Fluoro - AChE™*

Fluorescence Assay for Monitoring Red Blood Cell Acetyl cholinesterase Activity

PROTOCOL

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Introduction

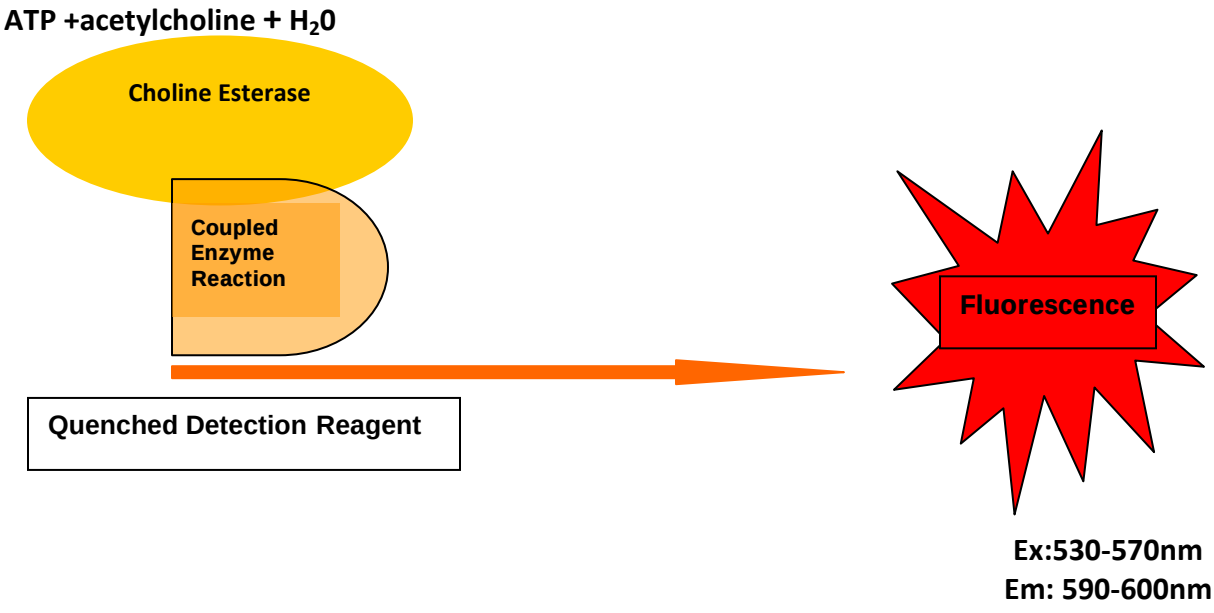
Exposure to chemical nerve agents, pesticides and certain drugs (anesthetics, cocaine and therapeutical drugs) reduces the activity of red blood cell (RBC) acetyl cholinesterase (AChE). The RBC-AChE can be used as a biomarker to monitor suppressed and or increased AChE function in the peripheral and central nervous system [9].

Acetyl cholinesterase (AChE) is one of the most important enzymes involved in nerve transmission. The enzyme is bound to cellular membranes of excitable tissue (synaptic junction, endoplasmic reticulum, etc) [1-3]. Acute toxicity to humans and animals through inhibition of AChE by both nerve gases and an important class of pesticides has long been a field of intensive scientific investigation [4,5]. AChE inhibitors have also been used clinically as Alzheimer's treatments (*e.g.*, tacrine (tetrahydroaminoacridine)) [6] and are the subject of increasing interest in various disease processes and treatment strategies [7,8]. However, both environmental detection of AChE inhibitors and development of modulators of AChE enzymatic activity as drugs have been hampered by the difficulty and complexity of the current assay methods.

Assay Principle

We have developed a highly sensitive, very rapid, extremely simple assay to determine acetylcholinesterase activity in RBC's, using the natural substrate, acetylcholine. Additionally, by using specific inhibitors, the kit can be used to detect AChE activity in a variety of samples. A series of coupled enzyme reactions quickly translates the presence of active AChE into a change in the fluorescence of a quenched detection reagent.

Figure 1



I: Kit Contents: Cat# AChE100-2; 100 Tests

1. **Component A**: 5.5mL.....Part# 3042
   Detection Reagent Diluent
2. **Component B**: 5.5mL.....Part# 3043
   Coupled enzyme reagent.
3. **Component C:** 1 vial detection reagent.....Part# 4016
4. **Component D**: 5X Reaction Buffer, 20ml.....Part# 3011
5. **Component E:** Acetylcholine.....Part# 7012
6. **Component F:** Human Red Blood Cell Acetyl cholinesterase,1.25 Units.....Part# 6020

II: Materials and Equipment Needed:

1. Black 96 well plates for fluorescence plate reader.
2. Fluorescence plate reader.
3. Optional Cholinesterase specific inhibitors. Follow all warnings and precautions.
   1. Acetyl cholinesterase: Sigma Cat# A9013: BW 284c51
   2. Butyryl cholinesterase Sigma Cat# T1505: iso-OMPA
4. Triton X-100: Sigma Cat# X100.

III. Warnings and Precautions:

1. **For Research use only. Not for use in diagnostic procedures.**
2. Practice safe laboratory procedures by wearing protective clothing and eyewear.
3. The fluorescent product of the detection reagent is not stable in the presence of thiols (DTT or 2-mercaptoethanol will interfere with the assay. See technical note#1 below.
4. NADH and glutathione (reduced form: GSH) will interfere with the assay.
   See Technical note #1.
   If you are going to use your own buffer keep the pH between 7.0-8.0.

IV: Reagent set up:

1. **Component A**: Frozen liquid, thaw and aliquot into single-use vials. Freeze at –70^{0}C.
2. **Component B**: Frozen liquid, thaw and aliquot into single-use vials. Freeze at –70^{0}C.
3. **Component C**: Dried detection reagent, reconstitute by adding 110 uL DMSO. Aliquot into single-use vials. Freeze at –70^{0}C.
4. **Component D**: 5X Reaction Buffer. Store 5X and 1X at 4-8^{0}C. Dilute 1:5 with Di water to make a 1X solution.
5. **Component E:** Acetylcholine Chloride. MW 181.66. Make a stock 0.5M solution in Di water by adding 9.083 mg Acetylcholine Chloride to 100uL of Di water.
   This solution should be prepared fresh just prior to performing assay, as acetylcholine is highly unstable in solution. Any unused solution should be discarded after the experiment.
   Cautionary Notes:
   1. **Avoid repeated freeze thaw cycles of all components.**
   2. **Keep components out of direct light (such as in a bio-safety hood).**
6. **Component F:** Human Red Blood Cell Acetyl cholinesterase.....Part#6020
   RBC-AChE at 5 units/mL. Dilute in 1X reaction buffer to construct a standard curve. Aliquot into single use vials and freeze at –70^{0}C.

V: Assay Set-Up

1. Thaw Components A and B in the dark and let them come to room temperature before starting assay.

2. Make a working stock solution of component A by pipetting reconstituted component C (detection reagent) using the following formula: for every 1mL of component A add 10uL of component C, this can be stored for several weeks, if aliquoted into single use vials.
Note: Keep protected from direct light.

3. Make a working stock solution of component B by adding acetylcholine from the stock solution prepared in Step IV above (0.5M) to make a final concentration of 1-2mM. If using competitive inhibitors the acetylcholine concentration must be lowered. This concentration must be determined empirically and optimized for each application. Also set up a **negative control** with
no acetylcholine. This will be used to determine background signal of the quenched detection reagent in each sample type.

4. Sample preparation:

A. Wash Red blood cell (RBC) preparation with PBS several times and after the final wash re-suspend RBC in 3 volumes of 1X reaction buffer + 1% TritonX-100 (for example to 1mL of packed RBC's add 2mL of 1X reaction buffer + 1% TritonX-100)

B. Tissue samples can be homogenized in 1X Reaction buffer. It is recommended to add protease inhibitors to the homogenates. Homogenates can be further diluted in 1X reaction buffer and are ready for the assay or can be frozen at -70^0C for later analysis. Note: avoid using serine protease inhibitors as they will also inhibit AChE.

C. Cells can be lysed with 1X reaction buffer supplemented with 1% Triton X-100. Before lysing wash cells with PBS several times and after the final wash re-suspend cell pellet add adding 500uL of 1X reaction buffer + 1% TritonX-100. Gently vortex cells and let stand for 10 minutes at 4-8^{0}C to lyse them. Lysates are ready for the assay or can be frozen at -70^0C for later analysis. Lysates can be further diluted in 1X reaction buffer prior to the assay.

D. Saliva. Collect saliva and centrifuge to remove cells and debris. Collect the clear supernatant for the assay. Supernatant can be can be frozen at -70^0C for later analysis.

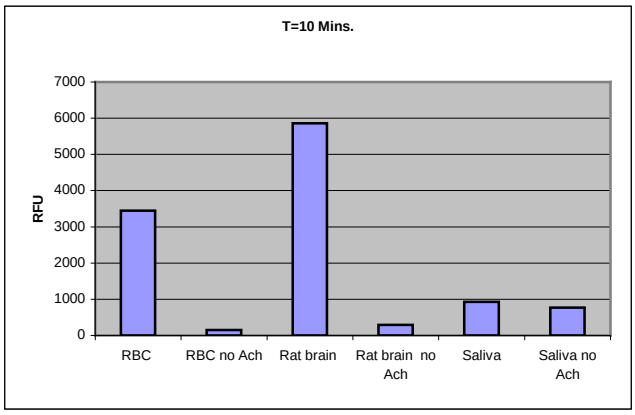
VI: Assay: It is important to read the Technical Notes before starting.

1. The basic steps in the assay are: 50uL of working stock component A + 50uL of working stock component B+ 10-50 uL of sample. Incubate and measure fluorescence. If needed, specific inhibitors can be added to the samples, prior to adding them to the assay, to block acetyl or butyrylcholinesterase.
Control 1: (optional). Run a control with no acetylcholine in component B + A + sample. This control should be run for each type of sample and dilution. This control will help determine if the sample has thiols that will react with the quenched detection reagent (see technical note 1) giving a high background. It is recommended that this control be run with saliva samples (technical note #1).
Control 2: (no sample control) Run a control with component B (+ acetylcholine) + A+ 1X reaction buffer. Use the same volume of reaction buffer as in the sample. (If using 50uL of sample per reaction, use 50uL of 1X reaction buffer to keep reaction volume in sample and control wells constant). This control will measure natural occurring acetylcholine degradation in aqueous solutions and represents the background signal.

2. Take several readings to determine optimal signal time point. Ex;530-570nm, Em: 590-600nm.

VII: Results

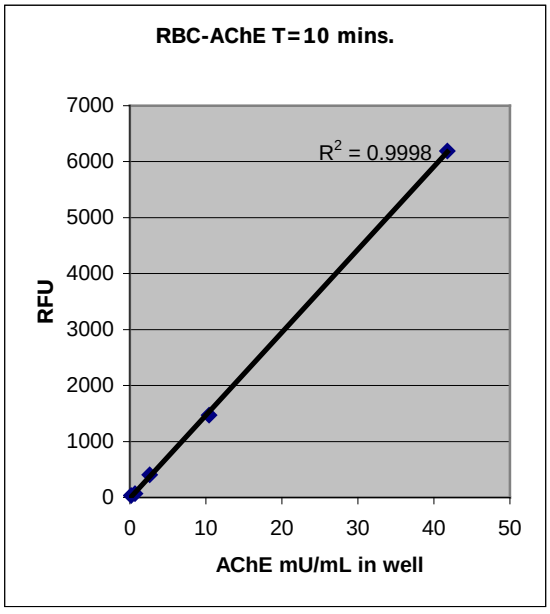
For demonstration purposes only.



Time=10 min	Dilution	Volume used	ug protein/well	RFU *
RBC	1:1000	10uL	5	3450
RBC no Ach	1:1000	10uL	5	152
Rat brain	1:500	10uL	20	5864
Rat brain no Ach	1:500	10uL	20	296
Saliva	neat	10uL	ND	925
Saliva no Ach	neat	10uL	ND	772

Fig 1: Protein concentration for each sample was determined using the BCA Protein Assay Kit (Pierce). The lysates were diluted in 1X reaction buffer and the indicated volumes add to the Fluoro: AChE reaction (component A+B). Samples were incubated at room temperature in the dark for 10 minutes. Fluorescence was measured in a 96 well plate reader Ex: 530nm Em: 590nm. (ND= Not determined). Acetylcholine: 1mM final.

*Control 2 (background) subtracted from signal.



RBC-AChE mU/mL in well	RFU	SD RFU
167.000	9999	0.00
41.750	6375	121.70
10.438	1659	9.00
2.609	590	9.81
0.652	256	7.23
0.163	222	8.72
0	186	8.08

Fig.2 Red Blood Cell AChE (RBC-AChE) was purified and protein concentration determined using the BCA Protein Assay Kit (Pierce). The RBC-AChE was titrated in 1X reaction buffer and activity determined using the Fluoro:AChE kit. Acetylcholine concentration = 1mM final. In the graph the background value has been subtracted (0 RBC-AChE) to generate standard curve.

VIII: Technical Notes

1. The quenched detection reagent reacts with thiols, reducing reagents (DTT, 2-mercaptoethanol), NADH and GSH. Diamide (Sigma Cat# D3648) can be added to the samples to block these groups. Add diamide to a final concentration of 10mM and incubate samples for 15-30 minutes at room temperature prior to adding them to the assay.
2. Acetyl cholinesterase activity can be inhibited in the sample by adding BW 284c51: Sigma Cat# A9013 prior to adding sample to the assay.
   Consult Sigma on optimal concentration (caution: BW 284c51is very toxic read MSDS before handling).
3. Butyryl cholinesterase activity can be inhibited in the sample by adding iso-OMPA: Sigma Cat# T1505 prior to adding sample to the assay.
   Consult Sigma on optimal concentration (caution: iso-OMPA is very toxic read MSDS before handling).

Ordering information

Catalog #	Size	Price (US$)
ACHE 100-2	100	395
ACHE 100-3	500	1595

References

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(4) HA Berman and MM Decker**.** Kinetic, equilibrium, and spectroscopic studies on dealkylation ("aging") of alkyl organophosphonyl acetylcholinesterase. Electrostatic control of enzyme topography. J. Biol. Chem., Aug 1986; 261: 10646-10652 .

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