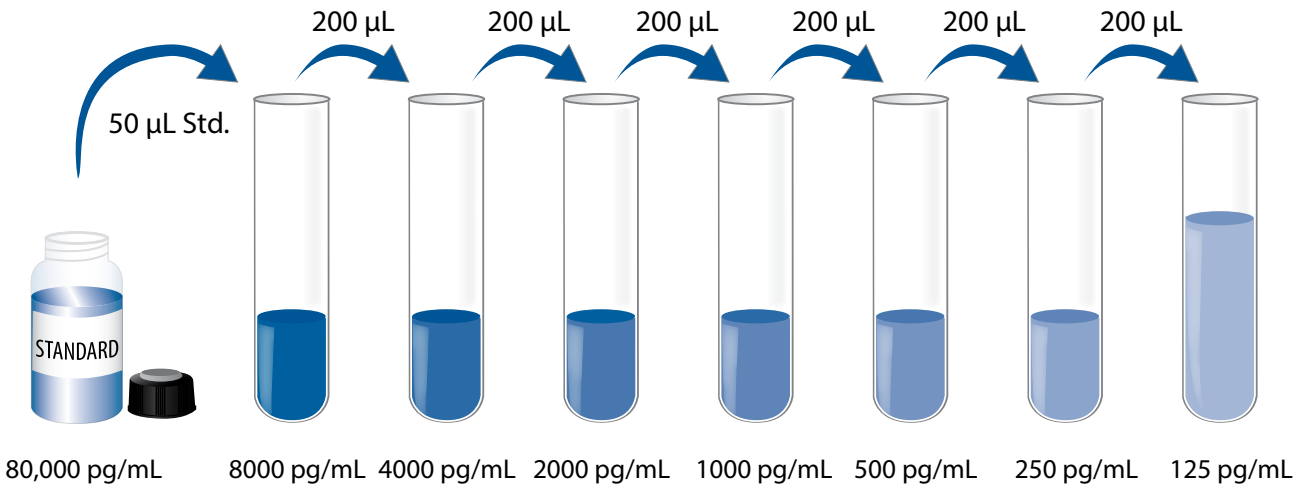
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-26 (diluted 1:4) - Add 10 mL of Calibrator Diluent RD5-26 Concentrate to 30 mL of deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5-26 (diluted 1:4).

Human ACHE Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human ACHE Standard with deionized or distilled water. This reconstitution produces a stock solution of 80,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5-26 (diluted 1:4) into the 8000 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 8000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:4) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1-63 to each well.
4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human ACHE Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See the Sample Preparation section.

CALCULATION OF RESULTS

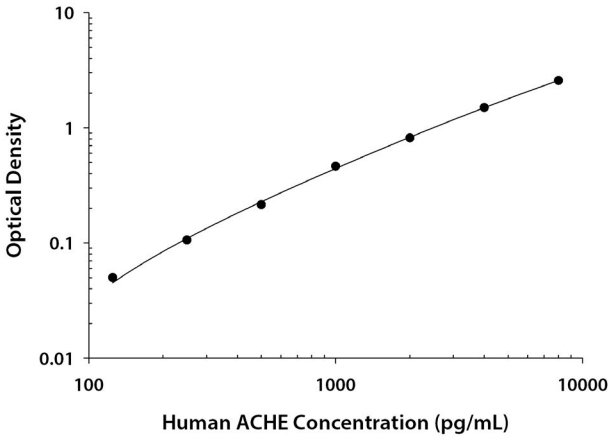
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human ACHE concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected
0	0.050 0.067	0.059	—
125	0.101 0.116	0.109	0.050
250	0.160 0.169	0.165	0.106
500	0.271 0.277	0.274	0.215
1000	0.504 0.540	0.522	0.463
2000	0.849 0.902	0.876	0.817
4000	1.546 1.562	1.554	1.495
8000	2.611 2.650	2.631	2.572

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	476	1333	2646	482	1353	2731
Standard deviation	14.6	44.4	222	34.3	79.3	81.7
CV (%)	3.1	3.3	8.4	7.1	5.9	3.0

RECOVERY

The recovery of human ACHE spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	103	87-113%
Cell Lysis Buffer (n=4)	99	86-111%
Serum* (n=4)	101	95-114%
EDTA plasma* (n=4)	102	93-119%
Heparin plasma* (n=4)	95	84-110%

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Twenty-three assays were evaluated and the minimum detectable dose (MDD) of human ACHE ranged from 11.5-43.7 pg/mL. The mean MDD was 24.8 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against highly purified CHO cell-derived recombinant human ACHE produced at R&D Systems.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human ACHE were diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Cell lysis buffer (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	101	106	102	101	98
	Range (%)	96-103	103-112	98-105	98-104	94-106
1:4	Average % of Expected	102	105	100	100	99
	Range (%)	95-108	99-110	92-107	97-102	97-105
1:8	Average % of Expected	105	103	99	97	99
	Range (%)	96-110	98-107	86-109	93-104	87-108
1:16	Average % of Expected	106	102	104	102	101
	Range (%)	96-114	98-107	88-113	95-106	90-108

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human ACHE in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=36)	1374	832-2251	336
EDTA plasma (n=36)	1278	787-2256	338
Heparin plasma (n=36)	1186	755-2050	313

Cell Culture Supernates - Peripheral blood leukocytes (1 x 10^6 cells) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 1 and 5 days. Aliquots of the cell culture supernates were removed and assayed for human ACHE. No detectable levels were observed.

Tissue Lysates - Tissues were rinsed with PBS, cut into 1-2 mm pieces, and homogenized with a tissue homogenizer in PBS. An equal volume of RIPA buffer containing protease inhibitors was added and tissues were lysed at room temperature for 30 minutes with gentle agitation. Debris was then removed by centrifugation. Aliquots of the lysates were removed and assayed for human ACHE.

Tissue	pg/mg of lysate
Human brain motor cortex	474
Human cerebellum	9530
Human hypothalamus	11,374
Human skeletal muscle	3219

SPECIFICITY

This assay recognizes natural and recombinant human ACHE.

The factors listed below were prepared in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors in a mid-range recombinant human ACHE control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:	Recombinant mouse:	Other proteins:
BCHE	ACHE	human Amyloid β-Peptide (1-40) human Amyloid β-Peptide (1-42)

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