Laboratory Procedure Manual

Analyte:	*Active Butyrylcholinesterase (BChE)*
Matrix:	Human Plasma, Serum, and Blood
Method:	Absorption Spectroscopy
Method No.:	2543
Revised:	**08/09/2021**
As performed by:	*Emergency Response Branch* *Division of Laboratory Sciences* *National Center for Environmental Health*
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Important Information for Users

The Division of Laboratory Sciences periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

Data File Name	Variable Name	SAS Label	CLIA Approved
BCHE	LBXBA	Butyrylcholinesterase Activity (U/mL)	Yes

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

Clinical Relevance

Organophosphorus nerve agents (OPNAs) are small synthetic molecules that are highly toxic to humans. Shortly after dermal or inhalational exposure to these agents, the body's cholinesterase activity is severely diminished due to the covalent binding of OPNAs to the active site of these enzymes. Acetylcholinesterase (AChE) is an enzyme found on red blood cells, at neuromuscular junctions, and in cholinergic synapses in both the central and peripheral nervous systems. It is responsible for turning off synaptic transmission by hydrolyzing the neurotransmitter acetylcholine (ACh). When AChE activity is inhibited by OPNAs, ACh continues to activate receptors on postsynaptic neurons and muscles, leading to continual and rapid muscle twitching, hyperglandular secretion, neuromuscular paralysis and potentially death.[1,2]

Test Principle

OPNAs inactivate cholinesterases by forming covalent adducts, resulting in nervous system impairment.[3] Though nervous system impairment is primarily a result of AChE inhibition, a second type of cholinesterase, butyrylcholinesterase (BChE), is also inhibited by OPNAs. BChE, a naturally occurring plasma protein, was chosen as an indicator of reduced cholinesterase activity because it is present at higher concentrations than AChE in the blood.[4] Furthermore, within the blood system, AChE is found bound to the surface of red blood cells while BChE is freely circulating. BChE can be obtained directly from either serum or plasma which can be used interchangeably for measuring BChE activity.

The method described here quantifies BChE activity in human serum/plasma using absorption spectroscopy to monitor the rate of substrate hydrolysis.[5,6] Samples are incubated with butyrylthiocholine (BTCh) and dithiobisnitrobenzoic acid (DTNB). During this incubation, active BChE will hydrolyze BTCh into butyrate and thiocholine. The thiol generated by the formation of thiocholine rapidly reacts with DTNB cleaving the disulfide bond to form nitrothiobenzoate (NTB) which presents an absorption band centered at 412 nm. Monitoring the absorption at 412 nm during the incubation provides information on the rate at which NTB is generated which occurs at a 1:1 ratio with the formation of thiocholine. This method can detect and report BChE activity as low as 0.02 U/mL, which is equivalent to 0.4% activity of the reference range. This reportable limit is below the accepted 20-25% activity threshold for clinical significance.[7] BChE activity quality control (QC) plasma pools were characterized as part of method validation.

2. SAFETY PRECAUTIONS

Reagent Toxicity of Carcinogenicity

BTCh and DTNB are not known to be toxic or carcinogenic. However, universal precautions should be followed, and personal protective equipment should still be used including gloves and safety glasses when handling biological fluids.

Human serum, plasma, and blood specimens are considered biological hazards and should be handled in a biological safety cabinet to prevent exposure to bloodborne pathogens and other potential hazards. Blood and serum controls should be purchased prescreened for bloodborne pathogens.

Freshly prepared 70% isopropyl alcohol (IPA) or 10% commercial bleach in water should be used to decontaminate all work surfaces after handling specimens. To decontaminate a spill containing biological fluids, spray this mixture directly onto the spill, and clean the surface thoroughly with absorbent towels. Contaminated materials should be autoclaved prior to disposal.

Radioactive Hazards

None.

Microbiological Hazards

Follow Universal Precautions. Because of the possibility of being exposed to various microbiological hazards, appropriate measures should be taken to avoid any direct contact with biological specimens. Generally, a Hepatitis B vaccination series is recommended for health care and laboratory workers.

Workstation cleanup should involve the use of fresh 70% isopropyl alcohol (IPA) or 10% bleach. Spray this mixture on any spills, rub thoroughly. Contaminated materials should be autoclaved prior to disposal.

Mechanical Hazards

There are minimal mechanical hazards when performing this procedure using standard safety practices. Laboratory workers should read and follow the manufacturer’s information regarding safe operation of the equipment.

Protective Equipment

Standard safety precautions should be followed when performing this procedure, including the use of a lab coat, safety glasses, durable gloves, and a chemical fume hood and/or biological safety cabinet. Refer to the laboratory Chemical Hygiene Plan and DLS safety policies and procedures for details related to specific activities, reagents, or agents.

Training

Training and experience in the use of the BioTek microplate reader should be obtained by users of this procedure. Users should be trained appropriately by an experienced operator of the instrument, be required to read the operation manuals, and demonstrate safe laboratory techniques before performing the method.

Personal Hygiene

Follow Universal Precautions. Care should be taken when handling chemicals or any biological specimen. Routine use of gloves and proper hand washing should be practiced. Refer to the laboratory Chemical Hygiene Plan and DLS safety policies and procedures for details related to specific activities, reagents, or agents.

Disposal of Wastes

Waste materials must be disposed of in compliance with laboratory, Federal, State, and Local regulations. Solvents and reagents should always be put to waste in an appropriate container clearly marked for waste products and temporarily stored in a ventilated chemical cabinet. All disposable items that come in direct contact with specimens are to be placed in a biohazard autoclave bag that should be kept in appropriate containers until autoclaved. Wipe down all surfaces with a freshly prepared 10% bleach or 70% IPA solution when work is finished. Any other non-disposable glassware should be washed and recycled or disposed of in an appropriate manner. The empty vials which contained the standards are hazardous waste and should be discarded appropriately.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Software and Knowledge Requirements

This method has been validated using a BioTek Synergy Neo2 microplate reader that includes Gen5 data analysis software and reagent injectors. After processing, the data is exported to a DLS laboratory information management system (STARLIMS). Statistical analysis of data is performed using Statistical Analysis System (SAS) software. Knowledge of and experience with these software packages (or their equivalent) are required to utilize and maintain the data management structure.

Specimen Information

Samples are aliquoted and barcoded by the Incident Response Laboratory (IRL) located on the 4th floor of building 110 of CDC's Chamblee Campus. The IRL enters sample information into STARLIMS and provides each sample with a barcoded sample ID (also referred to as a sample name). Physical samples are tracked by the IRL using standardized chain of custody forms.

Information pertaining to specimens is entered into the instrument manually or using a barcode scanner. Data that are manually entered include the sample identification number and standard number. After analysis is complete, the data is processed and then transferred to STARLIMS.

Data Maintenance

All sample and analytical data are checked after being entered into the instrument database for transcription errors and overall validity. Both the instrument and data storage directories should be routinely backed up onto a suitable network attached storage device or other storage medium. Either the supervisor or the local area network manager should be contacted for emergency assistance.

Information Security

Information security is managed at multiple levels. The information management systems that contain the final reportable results are restricted through user ID and password security access. The computers and instrument systems that contain the raw and processed data files require specific knowledge of software manipulation techniques and physical location. Site security is provided at multiple levels through restricted access to the individual laboratories, buildings, and site.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

Specimen Instructions

No special instructions such as fasting, special diets, etc. are required.

Specimen Collection

Plasma or serum specimens are collected from subjects using standard collection methods. In the case of suspected OPNA exposure, plasma/serum should be collected as soon as possible after the incident, preferably within 48 hours. Plasma/serum specimens should be frozen upright as soon as possible and stored on dry ice for shipping. Freezing is needed for accurate activity assessment to avoid reactivation by spontaneous hydrolysis and reduces the potential for specimen leakage. All plasma and serum specimens should be stored frozen until analysis. When separating plasma or serum from whole blood is not possible prior to shipping, whole blood should be shipped refrigerated at 4 ºC, and plasma should be separated from blood cells immediately upon receipt. Analyte degradation may occur when shipping whole blood, even with refrigeration. Special care must be taken in sample packaging to protect the specimen collection vessels from breakage during shipment.

Specimen Handling

Serum and plasma specimens are thawed and aliquoted; the residual specimens are then stored again at -20 ºC or lower until needed. Whole blood specimens should be centrifuged at 3,000 x g at 20 ºC to collect plasma. If the blood will not separate, then a 2-fold dilution of the sample should also be analyzed to reduce the possibility of excess hemoglobin in the sample preventing accurate analysis. Heat treatment to >50ºC for sterilization is not recommended and will result in denaturation of the enzyme and loss of activity.

Specimen Quantity

A minimum of 10 µL of plasma/serum or 50 µL blood is needed for analysis.

Unacceptable Specimens

The criterion for unacceptable specimens includes a low volume (< 10 µL specimen). In addition, maintenance of temperature during shipment should be verified by examining the shipment for the presence of unmelted ice in the frozen ice packs. Internal temperature of the shipping materials should also be measured directly.

A description of reasons for each rejected sample should be recorded on the sample transfer sheet such as low sample volume (< 10 µL specimen), leaking or damaged container, prolonged storage as whole blood (> 21 days as whole blood), or warm specimens (> 20 ºC for 14 days). Special exceptions to the sample rejection criteria can be made with approval by the division toxicologist.

Sample IDs should also be verified with the chain of custody information prior to accepting the release of samples from the IRL to the analyst. If any discrepancies in sample information are found they should be notified to the IRL staff for correction.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

Reagents and Sources

Reagents and sources used during the development and validation of this method are listed in Table 1. Reagents for use with this method should be equivalent to those listed if they are obtained from other sources.

Table1. Reagents and Sources

Reagent	Source*
Phosphate Buffer (PB)*	EQM Research, Inc. (Cincinnati, OH)
Dithiobis Nitrobenzoic Acid (DTNB)*	EQM Research, Inc. (Cincinnati, OH)
Butyrylthiocholine (BTCh)*	EQM Research, Inc. (Cincinnati, OH)
Non-Fat Dry Milk (NFD)*	LabScientific, Inc (Livingston, NJ)
Protexia (BChE Enzyme)*	PharmAthene (Anapolis, MD)
Normal Human Plasma*	Tennessee Blood Services (Memphis, TN)
Normal Human Serum*	Tennessee Blood Services (Memphis, TN)
Type I Ultra-Pure Water*	Generated in-house using an Aqua Solutions Type I RO+DI System

* Or equivalent

Reagent Preparation

Reagents

a. Phosphate Buffer (PB), 690 mM (PN: 701), diluted 10-fold with deionized water for a final concentration of 69 mM, pH 7.4.

b. One vial Dithiobis Nitrobenzoic Acid (DTNB), in dry powder form (PN: 703), mixed with 11 mL deionized water for a final concentration of 3.0 mM.

c. One vial Butyrylthiocholine (BTCh), in dry powder form (PN: 705), mixed with 6 mL deionized water for a final concentration of 20.0 mM.

Blocking Solution

Weigh out 1.0 g of NonFat Dry Milk and resuspend in 25.0 mL of deionized water to create a 4% NFD milk solution. Split the volume equally (12.5 mL each) into 2 Amicon Ultra-15 100k centrifugal filters (PN: UFC910024) and centrifuge at 4,000 RPMs for 60 minutes in a swinging bucket rotor. Combine the filtrate from both tubes into a single container.

Function Check

A calibrated test plate, which is re-certified by the manufacturer on a yearly basis, serves as the function check for this method. The test plate consists of multiple positions that contain filters which absorb at specific wavelengths and optical densities. Analysis of the test plate provides verification of the instrument's positional accuracy as well as its ability to accurately detect absorbance at specified wavelengths.

Standards Preparation and Sources

For long-term storage, these materials should be stored at -20 °C or lower. However, avoid excessive (n= > 4) freeze thaw cycles as repeated cycles will reduce enzyme

activity. All quality control materials should be stored at -70 °C.

Calibration Standards

The calibration standards are diluted from a stock solution of Protexia (rBChE enzyme). A certificate of analysis from Battelle Memorial Institute reported an enzymatic activity of 16,1040 U/mL at 25 °C of the stock solution. The calibration standards (described in Table 2) are diluted in blocking solution (filtered 4% NFD milk) and analyzed for activity to establish a calibration curve for quantification (described in Section 7).

Table 2. Analytical Standards Concentration

Standard Identifiers	BChE Activity (U/mL)
BChE#-Std 1	9
BChE#-Std 2	7
BChE#-Std 3	5
BChE#-Std 4	2.5
BChE#-Std 5	1.2
BChE#-Std 6	0.5
BChE#-Std 7	0.1
BChE#-Std 8	0.05; LRL

Quality Control Materials

a. QC High: Unadducted plasma or serum.

b. QC Mid: A 1:2 dilution of QCH in matrix blank.

c. QC Low: A 1:20 dilution of QCH in matrix blank.

d. Matrix Blank: The matrix blank QC is prepared using thermal inactivation. Plasma or serum matrix blank is prepared by heating unadducted plasma or serum, in 15-mL falcon tubes, to 60 °C for 15 minutes in a water bath to facilitate flocculation of fibrinogen. Following the initial incubation, the plasma or serum is transferred to 1.5 mL microcentrifuge tubes then centrifuged for 10 minutes at 10,000 xg. The plasma or serum (supernatant) is transferred to a new 15-mL falcon tube, being careful not to disturb the pellet. The plasma or serum is heated to 68 °C for 5 minutes in a water bath to inactivate BChE. Note: excessive heating of plasma or serum, beyond the conditions outlined above, may result in irreversible solidification of the plasma. Additionally, the matrix needs to be at the level or below the level of the water in the water bath. Any matrix that is not in contact with the water bath will not heat properly.

Other Materials and Supplies

Materials, supplies, and resources used during the development and validation of this method are listed below. Any equivalent product can be used in place of those listed below:

a. Pipettors with Adjustable Volumes: 2-20 µL, 20-200 µL, and 100-1000 µL (Rainin, Oakland, CA or equivalent).

b. Multichannel Pipettors with Adjustable Volumes: 20-200 µL and 100-1000 µL (Eppendorf Co., Westbury, NY or equivalent).

c. Easypet Serological Pipette Dispenser (Eppendorf Co., Westbury, NY or equivalent)

d. Fisher Brand Mini Vortexer (Fisher Scientific, Pittsburgh, PA or equivalent).

e. Falcon Polypropylene Conical Tubes, 15 mL and 50 mL (Fisher Scientific, Fair Lawn, NJ or equivalent).

f. MixMate Plate Mixer (Eppendorf, Hamburg, Germany, or equivalent).

g. Centrifuge 5810 R with Microplate Bucket Rotor (Eppendorf, Hamburg, Germany, or equivalent).

h. Protein LoBind 96 Well Deepwell Plates (Eppendorf, Hamburg, Germany, or equivalent).

i. Fisher Brand 96-Well Clear Flat-bottom Plates (Fisher Scientific, Pittsburgh, PA or equivalent).

j. Bio-Tek Synergy Neo2 Microplate Reader (BioTek, Winooski, VT or equivalent).

Instrumentation

The BChE Activity assay is performed on a Bio-Tek Synergy Neo2 Microplate reader (Winooski, VT) which is equipped with reagent injectors. A microplate reader with appropriate sensitivity and analysis capability can be used for this method following instrument comparison or materials characterization.

Configuration

The microplate reader procedure is described in Table 3.

Table 3. Microplate Reader Procedure

Parameter	Neo2 Setting
Temperature: Incubator	25+ 0.5 °C
Temperature: Preheat	TRUE
Shake: Intensity	567 cpm (Linear)
Shake: Duration	10 minutes
Dispense: Dispenser	2
Dispense: Prime: Priming	Before dispense step
Dispense: Prime: Volume	20 µL
Dispense: Dispense: Volume	20 µL
Dispense: Dispense: Rate	225 µL/sec
Kinetic: Runt Time	5 minutes
Kinetic: Interval	1724 seconds
Kinetic: Shake: Intensity	Medium
Kinetic: Shake: Duration	10 seconds
Kinetic: Read: Wavelength	412nm
Kinetic: Read: Read Speed	Sweep
Kinetic: Read: Pathlength Corr.	TRUE

Microplate Reader Instrument Control Program

Create a suitable instrument control program for the instrument based on the parameters described above. For the current method development and validation, a method titled “BChE Activity - BChE4 Materials.prt” was created using the Gen5 software to control the microplate reader.

Instrument Data Processing Program

Create a suitable data processing program to define the criteria for evaluation and processing of the raw data. Refer to the software manuals for the details and requirements of creating a valid processing method.

The “BChE Activity - BChE4 Materials” protocol includes data reduction steps to process raw data to standard units (U/mL) of activity. A unit is the amount of enzyme that will catalyze the transformation of 1 µmole of substrate per minute. Data reduction is performed in the following steps: Pathlength Correction > Background Subtraction > Mean Velocity (OD/min) > BChE Activity.

BChE Activity = (Dilution Factor * V_{mean}) / ε

BChE Activity = Units / mL

Dilution Factor = 100

V_{mean} = Mean Velocity = ΔOD / min

ε = DTNB Molar Extinction Coefficient[5] at 412 nm = 13.6 M^{-1} cm^{-1}

Comparison of Method Performed in Multiple Instruments

An instrument comparison was completed according to DLS Policies and Procedures Manual (version 7.2, section 10.2) and results are shown in Table 4. As stated in the manual, *"At least once every six months, a set of at least five samples spanning the reportable range of the analyte(s) of interest are run on both instruments…The Pearson correlation coefficient of the compared results should be >0.95, if not, appropriate remedial action should be taken."*

Table 4. Instrument Comparison Results

	Synergy Neo2 Belle vs Synergy Neo2 Mrs Potts	
	Slope	R^2
Quality Controls	y = 1.0057x	1
Standards	y = 0.9997x	1
Random Samples (n=15)	y= 0.9845x	0.9996

Regarding this method, 15 pre-characterized random samples were tested on two instruments (Table 5). The values for the samples, quality controls, and calibrators were compared.

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Table 5. Instruments Available for the Test Method

Instrument Name	Instrument Component	Serial Number
Belle	Synergy Neo2	1807276
Mrs Potts	Synergy Neo2	2009174

The ongoing instrument comparison results will use pre-characterized materials and will be maintained in the quality control notebook for the method.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

Calibration Verification

A calibration curve is run with each analytical batch. The calibration curve is constructed from the 8 calibration standards (Table 2). Additional verification is conducted by quantifying previously characterized QC samples against the calibration curve and statistically comparing the calculated results to known values.

Calibration Curve

Data Collection

A calibration curve is constructed using an 8-point curve of calculated concentration versus the known concentration.

Calculation of Curve Statistics

The slope and y-intercept of the calibration curve are determined by linear least squares regression (with 1/y weighting), using the Gen5 software (or equivalent).

Evaluation of Curve Statistics

The R^2 value for the curve must be greater than 0.98. Linearity of the standard curve should extend over the entire standard range with 1/y weighting. The y-intercept should not be significantly different from 0; if it is, the source of the bias should be identified. Possible sources include contamination or improper storage of any of the standard solutions.

Use of Calibration Curve

The lowest point on the calibration curve is the lowest reportable limit (LRL). The highest point on the calibration curve is the highest reportable limit and is above the expected range of results.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

Sample Receiving

Samples are aliquoted and barcoded by the IRL as described in *Section 3, Specimen Information*.

Upon receipt of specimens that do not meet acceptance criteria, the IRL notifies the Laboratory Chief (LC) and Branch Chief (BC) of sample receipt conditions. The LC and BC in consultation with the DLS Medical Officer/Clinical Consultant decide to reject or accept patient samples for testing. Acceptance or rejection of patient samples is notified via e-mail to the requester.

Sample Preparation

A. Calibration curve, QC materials and samples

1) Remove standards and QCs from the freezer and allow to equilibrate to room temperature.

2) In a new, protein LoBind, deep well plate labeled as ‘sample preparation plate’, add 40 µL of filtered 4% nonfat dried milk (blocking

solution) to each well of the first 2 columns (A1-H2). If additional samples will be analyzed (up to 84 unknowns per sample preparation plate), add 40 µL of blocking solution to 1 additional well per sample.

3) Centrifuge the sample preparation plate for 1 minute at 300 xg to accumulate blocking solution to the bottom of the well. Use an empty plate as a counterbalance.

4) Place the sample preparation plate on a MixMate shaker and mix for 5 minutes at 1,200 rpm to thoroughly coat the bottom of the wells.

5) Very briefly vortex all the standards, QCs, and samples to be analyzed.

6) Add 10 µL of each standard, QC, or sample to the corresponding well (shown below) already containing 40 µL of blocking solution. When adding standards, QCs, and samples avoid pipetting down the side of the well. Instead, pipette directly into the blocking buffer to avoid loss of BChE to the sides of the wells. When adding the standards, QCs, and samples pipette up and down 3 to 5 times to ensure complete expulsion of the sample.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	MB										
B	Std 2	QCH										
C	Std 3	QCM				**Additional**						
D	Std 4	QCL				**Samples**						
E	Std 5											
F	Std 6											
G	Std 7											
H	Std 8											

Figure 1. Sample Preparation Plate - Used to dilute standards, QCs, and samples in blocking buffer (Protein LoBind, Deep Well Plate). MB = matrix blank.

7) Place the sample preparation plate on a MixMate shaker and mix for 5 minutes at 1,200 rpms to thoroughly mix the samples and blocking solution.

8) While the sample is being mixed, prepare the analysis plate.

   a. In a new, optically clear, microtiter plate, add 150 µL of 69 mM Phosphate Buffer (pH 7.4) to one column for each column of the

deep well plate that contains at least one sample (example: if two columns of the deep well plate contain samples, the first two columns of the analysis plate should have phosphate buffer added).

b. Add 20 µL DTNB Reagent to each well containing Phosphate Buffer.

9) Once the sample preparation plate has finished shaking, use a multichannel pipette to add 10 µL of sample solution from column 1 of the sample preparation plate to column 1 of the analysis plate. Repeat the process, adding 10 µL of sample solution from column two of the sample preparation plate to column two of the analysis plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1	MB										
B	Std 2	QCH										
C	Std 3	QCM				**Additional**						
D	Std 4	QCL				**Samples**						
E	Std 5											
F	Std 6											
G	Std 7											
H	Std 8											

Figure 2. Analysis Plate – Used to monitor the optical change of each standard, QC, and sample to determine activity (Optically Clear, Microtiter Plate). MB = matrix blank.

Sample Analysis

A. Preliminary System Set-Up and Performance Check

While the sample preparation plate is being blocked, set up the microplate reader:

1) On the desktop of the instrument computer, double click the "***Gen5 3.10***" icon to launch the Gen5 software.

2) When the software loads the Task Manager window will automatically open. In the Task Manager window select "***Experiments***" in the left windowpane and then click on "***Create using an existing protocol…***" in the right windowpane. Select "***BChE Activity - BChE4 Materials***" as the

protocol. Note: the full file path for the protocol is "C:\\Users\Public\Public Documents\ Protocols\BChE Activity-BChE4 Materials.prt"

3) A system test must be completed prior to analyzing the plate. This can be done by selecting "***System" → "Diagnostics" → "Run System Test"***. It will have you choose the instrument that you would like to test. You will need to enter in your user ID when prompted.

4) A test plate must be run prior to analyzing the plate, as this acts as the function check for the run. This can be done by selecting *"**System" → "Diagnostics" → "Test Plates" → "Run".*** It will have you choose the instrument and test plate that you will be running that you would like to test. Select all of the wavelengths in the box by simply clicking each value. You will need to enter in your user ID when prompted.

5) Remove the faceplate of the dispenser and place the tip of syringe 2 in a tube containing an appropriate amount of BTCh reagent. Replace the dispenser faceplate. Note: approximately 4 mL of reagent is required for a full plate (84 samples).

6) Click on "***System***" in the top menu and select "***Instrument Control***" then select which instrument you will be using. *Note: The instrument nicknames **"Belle"** and **"Mrs. Potts"** are indicated on the list.*

7) On the Reader Control window, select the "***Dispenser***" tab.

8) Choose dispenser ***2*** from the drop-down menu, enter ***1150*** µL as the volume, ***225*** µL/sec as the rate, and click the "***Prime***" button.

9) When prompted, place the priming plate on the instrument tray carrier and click the "***Okay***" button.

10) Once priming is complete and the priming plate is ejected, examine the plate to verify that reagent ~60 µL was dispensed onto the plate.

11) On the Reader Control window, select the "***Pre-Heating***" tab.

12) Verify that the instrument is preheating to 25°C by ensuring the "Requested" temperature displays 25.0 and the "On" checkbox is checked.

13) Close the Reader Control window.

B. Final Set-Up and Operation

1) On the instrument computer, click the "***Procedure***" button located at the top of the screen.

2) Double click the "***Dispense 20 µL using dispenser 2***" step and then click the button in top right of the submenu indicating which wells will receive

reagent. Select all wells that include samples then click the "**OK**" button. Click "***OK***" to close the dispense submenu.

3) Double click the "***Read: (A)412***" step and then click the button in top right of the submenu indicating which wells will be read. Select all wells that include samples then click the "**OK**" button. Click "***OK***" to close the read submenu.

4) Click "***OK***" to close the Procedure menu.

5) Verify the instrument has preheated to 25.0°C (±0.1°C). The current temperature of the instrument is displayed in the top middle of the screen.

6) Click the "***Read Now***" button at the top middle of the screen to begin the run. Make a note of any prompts that are listed then click "***OK***".

7) Verify that the priming trough is empty and has been properly oriented on the instrument tray carrier and then click "***Yes***".

8) When prompted, place the analysis plate on the instrument tray carrier with well A1 oriented at the back right corner of the tray carrier and click "***OK***".

9) The instrument will incubate the sample for 10 minutes before adding BTCh reagent to the selected wells and then monitoring the reaction for 5 minutes. Once the run is complete, the plate will be ejected from the instrument.

C. System Closeout

1) Remove the plate from the instrument and replace it with the priming plate.

2) Click on "***System***" in the top menu and select "***Instrument Control***" then select which instrument you will be using. *Note: The instrument nicknames **"Belle"** and **"Mrs. Potts"** are indicated on the list*

3) On the Reader Control window, select the "***Dispenser***" tab.

4) Choose dispenser ***2*** from the drop-down menu, enter ***2000*** µL as the volume, ***225*** µL/sec as the rate, and click the "***Purge***" button.

5) Once the purging has finished, remove the faceplate of the dispenser and move the tip of syringe 2 from the BTCh reagent to the water wash tube. The BTCh reagent can be stored at RT for future use.

6) Enter ***5000*** µL as the volume, ***225*** µL/sec as the rate, and click the "***Prime***" button.

7) Once priming has finished, remove of the tip of syringe 2 from the water. Replace the dispenser faceplate. (Note: the tip of syringe 2 should now be open to the air which is how the instrument will be stored)
8) Enter ***2000*** µL as the volume, ***225*** µL/sec as the rate, and click the "***Prime***" button.
9) When priming has finished, remove and empty the priming plate.
10) Empty and then replace the priming trough.
11) Close the Reader Control window.
12) Click the "***Export***" button at the top of the screen and choose the "***Power Export (Excel)***" option.
13) Click the "***OK***" button and when prompted chose a directory to save the excel file.
14) The generated Excel file should be annotated with the appropriate sample names and can then be uploaded into STARLIMS.

Processing of Data

All raw data files are quantified using the Gen5 (or equivalent) software. The data processing results are saved in an experiment file which allows for the creation of an Excel file for upload into STARLIMS. The experiment file shows the calibration curves used as well as their respective correlation coefficient (R) values.

1) In the ***Statistics*** window, ensure STARLIMS Export is displaying data.
2) Right click and select Quick Export.
3) The generated Excel file should be annotated with the appropriate sample names and uploaded into STARLIMS.
4) Export the data to an electronic database or print a hard copy of the long report. If possible, print all data on one page, in landscape format.

Analyzing and Storing the Plate Reader Data

Before and after analysis, save experiment in the designated folder on the instrument computer. Use ERB naming guidelines when saving experiment.

Calibration Standards Processing

Verify that the coefficient of determination (R^2) value of the calibration curve is greater than 0.98. Examine any results which do not qualify to determine underlying causes including undeleted outliers and a nonlinear curve.

Quality Control Samples Processing

Each analytical batch includes four QCs (QCH: unadducted plasma; QCM: a mixture of normal plasma with heat inactivated plasma; QCL: heat inactivated plasma with a small percentage of normal plasma; and Matrix Blank: heat inactivated plasma). Acceptability of results for the entire analytical batch is dependent upon the agreement of the results from these control materials within ranges established during method validation.

For control materials processing, examine each QC result and compare to the statistical data from the characterization runs (described in *Section 10, Table 7*). The following criteria are the minimum requirements for evaluation of the QC results: A QC result is considered “out of control” if the concentration is outside of the three times the standard deviation (3σ) confidence interval. A QC result is considered “out of control” if it is the second of two consecutive standardized run results that are both outside the same side of the 2σ confidence interval. A QC result is considered “out of control” if it is ≥ the 10th value on the same side of the characterized mean for that QC level and it is outside of the 2σ confidence interval. This is known as the 10 X-bar rule. A QC result is considered “out of control” if it is the second of two consecutive standardized run results differ by more than 4σ.

9. REPORTABLE RANGE OF RESULTS

Linearity limits

The reportable range of results for BChE activity in this method is given in Table 6. The lowest reportable limit (LRL) is the concentration of the lowest calibrator. The highest reportable limit is the highest linear calibrator.

Table 6. Calibration Curve Characteristics for BChE Activity (n = 26 Calibration Curves)

Analyte	Lowest Reportable Limit (U/mL)	Coefficient of Determination (R^2)	Mean for Lowest Calibrator ± 1σ (U/mL)
BChE Activity	0.05	>0.98	0.0451±0.0037
Analyte	**Highest Reportable Limit (U/mL)**	**Coefficient of Determination (R^2)**	**Mean for Highest Calibrator ± 1σ (U/mL)**
BChE Activity	9.0	>0.98	8.8280±0.2199

10. QUALITY CONTROL (QC) PROCEDURE

Quality Assessment

Quality assessment procedures follow standard practices. Daily experimental checks are made on the stability of the analytical system, blanks and standards before each analytical run. One quality control set will be included on every plate (84 patient samples) and must fall within three standard deviations of the mean shown in Table 7 to ensure accurate measurements. All data entry errors are evaluated by the supervisor and corrected only after consultation with the analyst and positive identification of the correct information.

Establishing QC Limits

Quality control limits were established for each QC pool through validation of 26 analytical batches each containing a matrix blank and three quality control samples. Statistical information on the mean, standard deviation, coefficient of variation, and confidence limits are calculated for the validation data set. Individual quality control charts for the characterization runs are created and examined, and quality control limits are used to insure analytical stability.

The following criteria are the minimum requirements for evaluation of the QC validation results. An analytical run is considered “out-of-control” if:

1) A QC result is considered “out of control” if the concentration is outside of the three times the standard deviation (3σ) confidence interval.

2) A QC result is considered “out of control” if it is the second of two consecutive standardized run results that are both outside the same side of the 2σ confidence interval.

3) A QC result is considered “out of control” if it is ≥ the 10th value on the same side of the characterized mean for that QC level and it is outside of the 2σ confidence interval. This is known as the 10 X-bar rule.

4) A QC result is considered “out of control” if it is the second of two consecutive standardized run results differ by more than 4σ.

Table 7. Mean QC Concentration and Confidence Limits (in U/mL).

QC Pool	Analyte	Mean - 3σ	Mean - 2σ	Mean	Mean + 2σ	Mean + 3σ
BChE4-QCH	BChE Activity	2.5603	2.7102	3.009	3.3095	3.4594
BChE4-QCM	BChE Activity	1.3001	1.3814	1.5438	1.7062	1.7875
BChE4-QCL	BChE Activity	0.1264	0.1348	0.1515	0.1683	0.1767

If the QC result is “out-of-control,” the cause of the failure must be determined and corrected. No results from the associated run may be reported. Additional QC criteria may be added if consistent with laboratory policy.

The statistical data calculated for the validation data set are used as control parameters for subsequent runs of unknown samples.

Proficiency Testing

The Proficiency Testing (PT) scheme for this method is administered by the Emergency Response Branch. This internal PT consists of a random number of pre-characterized samples (5-10 samples) prepared by the supervisor.

The internal PT samples are analyzed three times per calendar year and are reported withing two weeks of receipt. Samples are analyzed in accordance with the documented method. Analytical results are reviewed by a Quality Control (QC) Officer. These results are then submitted by the analyst to the PT Coordinator for grading.

A successful PT challenge requires a grade of 80% or above per analyte in order to pass the challenge. It is required that two out of the three challenges are passing. The PT report is provided within 45 days of the end of the PT Challenge. The PT report is signed by the Branch Chief and Analyst. A corrective action report is required for any PT failures (<80% of the Z-scores per analyte are acceptable).

The report is kept in the QC notebook.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

Analyte Activity in Standards or QC Materials

If an inordinately large amount of analyte activity is measured in one of the calibration standards or QC materials, but this is not seen in the remainder of the samples, this indicates a contamination of this particular sample. The source of this incident should be investigated to prevent repeat occurrences, but no further action is required.

Analyte Activity in all Samples

If an inordinately large amount of BChE activity is present in all measurements for a particular day, it is likely that one or more of the reagent solutions are contaminated. If necessary, prepare new solutions.

QC Sample Outside of Acceptable Criteria

If the activity of one or more of the control materials falls outside the 3σ confidence limits, one of the above causes is likely. Follow the steps outlined above to isolate and correct the problem. No analytical results are reported for runs not in statistical control. Before beginning another analytical run, several QC materials (in the case of QC failure) or calibration verification samples (in the case of calibration failure) should be analyzed. Once calibration and/or quality control have been reestablished, analytical runs may be resumed.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Care is required to prevent contamination of QC materials, standards, and samples.

Possible Interferences

Carefully monitor the kinetic curves for unknown interferences. DTNB is sensitive to free thiols which result in an increased background signal if present. Additionally, absorbance at 412 nm is sensitive to hemoglobin, which in excess, could obscure the signal generated by the reaction.

Limitations of the Method

This activity assay measures the BChE activity of a sample and not necessarily inhibition of BChE activity. Low activity results could be obtained due to a low amount of BChE present or an isoform of BChE with reduced activity. Additionally, BChE could become inactivated due sample handling or processing.

13. REFERENCE RANGES (NORMAL VALUES)

BChE activity at 37°C was measured for 192 matched-set serum and plasma samples and 96 whole blood samples (Tennessee Blood Services, Memphis, TN). These were anonymous samples donated by people who were presumably unexposed to OPNAs. The average BChE activities were as follows: serum, 4.491±0.791 U/mL; plasma, 4.447±0.810 U/mL; whole blood, 4.378±0.677 U/mL

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Activity less than 3σ below the mean of normal values should be assumed to be consistent with inactivated BChE and reviewed in the context of available epidemiological information by the Division Toxicologist.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

BChE activity is known to be stable for up to 3 years in serum/plasma stored at -70 ^{o}C. Specimens may reach and maintain ambient temperature while preparing the samples for analysis. If the measurement is delayed until the next day, samples should be frozen at -70 °C. Heating samples above room temperature is not recommended and may result in loss of activity or spontaneous adduct hydrolysis.

Stability

The stability of BChE was completed by using stock solutions prepared by the University of Nebraska Medical Center (UNMC). The stability of BChE in matrix was assessed using freeze-thaw cycles, storage at room temperature (bench-top), long-term storage at -70 °C, and storing processed samples at room temperature. Dilution of UNMC stock solutions at two concentration levels within the reportable range were prepared in matrix. Initial measurements in matrix for the two concentration levels were 3.5 and 0.35 U/mL. Stability results in matrix are shown in Appendix A, Table A3. All measurements were done in triplicate and all individual assessment results were within $\pm$15% of the nominal concentration except for processed samples where results were outside ± 15% (see *Appendix A, Method Performance*).

Freeze-thaw Cycle Stability

The freeze-thaw cycle stability for BChE was determined by assessing a minimum of four freeze-thaw cycles. The solutions were frozen four times at -70 °C and then thawed at room temperature.

Bench-top Stability

The length of times used for the short-term stability of BChE was determined by the typical time needed to handle study samples. The samples were stored at room temperature for one day. After this time, samples were prepared following the method's sample preparation protocol and analyzed by the spectrophotometer.

Processed Sample Stability

The short-term stability of BChE was determined from the processed samples (ready for instrument analysis). The processed samples were stored in the hood at room temperature for 24 hours. After this time, the samples were analyzed, and results were compared to initial measurement values (t_0).

Long-term Stability

The length of time to determine long-term stability of BChE in matrix was based on the time between date of first sample collection and date of last sample

analysis. The initial assessment for long-term stability of the samples was completed by storing samples at -70 °C for one month.

16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

Two test systems (Table 5)) were validated for this test and are available for sample analysis. If a test system fails, the other test system can be used while the failed test system is repaired and returned to a service status. A comparison of these two test systems was conducted and the process is described in Section 6.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Results are generally reported to 3 significant digits. In addition to activity measurements of unknowns, results of quality control activity should accompany all results.

Within the method development and analysis group at the CDC, the run data processed within instrument control software are exported to an Excel file. The data from the Excel file is then transferred to a database called DLS STARLIMS. The purpose of DLS STARLIMS is to track the life cycle of the specimen and its analytical results through sample acquisition, analysis, quality control/assurance, and reporting and to archive analytical results for later retrieval. DLS STARLIMS is DLS's web-based laboratory information management system.

Upon completion of laboratory testing, results are to be reviewed by all levels of supervision up to and including the Branch Chief. The Laboratory Chief is responsible for review of the quality control results to assure the measurement process was in-control for each of the patient analyses. Quality control results must be approved by the Laboratory Chief and one of the DLS statisticians or other specified QC officer. The DLS statisticians (or QC officer) therefore provide an additional quality assurance review.

Review and approval of the results by appropriate persons are documented in DLS STARLIMS. Review by the Laboratory Chief is to include an evaluation of the consistency of patient results with information which may be available on the patient (e.g., age, sex).

Results along with the record of results approval is kept for at least two years in DLS STARLIMS. All laboratory reports must be signed by the Division Director or his designate before release. One exception is in the case of studies or surveys that span months or years and involve multiple reports of data (e.g., National Health and

Nutrition Examination Survey). In this case, each data set reported should be approved by the Laboratory Chief, a DLS statistician (or QC officer) and the Branch Chief but does not require approval by the Division Director.

Laboratory reports are to be released only to the authorized requestor. Each method includes a description of actions to be taken when results are sufficiently high or low to constitute atypical levels ("panic values"). The requestor is to be contacted immediately with such results.

In addition to the written laboratory report, the data may be transmitted electronically (i.e. e-mail, CD, or DVD) in a suitable format; for example, a spreadsheet (e.g., Excel), a SAS data set, or an ASCII file. The requestor may verify the accuracy of an electronic transmission of the data by comparing it with the hardcopy version.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

If any serum or plasma specimen remains after analysis, this material should be returned to storage at -70 °C.

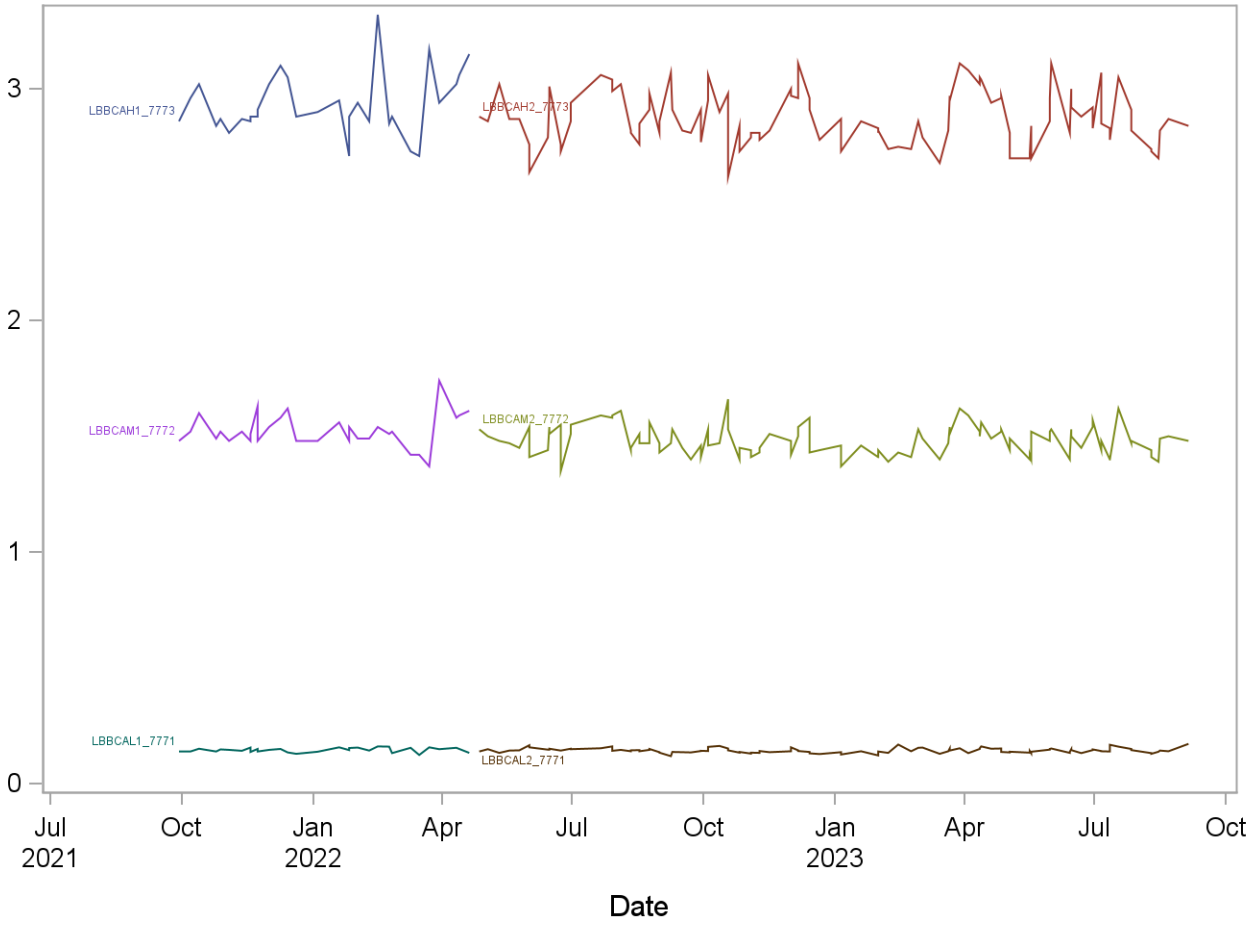
Standard record keeping (e.g., database, notebooks, data files, etc.) is used to track specimens. It is recommended that records be maintained for 2 years, including related QA/QC data, and that duplicate records be kept off-site in electronic format.

19. SUMMARY STATISTICS AND QC GRAPHS

Please see the following pages.

August 2021-August 2023 Summary Statistics and QC Chart
LBXBA (Butyrylcholinesterase Activity (U/mL))

Lot	N	Start Date	End Date	MEAN	Standard Deviation	Coefficient of Variation
LBBCAH1_7773	31	29SEP21	20APR22	2.932	0.135	4.6
LBBCAH2_7773	104	27APR22	05SEP23	2.872	0.114	4.0
LBBCAL1_7771	31	29SEP21	20APR22	0.146	0.010	6.6
LBBCAL2_7771	104	27APR22	05SEP23	0.143	0.010	7.0
LBBCAM1_7772	31	29SEP21	20APR22	1.525	0.072	4.7
LBBCAM2_7772	104	27APR22	05SEP23	1.480	0.062	4.2



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Appendices

Appendix A: Method Performance

Table A1. Accuracy using spike recovery

Accuracy using Spike Recovery

Recovery = (final concentration – initial concentration)/added concentration
Recovery should be 85-115% except at 3*LOD where can be 80-120%

Method name: BChE Activity
Method #: 2543v2
Matrix: Serum & Plasma
Units: U/mL
Analyte: **BChE Activity**

		Sample 1 - Plasma					Sample 2 - Serum					Mean recovery (%)	SD (%)
	Replicate	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)	Spike concentration	Measured concentration Day 1	Day 2	Mean	Recovery (%)		
Sample	1	0	0.00	0.00	0.0		0	0.00	0.00	0.0		104.8	7.2
	2		0.00	0.00				0.00	0.00				
	3		0.00	0.00				0.00	0.00				
Sample + Spike 1	1	3.500	3.926	3.421	3.7	104.4	5.000	4.693	5.257	5.1	101.3		
	2		3.556	3.631				4.746	4.663				
	3		3.839	3.551				6.276	4.746				
Sample + Spike 2	1	1.750	2.145	2.152	2.0	114.7	3.500	3.553	3.555	3.5	99.0		
	2		2.007	1.813				3.454	3.189				
	3		2.079	1.848				3.681	3.349				
Sample + Spike 3	1	0.350	0.396	0.369	0.39	112.2	0.700	0.691	0.702	0.68	97.3		
	2		0.395	0.381				0.601	0.671				
	3		0.427	0.389				0.727	0.694				

Table A2. Method precision

Precision

Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name: BChE Activity
Method #: 2543v2
Matrix: Plasma
Units: U/mL
Analyte: **BChE Activity**

Plasma	QCH					
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	4.39	4.27	4.33	3.78E-03	3.78E-03	37.5
2	4.47	5.00	4.74	6.94E-02	6.94E-02	44.8
3	4.59	4.60	4.60	3.03E-05	3.02E-05	42.3
4	4.80	4.46	4.63	2.87E-02	2.87E-02	42.8
5	4.42	4.27	4.35	5.85E-03	5.85E-03	37.8
6	4.75	4.75	4.75	2.25E-06	2.25E-06	45.1
7	4.69	4.33	4.51	3.15E-02	3.15E-02	40.7
8	4.59	4.34	4.46	1.59E-02	1.59E-02	39.8
9	4.37	4.32	4.35	5.29E-04	5.29E-04	37.8
10	4.53	4.19	4.36	2.84E-02	2.84E-02	38.0
Grand sum	90.122	**Grand mean**	4.5061			

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev (%)**
Within Run	0.368266	0.0368266	0.191902579	4.26
Between Run	0.4762058	0.052911756	0.08968042	1.99
Total	0.8444718		0.211823459	**4.70**

Plasma	QCL					
Run	**Result 1**	**Result 2**	**Mean**	**SS 1**	**SS 2**	**2*mean^2**
1	0.266	0.250	0.26	6.40E-05	6.40E-05	0.133
2	0.227	0.263	0.25	3.24E-04	3.24E-04	0.120
3	0.252	0.238	0.25	4.90E-05	4.90E-05	0.120
4	0.225	0.227	0.23	1.00E-06	1.00E-06	0.102
5	0.251	0.234	0.24	7.23E-05	7.22E-05	0.118
6	0.254	0.224	0.24	2.25E-04	2.25E-04	0.114
7	0.275	0.207	0.24	1.16E-03	1.16E-03	0.116
8	0.262	0.244	0.25	8.10E-05	8.10E-05	0.128
9	0.255	0.236	0.25	9.03E-05	9.03E-05	0.121
10	0.247	0.266	0.26	9.03E-05	9.03E-05	0.132
Grand sum	4.903	**Grand mean**	0.24515			

	Sum squares	**Mean Sq Error**	**Std Dev**	**Rel Std Dev**
Within Run	0.0043	0.0004	0.0207	8.46
Between Run	0.0016	0.0002	0.0000	0.00
Total	0.0059		0.0207	**8.46**

Table A3. Stability of BChE Activity

Stability

Freeze and thaw stability
Description: four times frozen at -80°C and then thawed (4 freeze-thaw cycles)
Bench-top stability
Description: original samples (not prepared for instrument analysis) stored at room temperature for 1 day
Processed sample stability
Description: processed samples (ready for instrument analysis) stored at room temperature for 24 hours
Long-term stability
Description: samples stored at -80°C for 30 days

All stability sample results should be within ±15% of nominal concentration

Method name: BChE Activity
Method #: 2543v2
Matrix: Plasma
Units: U/mL
Analyte: **BChE Activity**

Quality material 1	**Initial measurement**	**Four freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-term stability**
Replicate 1	3.926	3.432	3.814	4.308	3.715
Replicate 2	3.556	3.395	3.236	4.529	3.603
Replicate 3	3.839	3.353	3.429	5.147	3.500
Mean	3.774	3.393	3.493	4.661	3.606
% difference from initial measurement	--	**-10.1**	**-7.4**	**23.5**	**-4.4**

Quality material 2	**Initial measurement**	**Four freeze-thaw cycles**	**Bench-top stability**	**Processed sample stability**	**Long-term stability**
Replicate 1	0.396	0.376	0.379	0.684	0.410
Replicate 2	0.395	0.395	0.418	0.635	0.372
Replicate 3	0.427	0.415	0.382	0.578	0.405
Mean	0.406	0.395	0.393	0.632	0.396
% difference from initial measurement	--	**-2.6**	**-3.2**	**55.7**	**-2.5**

Table A4. Method limit of detection, specificity and fit for intended use.

LOD, specificity and fit for intended use

Method name: BChE Activity
Method #: 2543v2
Matrix: Plasma
Units: U/mL

Analytes	Limit of Detection (LOD)	Interferences successfully checked in at least 50 human samples	Accuracy, precision, LOD, specificity and stability meet performance specifications for intended use
Active BChE	0.02	yes	yes

Appendix B: Method Ruggedness

Five Tested Parameters	Final Method Level	Result for analyte (U/mL)	lower level	Result for analyte at lower level	higher level	Result for analyte at higher level
Time of Shaking after Sample Addition	5min	3.93	2 min	4.27 (109%)	7 min	4.26 (108%)
Time Since DTNB has been made	0 days	4.25	--	--	12 days	4.56 (107%)
Phosphate Buffer Concentration	69 mM	4.40	0.69 mM	4.33 (98%)	690 mM	4.50 (102%)
Blocking Buffer Volume	40 µL	4.42	20 µL	>3.5	60 µL	4.79 (108%)
BTCh Reagent	20mM	4.71	13.3mM	4.69 (100%)	26.7 mM	4.59 (98%)
Five Tested Parameters	**Final Method Level**	**Result for analyte (U/mL)**	**lower level**	**Result for analyte at lower level**	**higher level**	**Result for analyte at higher level**
Time of Shaking after Sample Addition	5min	0.269	2 min	0.257 (96%)	7 min	0.242 (90%)
Time Since DTNB has been made	0 days	0.243	--	--	12 days	0.258 (106%)
Phosphate Buffer Concentration	69 mM	0.279	0.69 mM	0.237 (85%)	690 mM	0.243 (87%)
Blocking Buffer Volume	40 µL	0.257	20 µL	0.333 (130%)	60 µL	0.284 (111%)
BTCh Reagent	20mM	0.253	13.3mM	0.266 (105%)	26.7 mM	0.262 (104%)