# Laboratory Procedure Manual

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|---|---|
| *Analyte:* | Butyrylcholinesterase concentration (BChE) |
| *Matrix:* | Plasma, serum, and blood |
| *Method:* | UHPLC-MS/MS |
| *Method No.:* | 2512 |
| *Revised:* | **09/07/2022** |
| *As performed by:* | *Emergency Response Branch*<br>*Division of Laboratory Sciences*<br>*National Center for Environmental Health* |
| *Contact:* | *Elizabeth Hamelin, M.S.*<br>*Branch Chief*<br>*Phone: 770-488-7082*<br>Email: eph3@cdc.gov |

## 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 | LBXCH | Butyrylcholinesterase Con (ng/mL) | Yes |

# 1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

## Clinical Relevance

Organophosphorus nerve agents (OPNA) are a class of chemical warfare agents first synthesized just prior to World War ll. OPNAs have little or no use except as chemical weapons; therefore, they are classified as Schedule 1 chemical warfare agents in the Chemical Weapons Convention treaty administered by the Organisation for the Prohibition of Chemical Weapons [1]. Even though use of chemical weapons is prohibited, large scale sarin (GB) gas attacks have been documented in Matsumoto in 1994 [2], Tokyo in 1995 [3], and Syria in 2013 [4]. OPNAs bind to the active site of cholinesterases and diminish normal enzymatic activity. Symptoms of OPNA exposure are directly related to cholinesterase inhibition and include rapid muscle twitching, neuromuscular paralysis, and ultimately death [5]. The extreme toxicity, relative ease of synthesis, and existence of legacy stockpiles highlight the need for quantitative methods to detect OPNA exposure in clinical samples [6].

OPNAs have short lifetime in the body due to their chemical reactivity; therefore, measuring live agents in clinical samples is difficult for confirming exposure. OPNAs hydrolyze to form specific acid metabolites that can be detected in urine and blood; however, hydrolysis products are often eliminated within several days after exposure [7]. OPNAs also react with proteins to produce covalent adducts that can persist for several weeks after an exposure event. Several protein-based methods for confirming OPNA exposure have been developed, including the Ellman activity assay [8], fluoride reactivation [9], albumin adducts [10], and adducts to butyrylcholinesterase (BChE) [11] each with varying levels of specificity.

The Ellman assay monitors cholinesterase activity making it a fast but non- specific assay [8]; furthermore, spontaneous regeneration of cholinesterase activity [12] through hydrolysis of the active site adduct makes the method unreliable as a long-term detection method. Fluoride reactivation is very sensitive, but degradation through partial hydrolysis of the OP adduct, also known as aging, eliminates the possibility of refluoridation [13]. Adducts to tyrosine from albumin are reliable biomarkers that do not age; however, OPNAs specifically bind to the cholinesterase active site where the enzymatic reaction can occur 500 times faster than the nucleophilic reaction with tyrosine residues [14]. BChE is a convenient and reliable biomarker because it is easily extracted from blood, has a high reaction rate with OPNAs, and has a relatively long half-life.

The presented work reports a sensitive and accurate method for quantifying unadducted BChE in human blood. A decrease in unadducted BChE levels could suggest the presence of OP adducts. Orthogonal methods are available to run in conjunction with this method to identify the specific OPNA adduct. The intention of this method is to provide a rapid screen to evaluate potential samples with lower BChE levels which may be a result of OPNA exposure.

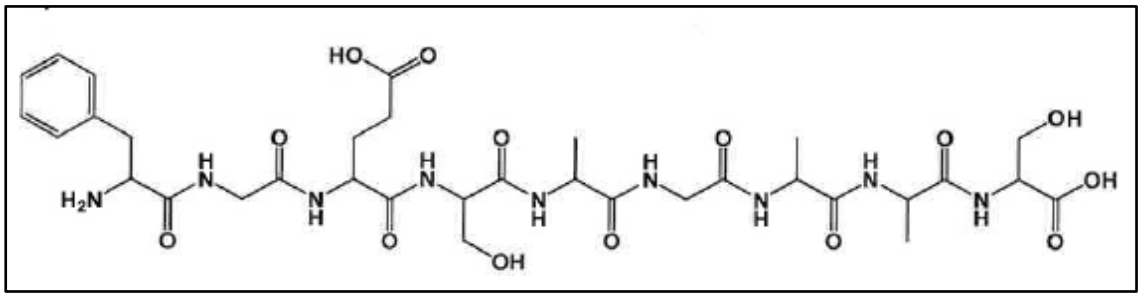



**Figure 1.** Structure of unadducted butyrylcholinesterase (BChE) nonpeptide biomarker (FGESAGAAS).

## Test Principle

OPNAs form covalent adducts with BChE, a naturally occurring plasma protein. BChE has been chosen as an analytical biomarker of OPNA exposure because it is freely circulating in the blood and is present at higher concentrations than acetylcholinesterase [15]. BChE can be obtained directly from either serum or plasma, and the two matrices can be used interchangeably for measuring OPNA adducts to BChE. The method described here utilizes immunomagnetic separation liquid chromatography- tandem mass spectrometry (LC-MS/MS) to quantify unadducted BChE [16]. Unadducted BChE quickly identifies samples with reduced levels of the endogenous protein. Samples with reduced BChE levels can be further investigated using orthogonal methods to evaluate for potential OPNA exposure since reduced BChE levels could indicate OP exposure.

Immunomagnetic beads conjugated to monoclonal BChE antibodies specifically purify unadducted and OP adducted BChE protein from serum/plasma. BChE protein bound to the bead is digested with pepsin to yield a nine amino acid biomarker peptide containing the BChE active site (Figures 1 & 2). Laboratory methods have been automated to increase throughput while maintaining a high degree of ruggedness and precision [16]. The use of automation during sample preparation decreases the errors that may be introduced when manually processing samples. Unadducted BChE has a unique chemical structure that can be chromatographically separated from adducted BChE and detected by tandem mass spectrometry. Native and isotopically labeled peptides corresponding to the pepsin digest biomarkers for unadducted BChE are used as calibrators and internal standards. Isotopically labeled internal standard and synthetic peptide calibrators are added prior to pepsin digestion. The method can detect unadducted BChE as low as 2 ng/mL.

After using immunomagnetic separation to remove unadducted and adducted BChE from clinical samples, the BChE-depleted serum/plasma can be stored for additional analyses with supervisor approval.

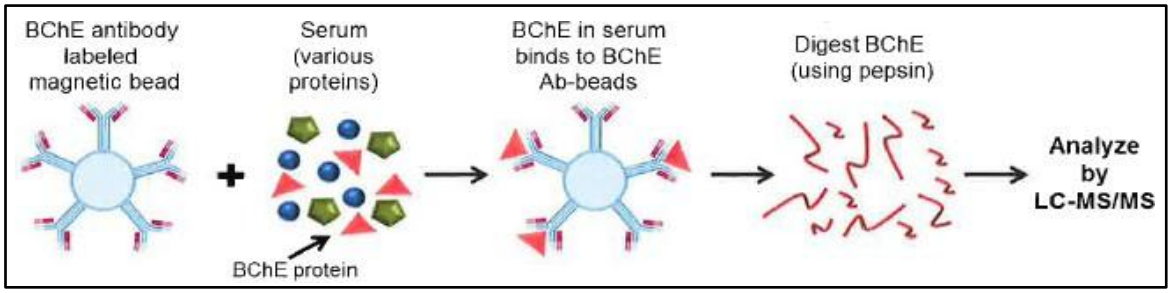



**Figure 2.** Schematic of the extraction of butyrylcholinesterase (BChE) protein from matrix using immunomagnetic separation. Upon BChE extraction, the protein is digested using pepsin, and the BChE nonapeptide is analyzed by LC-MS/MS.

## 2. SAFETY PRECAUTIONS

### Reagent Toxicity of Carcinogenicity

Universal precautions should be followed, and personal protective equipment such as gloves, lab coats, and safety glasses should always be used while handling these reagents. Safety Data Sheet documents (SDS) for these chemicals are readily accessible as hard copies in the lab.

### 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. Avoid direct contact with the mechanical and electronic components of the sample preparation robot, the liquid chromatograph, and the mass spectrometer, unless all power to the instrument is off. Generally, mechanical and electronic

maintenance and repair should be performed only by qualified technicians.

### 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 an HPLC system and a triple quadrupole mass spectrometer is necessary. Users should be trained by an experienced operator of the instrument, required to read the operation manuals, and demonstrate safe 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 the Sciex 6500+ triple quadrupole mass spectrometer that includes a database system as part of the Analyst/MultiQuant operating system. After processing, the data are

uploaded into DLS STARLIMS for data management. Statistical analysis of data is performed using Statistical Analysis System (SAS) software (SAS Institute, Cary, NC). 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.

Sample IDs are entered into the worklist via barcode scanner or manually. The barcoded sample IDs are provided on the chain of custody form. After analysis is complete, the data is processed and then transferred to DLS STARLIMS electronically.

### Data Maintenance

All methods, worklists, reports, and data (raw and processed) are backed up onto the network attached storage (NAS) server after business hours each night. All data on the NAS server is further backed up to the CDC encrypted network drive once per week. Additional backup storage solutions may include secure solid-state drive. The supervisor should be contacted for emergency assistance.

### Information Security

Information security is managed at multiple levels. Access to the instrument computer and STARLIMs are restricted and contain the final reportable results which are restricted through user ID and password security access. 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

In the case of suspected OPNA exposure, blood should be collected as soon as possible after suspected exposure due to the speed of metabolism for these agents. Blood samples are collected from subjects using standard collection methods. Separate plasma or serum from whole blood when possible prior to shipping. Plasma and serum samples should be frozen upright as soon as possible and stored on dry ice for shipping. When separating plasma or serum from whole blood is not possible prior to shipping, whole blood should be shipped at 4 ºC, and plasma should be separated from blood upon receipt.

Freezing reduces the potential for sample leakage. Special care must be taken in sample packaging to protect the sample collection vessels from breakage during shipment. All plasma and serum samples should be stored at -20 ºC or lower until analysis. Whole blood should be refrigerated at 4 ºC.

## Specimen Handling

Blood should be collected as soon as possible after suspected exposure. Care must be taken to ensure that samples are kept frozen (at -20 ºC or lower) during storage and shipment. If possible, samples should be shipped and picked up immediately so that they can be analyzed as quickly as possible.

## Specimen Quantity

A minimum of 50 µL aliquot of plasma, serum, or blood is required for this assay.

## Unacceptable Specimens

The criteria for unacceptable samples include insufficient sample volume (less than 50 µL) and suspected contamination such as a leaking or damaged sample container. Internal temperature of the shipping materials should be noted. A description of reasons for each rejected sample should be recorded on the chain of custody, initial and dated by the analyst. Special exceptions to the sample integrity rejection criteria can be made with approval by the Laboratory Director or Clinical Consultant. Sample IDs are verified with the chain of custody information by the analyst 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.

**Table 1.** Reagents and Sources.

| Reagent* | Grade | Source* |
|---|---|---|
| Acetonitrile | HPLC | MG Scientific (Pleasant Prairie, WI) |
| Water | HPLC | MG Scientific (Pleasant Prairie, WI) |
| Formic acid (~98% purity) | LCMS | Sigma-Aldrich Co. LLC. (St. Louis, MO) |
| Ultra-pure deionized water from in-house purification system | -- | Aqua Solutions (Jasper, GA) |
| Phosphate buffered saline with tween 20 (PBST; #P3563) | -- | Sigma-Aldrich Co. LLC. (St. Louis, MO) |
| Dimethyl pimelimidate dihydrochloride (DMP; #D8388) | -- | Sigma-Aldrich Co. LLC. (St. Louis, MO) |
| 10x concentrate of 0.2 M Tris buffered saline (TBS; #T5912) | -- | Sigma-Aldrich Co. LLC. (St. Louis, MO) |
| Triethanolamine Buffer Solution (#T0449) | -- | Sigma-Aldrich Co. LLC. (St. Louis, MO) |
| Pepsin from Porcine Gastric Mucosa (#P6887) | -- | Sigma-Aldrich Co. LLC. (St. Louis, MO) |
| BChE Monoclonal Antibodies from Clone 3EB (#HAH 002-001) | -- | AntibodyShop (Gentofte, Denmark) |
| Protein G Dynabeads for immunoprecipitation (#10004D or #10009D) | -- | Invitrogen (Carlsbad, CA) |
| BChE nonapeptide | -- | Battelle Memorial Institute (Columbus, OH) |
| $^{13}C_9$-BChE nonapeptide | -- | Battelle Memorial Institute (Columbus, OH) |

| | | |
|---|---|---|
| Tetraisopropyl pyrophosphoramide (iso-OMPA; #T1505) | -- | Sigma-Aldrich Co. LLC. (St. Louis, MO) |
| Purified butyrylcholinesterase (BChE; #P06376) | -- | University of Nebraska Medical Center (UNMC; Omaha, NB) |
| Plasma | -- | Tennessee Blood Services (Memphis, TN) |

## Reagent Preparation

### A. Peptide stock solutions

1 mg/mL stock solution of BChE nonapeptide: BChE powder nonpeptide was brought to room temperature. About 2.5 mg of the peptide was carefully weighed on a calibrated analytical balance (readability 0.01 mg) into a 5 mL cryovial. The exact mass was recorded. About 5 mL of 0.6% formic acid in HPLC-grade water was added to the 5 mL cryovial containing BChE nonpeptide. The exact weight was recorded mbof the solution. This process was performed in triplicate. Each individual BChE stock solution (~500 ppm each) was thoroughly vortexed. A portion of each BChE stock solution was added to 0.6% formic acid to construct a final pool containing 50 mL of 1 ppm BChE nonapeptide in 0.6% formic acid in HPLC-grade water. Peptide purity value which was provided by the vendor was factored into making the stock solution. Three individual preparations of BChE nonapeptide were initially made then pooled to eliminate prep error. The 1 ppm stock solution was used to make the calibrator curve.

10 mL of stock solution $^{13}C_9$-BChE nonapeptide (100x stock): BChE powder nonpeptide was brought to room temperature. About 2.5 mg of the peptide was carefully weighed on a calibrated analytical balance (readability 0.01 mg) into a 5 mL cryovial. The exact mass was recorded. About 5 mL of 0.6% formic acid in HPLC-grade water was added to the 5 mL cryovial containing $^{13}C_9$-BChE nonpeptide. The exact weight was recorded of the solution. This process was performed in triplicate. Each individual $^{13}C_9$-BChE stock solution (~500 ppm each) was thoroughly vortexed. A portion of each $^{13}C_9$-BChE stock solution was added to 0.6% formic acid to construct a final pool containing 10 mL of 25 ppm $^{13}C_9$-BChE nonapeptide in 0.6% formic acid in HPLC-grade water. Peptide purity value which was provided by the vendor was factored into making the stock solution. Three individual preparations of $^{13}C_9$-BChE nonapeptide were initially made then pooled to eliminate prep error. The 25 ppm

stock solution was used to make the final internal standard solution of 25 ppb in 0.6% formic acid in HPLC-grade water.

### B. BChE antibody conjugated to magnetic beads

Phosphate buffered saline with tween 20 (PBST): Combine one packet of dry powder PBST with 1 L water for a final concentration of 10 mM sodium phosphate, 138 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20.

5.4 mg/mL dimethyl pimelimidate dihydrochloride (DMP) in triethanolamine (TEA) solution: Add 86 mg of DMP to 16 mL TEA.

Tris Buffered Saline (TBS): Make a 10:1 dilution by adding 50 mL of 10x concentrated TBS to 450 mL of water.

### C. Mobile Phase

Mobile Phase A (MPA): 0.1% formic acid in water. Add 0.5 mL of LCMS-grade formic acid to 500 mL of HPLC-grade water. Mix well.

Mobile Phase B (MPB): 0.1% formic acid in acetonitrile. Add 0.5 mL of LCMS-grade formic acid to 500 mL of HPLC-grade acetonitrile. Mix well.

### D. Buffer Solution

0.6% formic acid solution: Add 3 mL of LCMS-grade formic acid to 500 mL of HPLC-grade water.

### E. Pepsin Solution

2 mg/mL pepsin solution: Add 2 mg of pepsin to 1 mL 0.6% formic acid in HPLC-grade water.

### F. Function Check

A function check (FC) sample is used to ensure proper instrument performance and acceptable results from the check. This sample is run prior to each analytical run that contains patient, proficiency testing, study, or exercise samples, and prior to method readiness runs. The FC sample must be within the laboratory established limits, and the results are documented in the instrument notebook using the

ERB2023 log or an approved alternate log. Failure of an FC is documented in the instrument notebook, along with remedial action.

Refer to *Section 19, Summary Statistics and QC Graphs* for the preparation, use, storage conditions, and established limits of the FC material.

## Standards Preparation and Sources

All calibration and QC materials should be stored at -70°C or lower when not in use.

### A. Native Calibration Standard

Seven calibrators were prepared individually from the 1 mg/mL BChE stock solution *(Section 6, Reagent Preparation, A. Peptide stock solutions*) by gravimetric dilution in 0.6% formic acid to the final concentrations listed in Table 2.

**Table 2.** Analytical Standards Concentration

| Standard Identifiers | BChE Conc (ng/mL) |
|---|---|
| OP-BChE1-S1 | 2 |
| OP-BChE1-S2 | 10 |
| OP-BChE1-S3 | 25 |
| OP-BChE1-S4 | 50 |
| OP-BChE1-S5 | 100 |
| OP-BChE1-S6 | 150 |
| OP-BChE1-S7 | 200 |

*Peptide concentrations shown in Table 2 are for OP-BChE1 materials. Subsequent standard lot information will be shown in *Section 19, Summary Statistics and QC Graphs*.

### B. Isotopically Labeled Internal Standard

The 25 ppm $^{13}C_9$-BChE stock solution (*Section 6, Reagent Preparation, A. Peptide stock solutions*) was diluted in 0.6% formic acid in HPLC-grade water to make the final product of 25 ng/mL BChE.

### C. Quality Control Materials

The matrix blank (MB) and three quality control (QC) materials were

prepared in-house using the information in Table 3.

**Table 3.** Quality Control Concentrations

| QC Levels | BChE nonapeptide concentration (ng/mL)* | Protocol |
|---|---|---|
| OP-BChE1-MB | 0 | Add 5 mL of 1 µg/µL iso-OMPA to 500 mL plasma. Incubate at 37 ºC for 48 h with shaking at 700 rpm and filter through 22 µm filter |
| OP-BChE1-QCL | 10 | Add 110 µL of 1 µg/µL iso-OMPA to 90 mL plasma. Incubate at 37 ºC for 48 h with shaking at 700 rpm and filter through 22 µm filter |
| OP-BChE1-QCM | 65 | 25 µL purified BChE in 72 mL plasma. Incubate at 37 ºC for 48 h with shaking at 700 rpm and filter through 22 µm filter |
| OP-BChE1-QCH | 150 | 90 µL purified BChE in 73 mL plasma. Incubate at 37 ºC for 48 h with shaking at 700 rpm and filter through 22 µm filter |

*QC concentrations shown in Table 3 are for OP-BChE1 materials. Subsequent QC information will be shown in *Section 19, Summary Statistics and QC Graphs*.

## Other Materials and Supplies

Materials, supplies, and sources used during the validation and routine analysis of this method are listed below. Materials and supplies for use with this method should be equivalent to those listed if obtained from other sources.

**Table 4.** Other Materials/Supplies and Sources

| Item* | Source* |
|---|---|
| Adjustable single channel pipettes: 2-20 µL, 20-200 µL, and 100-1000 µL | Eppendorf Co, (Westbury, NY) |
| Adjustable multichannel pipettes: 2-20 µL, 20-200 µL, and 100-1000 µL | Eppendorf Co, (Westbury, NY) |
| Mini Vortex | Fisher Scientific (Fair Lawn, NJ) |
| Falcon Polypropylene Conical Tubes: 15 mL and 50 mL | Fisher Scientific (Fair Lawn, NJ) |
| Dynal Sample Mixer (#947-01) | Invitrogen (Carlsbad, CA) |
| DynaMag Magnet (#12301D) | Invitrogen (Carlsbad, CA) |
| KingFisher 96 Tip Comb (#97002534) | Thermo Scientific (Pittsburgh, PA) |

| | |
|---|---|
| KingFisher 96-well 200 µL shallow well plate (#97002540) | Thermo Scientific (Pittsburgh, PA) |
| 2 mL deep-well 96 Plate, V-Bottom (#AB0932) | Thermo Scientific (Pittsburgh, PA) |
| KingFisher Flex Magnetic Particle Processor (#5400630) | Thermo Scientific, (Waltham, MA, |
| 2 mL deep-well 96 Plate, U-Bottom (#12566121) | Fisher Scientific (Fair Lawn, NJ) |
| MixMate Plate Mixer | Eppendorf (Hamburg, Germany) |
| Magnetic plate for 96-well plates | |
| Protein precipitation plates (#90036 or 90037) | Fisher Scientific (Fair Lawn, NJ) |
| 96-Well PCR Plate (#95120401) | Eppendorf Co., (Westbury, NY) |
| Thermomixer R | Eppendorf Co., (Westbury, NY) |
| PlateLoc Heat Sealer (#G5585A) | Agilent Technology (Santa Clara, CA) |
| Acquity BEH Shield RP18 (50x1 mm, 1.7 um) (#186002851) | Waters Corp (Milford, MA) |
| Sciex 6500+ QQQ | Sciex (Framingham, MA) |
| Agilent 1290 Infinity series UHPLC | Agilent (Santa Clara, CA) |
| Porvair nitrogen evaporator | Porvair (Hampshire, UK) |

*or equivalent source or catalog number

## Instrumentation

This method uses liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis on an Agilent 1290 Infinity II UHPLC system (Agilent Technologies, Santa Clara, CA) interfaced with a Sciex 6500 or Sciex 6500+ triple quadrupole mass spectrometer. Use Analyst Data Acquisition Software version 1.7 (or equivalent) to control the instruments and automate analysis. Use MultiQuant version 3.0.3 (or equivalent) for data evaluation. Equivalent LC and MS systems can be used if properly characterized before use.

### A. UHPLC Configuration

The UHPLC configuration is described in Table 5, and the gradient program is given in Table 6. The gradient program should be optimized based on gradient delay volume.

**Table 5.** UHPLC Configuration

| Module | Parameter | Setting |
|---|---|---|
| 1290 Infinity II Binary Pump | Mobile Phase A | 0.1% formic acid in water |
| | Mobile Phase B | 0.1% formic acid in acetonitrile |
| | Flow Rate | 200 µL/min (0-1 min) and 250 µL/min (1.01-2 min) |
| | Run Time | 2 min |
| | Temperature | 4 °C |
| 1290 Infinity II Autosampler | Injection Volume | 2.0 µL |
| | Needle and back seat wash (Time) | 0.1% formic acid in acetonitrile (S1) |
| 1290 Infinity II Column Compartment (TCC) | UHPLC Column | 100% water (S2) (6 sec each) |
| | Column Temperature | 60 °C |

**Table 6.** UHPLC Gradient Program

| Time (min) | MPA | MPB | Flow Rate (µL/min) |
|---|---|---|---|
| 0.00 | 98 | 2 | 200 |
| 1.00 | 80 | 20 | 200 |
| 1.01 | 2 | 98 | 250 |
| 1.50 | 2 | 98 | 250 |
| 1.51 | 98 | 2 | 250 |
| 2.00 | 98 | 2 | 250 |

## B. Mass Spectrometer Configuration

The Sciex 6500+ triple quadruple mass spectrometer source settings (Table 7) and Selective Reaction Monitoring parameters (Table 8) are shown below.

**Table 7.** Mass Spectrometer Ionization Source Settings*

| Action | Setting |
|---|---|
| Ionization Type | TurboIonSpray |
| Ion Polarity Mode | Positive Ion |
| MS Mode | SRM |
| Dwell Time | 60 ms/channel |
| Collision Gas (CAD) | Medium (10 on 6500 instruments) |

| | |
|---|---|
| Curtain Gas (CUR) | 20 psi |
| Ion Source Gas (GS1) | 30 psi |
| Ion Source Gas (GS2) | 20 psi |
| Ion Spray Voltage (IS) | 5500 V |
| Temperature (TEM) | 250 ˚C |
| Declustering Potential (DP) | 60 V |

*Source settings should be optimized per instrument

**Table 8.** SRM Parameters

| Analyte Name | Precursor ion (m/z) | Product ion (m/z) | Collision Energy[a] (V) | Entrance Potential (V) | Collision cell Exit Potential (V) |
|---|---|---|---|---|---|
| BChE | 796.3 | 691.3[b] | 32 | 6 | 35 |
| | | 620.3[c] | 35 | 6 | 30 |
| BChE_IS | 805.4 | 700.3[b] | 34 | 10 | 30 |

a) Settings should be optimized per instrument. b) Ion used for quantitation. c) Ion used for confirmation.

### C. UHPLC Mass Spectrometer Instrument Control Program

Create a suitable instrument control program for the instrument based upon the parameters described above. For the method development and validation, a method was created using Analyst 1.7 (or equivalent) software to control the LC and MS system.

### D. 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 details and requirements for creating a suitable and valid processing method. These can be readily created using the MultiQuant 3.0.3 software (or equivalent) and an existing data file.

### E. Comparison of Method Performed in Multiple Instruments

This method is performed on a Sciex 6500+ QQQ coupled to an Agilent 1290 Infinity II UHPLC system. An equivalent LC-MS/MS system can be used for this method following an approved instrument comparison procedure.

**Table 9.** Instruments Available for the Test Method

| Instrument Name | Instrument Component | Serial Numbers |
|---|---|---|
| JQG | Agilent 1290 Infinity II UHPLC | DEBAS01691 (Multisampler)<br>DEBAC04623 (Column compartment)<br>DEBBB06090 (Bin pump) |
| | Sciex 6500+ QQQ MS | 20903190514 |

## 7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

### Calibration Verification

Calibration is performed as part of each analytical batch, and a calibration curve is constructed from the 7 calibration standards. 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

#### A. Data Collection

A calibration curve is constructed using a 7-point curve of relative response (i.e., ratio of analyte peak area to internal standard peak area versus the calibrator concentration).

#### B. Calculation of Curve Statistics

The slope and y-intercept of the calibration curve are determined by linear least squares regression with 1/x weighting using the MultiQuant 3.0.3 software (or equivalent).

#### C. Evaluation of Curve Statistics

The coefficient of determination value (R) for the curve must be greater than 0.990. Linearity of the standard curve should extend over the entire standard range. The intercept, calculated from the least squares fit of the data, should not be significantly different from 0; if it is, the source of this bias should be identified.

### Use of Calibration Curve

The lowest and highest points on the calibration curve define the reportable limit of results. The remaining points are distributed between these two extremes. For unknown samples that are above the highest calibration point,

an appropriate sample dilution (e.g.,1:2) with water can be made after Branch Chief approval so that the unknown value of a freshly prepared sample will fall within the calibration range of the assay.

## 8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

### Sample Receiving

The IRL will notify the Laboratory Chief and Branch Chief if samples do not meet acceptable criteria (*Section 3, Specimen Information* outlines the sample receiving process). The Laboratory and/or Branch Chief in consultation with the Lab Director and Clinical Consultant can 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. Prepare BChE antibody-coated magnetic beads (aka beads)

1) Vortex Protein G Magnetic Dynabeads and transfer 8 mL to a 50 mL tube. Example shown in *Figure 3, Image 1A*.
    - Note: Volume critical step. Ensure that the beads are in solution. Pipette carefully.

2) Apply DynaMag-15 magnet to the beads and carefully remove the supernatant without disturbing the beads. Example shown in *Figure 3, Image 1B*.

*3)* Add 16 mL of PBST solution to the beads. Vortex. Apply magnet to beads and remove supernatant without disturbing the beads. Repeat this step two more times. Example shown in *Figure 3, Image 1C.*

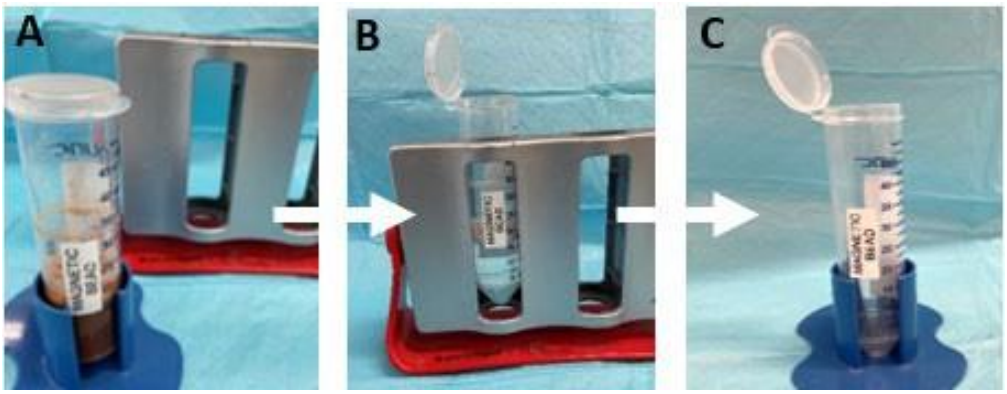



**Figure 3. Image 1A-C: Bead preparation** (1A: beads in solution; 1B: beads pulled out of solution using DynaMag-15 magnet; and 1C: supernatant removed without disturbing beads).

4) Add 32 mL of PBST solution and 1.6 mg of BChE monoclonal antibody solution (e.g., 1.6 mL of a 1 mg/mL antibody solution) to the beads. Vortex.

5) Incubate 2 hours at room temperature with rotation using the Dynal sample mixer on speed 20 (*Figure 4*).
   - Note: Overnight incubation is acceptable.


**Figure 4**. Beads on Dynal sample mixer (with balance tube).

6) Make DMP in TEA solution (86 mg DMP in 16 mL TEA). Vortex and set aside while beads continue to incubate.

7) After the 2-hour incubation (maximum overnight incubation), apply magnet to the beads and remove the supernatant without disturbing the beads.

8) Add 16 mL of TEA buffer to the beads, vortex, apply magnet,

and then remove the supernatant without disturbing the beads. Repeat this step one more time.

9) Add 16 mL of DMP in TEA buffer (prepared above) to the beads. Vortex.

10) Incubate beads for 30 minutes at room temperature with rotation using the Dynal sample mixer on speed 20.

11) Apply magnet and remove supernatant without disturbing the beads.

12) Add 16 mL of 1x TBS solution to beads. Vortex.

13) Incubate beads for 15 minutes at room temperature with rotation using the Dynal sample mixer on speed 20.

14) Apply magnet and remove supernatant.

15) Add 8 mL of PBST solution to the beads, vortex, apply magnet, and remove supernatant without disturbing the beads. Repeat this step one more time.

16) Briefly centrifuge vial containing beads and carefully extract all supernatant without disturbing beads.
    - Note: It is important to remove all excess solution before reconstituting beads in the next step.

17) Add 7.6 mL of PBST solution to the beads and vortex. This is the final BChE antibody conjugated bead solution. Store at 4 °C for up to 3 months.

18) Recommendation: Test beads prior to using on unknown samples by analyzing QCs and confirming that QCs fall within characterized limits
    - Note: Volume critical step. Pipette carefully.

## B. Prepare calibration curve, QC materials and samples

1) Thaw materials (stored at -70 °C)

   **Calibrators:** 7 vials (OP-BChE#-S1 through OP-BChE#-S7)
   **Quality Controls:** 3 vials (OP-BChE#-QCL, -QCM, and -QCH)

**Blank Control:** 1 vial (OP-BChE#-MB; plasma inhibited with iso-OPMA)
**Internal standard:** 1 vial (or 2 vials for a full 96-well plate) (OP-BChE#-ISTD)
**Samples:** provided by the IRL
- Note: # (lot number of materials)

2) Thoroughly vortex BChE antibody-coated magnetic beads (aka beads).
   - Note: Beads (preparation described in *Section 8, Sample Preparation, A. Prepare BChE antibody-coated magnetic beads [aka beads]*) can be prepared up to 3 months in advance and stored at 4ºC until use.
   - Recommendation: Test beads prior to using on unknown samples by analyzing QCs and confirming that QCs fall within characterized limits.

3) Pipette 75 µL of beads into a 96-well KingFisher shallow well plate for the same number of wells as the calibrators, QCs, and samples. Label plate "bead plate".
   - Note: Volume critical step. Pipette carefully.

4) Pipette 50 µL of **matrix blank (MB)** (into each well that will receive calibrators), **QCs**, and **samples** into each well of a 96-well 2 mL round bottom plate (see *Template A*). Label plate "sample plate".
   - Note: Volume critical step. Pipette carefully.
   -

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | MB | MB | SAMPLES (up to 84 per plate) | | | | | | | | | |
| B | | QCL | | | | | | | | | | |
| C | | QCM | | | | | | | | | | |
| D | | QCH | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

**Template A:** Sample plate layout.

## C. Transfer magnetic beads to sample plate using KingFisher processor

1) On the Kingfisher instrument: Select "Step 1: Add beads to sample" (under protein menu)

2) Follow KingFisher processor's prompts and load the appropriate plates as instructed; KingFisher tips sitting on tip plate (shallow

well plate), “bead plate” (shallow well plate with magnetic beads), and “sample plate” (deep well plate with samples). Example shown in *Figure 5*.


**Figure 5.** KingFisher plates for step C.

3) Once finished, follow the KingFisher processor’s prompts to remove the plates.

4) Cover the “sample plate” with adhesive foil and mix at 700 rpm for 45 minutes at room temperature on an MixMate plate shaker.

5) Discard KingFisher tips, tip plate, and “bead plate” into the biohazard waste container.

**D. Prepare digestion plate**

1) Prepare 1 mL of 2 mg/mL pepsin solution in 0.6% formic acid in HPLC-grade water at least 30 min prior to use.

    - Recommendation: Record enzyme weight, catalog number, and lot number

2) Add 10 µL of ISTD to each well of a KingFisher shallow 96-well plate except to well “A1” (which is a double blank sample). Add 10 µL of 0.6% formic acid to well “A1” only. Label plate as “digestion plate”.

    - Note: Volume critical step. Pipette carefully.

3) Add 50 µL of each calibrator or 0.6% formic acid in HPLC-grade water to the “digestion plate” (see *Template B*).

    - Note: Volume critical step. Pipette carefully.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | | | | | | | | | | | | |
| B | S1 | | | | | | | | | | | |
| C | S2 | | | | | | | | | | | |
| D | S3 | | | | | | | | | | | |
| E | S4 | | | | | | | | | | | |
| F | S5 | | | | | | | | | | | |
| G | S6 | | | | | | | | | | | |
| H | S7 | | | | | | | | | | | |

**0.6% formic acid in water**

**Template B:** Digestion plate layout for calibrators.

1) Add 10 µL of 2 mg/mL pepsin to each well (after it sat for 30 minutes) to the “digestion plate”.

## E. Transfer beads to digestion plate using KingFisher processor

1) Add 500 µL PBST to three 2 mL round-bottom plates. Label the three plates as “wash plates”
2) Once the 45-minute incubation is complete, carefully remove the PCR foil from the “sample plate”.
3) On the Kingfisher instrument: Select “Step 2: Bead Washes” (under protein menu).
4) Follow KingFisher processor’s prompts and load the appropriate plates as instructed; new KingFisher tips sitting on tip plate (shallow well plate), three “wash plates”, “sample plate” (contain samples with beads), and “digestion plate” (shallow well plate). Example shown in *Figure 6*.

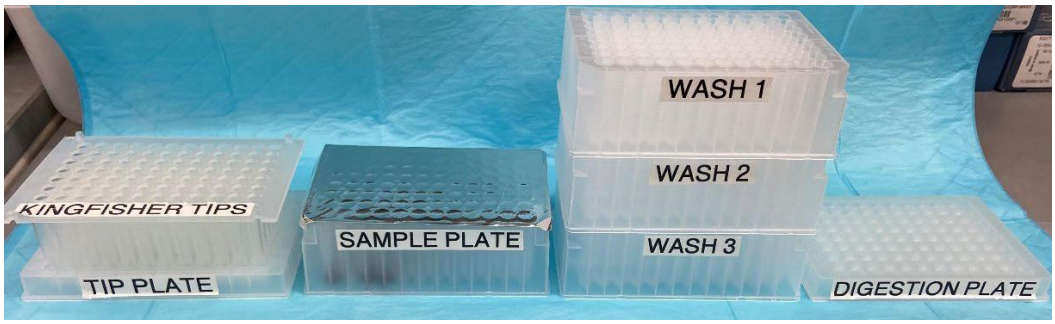



**Figure 6.** KingFisher plates for step E.

3) Once finished, follow the KingFisher processor's prompts to remove the plates.
4) Carefully apply magnetic plate to the digestion plate. This will help remove the beads from the KingFisher tips. Example shown in *Figure 7*.

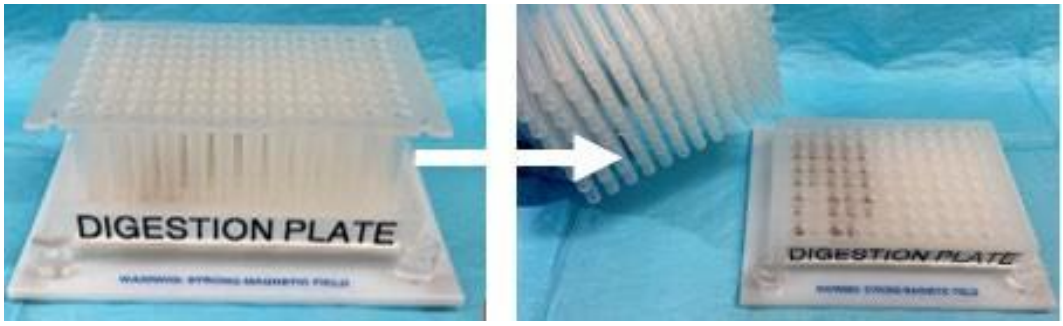



**Figure 7.** Removing KingFisher tips from digestion plate.

5) Cover the "digestion plate" with PCR foil and incubate at 37 °C for 30 min at 700 rpm on a Thermomixer R.
6) Discard KingFisher tips, two "wash plates", and "sample plate" into the biohazard waste container. Keep one "wash plate" for future step.
7) Once digestion is complete, briefly centrifuge the "digestion plate" to remove condensation from the PCR foil. Carefully remove the PCR foil.

## F. Remove beads from digestion plate using KingFisher processor

1) On the Kingfisher instrument: Select "Step 3: Remove beads from prep" (under protein menu).

2) Follow KingFisher processor's prompts and load the appropriate plates as instructed; new KingFisher tip sitting on tip plate (shallow well plate), one "wash plate", "digestion plate" (contain samples with beads). Example shown in *Figure 8.*

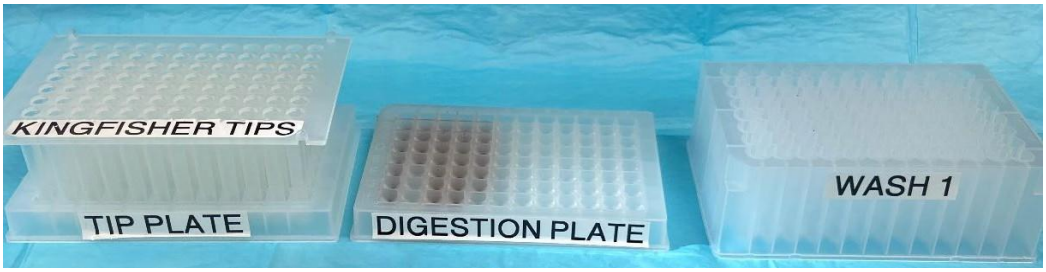



**Figure 8.** KingFisher plates for step F.

3) Once finished, follow the KingFisher processor prompts to remove the plates.

## G. Protein precipitation

1) Add 285 µL of acetonitrile to all wells of a 96-well protein precipitation plate that will receive sample.
2) Transfer the digested sample (~70 µL) to the protein precipitation plate. Use a 2 mL 96-well round bottom plate as the receiving plate.
3) Shake for 1 minute at 500 rpm on a MixMate plate shaker.
4) Pull the entire sample through the protein precipitation plate, eluting into the receiving plate using a vacuum manifold. Label receiving plate "BChE plate".

## H. Sample dry-down and reconstitution

1) Dry the "BChE plate" samples using a 96-well nitrogen evaporator.
    a. Recommendation: Use program "500 µL 75% ACN" on Porvair which dries samples for 45 minutes under nitrogen heated at 60 °C.

2) Reconstitute samples in 50 µL 0.6% formic acid in HPLC-grade water.

3) Transfer the reconstituted samples to a 96-well PCR plate and heat seal.

4) Load PCR plate onto LC-MS/MS system or store at -70 ºC until analysis.

## Sample Analysis

### A. Preliminary System Set-Up and Performance Check

1) Ensure LC solvent reservoirs contain adequate solvent to complete the run and are properly labeled. For example, 50 mL each of MPA and MPB will be required for an entire 96-well plate.

2) Condition the column at 200 µL/min at 100% MPB until the column compartment reaches 60 °C. Once at the desired temperature, condition the column at 200 µL/min at 2% MPB for at least 2 min.

3) Once the instrument is equilibrated, inject a FC standard. Compare values to the limits set during the FC characterization. Only proceed if the FC meets the criteria set during characterization.

### B. Final Set-Up and Operation

Set up a new batch using the appropriate analytical method, “2512_OP-BChE_Quantitiative Method”. Check that the number and autosampler location of the samples in the sequence correspond to the sample location on the worklist page.

### C. System Closeout

The instrument will go into standby mode 30 minutes after the final injection of the worklist.

## Processing of Data

All raw data files are quantified using the MultiQuant 3.0.3 (or equivalent) software. This file contains the area integrated for the analyte and the internal standard for each sample. The software will identify and auto-integrate each

peak based on the relative ISTD. Manual peak selection and integration is reserved for rare cases such as incorrect peak identification or exaggerated auto-integration. In these rare events, the peak should be manually integrated in a manner consistent with the ISTD and previous samples. When manually integrating peaks, the Division's Policy and Procedures Manual will be followed by the analyst. All manual integration events require additional quality review by a QC officer. The file shows the calibration curve used and its R value. The integrated peak areas for the analyte, retention time, and concentrations are also in the quantification file. After processing the data, a report can be generated for STARLIMS as an Excel file. The reviewed report file can be directly uploaded into STARLIMS.

### A. Analyzing and Storing the UHPLC-MS/MS Data

Create a new batch file using MultiQuant 3.0.3 (or equivalent) software. Add the desired samples to the batch, choose "OP-BChE_2512 Method", and analyze the batch. All samples in the batch will be processed and ready for review. The calibration line should not be forced through the origin and should have a linear 1/x fit.

Check the integration of each peak, and if necessary, integrate the peaks manually. The integrated analyte peak should be deleted if the retention time of the analyte peak is ±0.2 min from the ISTD retention time, if the confirmation ion is not present, or if the S/N is below the method's threshold. After all the peaks have been properly integrated save the quantitation file.

### B. Evaluation of Calibration Standards

Check the R value to make sure it is above 0.990. Examine any results to determine underlying causes, including undeleted outliers and nonlinear curve. Calibrators outside of 3 SD can be removed from the calibration curve. No more than one standard may be discarded without supervisor approval. A maximum of two standards can be discarded with supervisor approval. If either the high or low standard is dropped, the reporting limits must be adjusted accordingly.

### C. Evaluation of Quality Control Samples

Three QC samples and a matrix blank sample are included in each analytical run. All QC materials must be within the established limits for data to be eligible for reporting.

Examine each QC result and compare to the statistical data from the validation runs (described in *Section 10, Establishing QC Limits*). QC results are evaluated in SAS (SAS Institute, Cary NC) which uses the DLS QC program to assign a test-wise (aka experiment-wise) error rate based on the number of QC pools per run and to define the outlier rules. The SAS evaluation will generate Shewhart QC charts for each QC pool and each analyte and will flag outliers based on the DLS QC program rules. This method uses three QC pools and therefore follows the rules defined in Section 6.9 of the DLS Policy and Procedures Manual. The following criteria are the minimum requirements for evaluation of the QC results:

1) A QC result is considered “out of control” if the concentration is outside 3 sigma (σ).
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 2σ. This is referred to as the 2x2S rule.
3) A QC result is considered “out of control” if it is ≥ the $10^{th}$ value on the same side of the characterized mean for that QC level and it is outside of the 2σ. 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. This is known as the 4S rule.

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

### Calibration Standards Processing

The R-value must be greater than 0.990. The slope and y-intercept were observed during characterization and are expected to be 0.03 ± 0.02 and
<0.1, respectively. However, these values can change over time. Ultimately the QC concentration,

calibrator accuracy, and linearly of the calibration curve will validate the calibration.

### Quality Control Samples Processing

Three QC samples and a matrix blank sample are included in each analytical run and are known as quality control materials. The results of the quality control materials determine whether the analytical batch is “in control.” The QC samples are processed as unknowns and the calculated values are compared to the mean and limits established on these materials during the characterization. A batch is considered “in control” when the QC samples are within the range established during characterization and do not violate the additional rules outlined in *“Evaluation of Quality Control Samples”* section above.

## 9. REPORTABLE RANGE OF RESULTS

The lowest reportable limit (LRL) is the lowest calibrator, 2 ng/mL. The highest reportable limit is the highest linear calibrator, 200 ng/mL.

## 10. QUALITY CONTROL (QC) PROCEDURE

### Quality Assessment

Quality Assessment procedures follow the DLS Policies and Procedures Manual and standard practices. Daily experimental checks are made on the stability of the analytical system, and blanks and standards, as well as QC materials, are added to each day's run sequence. First, a FC sample is run at the beginning of each day to check the operation of the HPLC-MS/MS system. The retention time, peak area, peak height, width at half height, signal to noise, ion ratio, MS tune and calibration date, and efficiency are examined for BChE to ensure proper operating conditions. Column efficiency (N) is calculated, and the column back pressure is also recorded and linked to serial and lot numbers. This sample can also be used to detect any contamination in the system. One matrix blank and three QC levels are included in each run. 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

QC limits were established for each QC sample through characterization by ≥2 analysts over ≥20 analytical runs with no more than 2 per day. The means and standard deviations are used to calculate limits for each QC material. Individual QC charts for the characterization runs are created and examined, and QC limits are used to ensure analytical stability. The QC characterization statistics are listed in *Section 19. Summary Statistics & QC Graphs*.

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 for each analyte. Additional QC criteria may be added if consistent with laboratory policy. The statistical data calculated for the characterization 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 ERB Internal PT program. Five samples of unknown concentrations are analyzed during three separate PT challenges spaced throughout the year. The samples are prepared by the Chem Threat Laboratory Chief or their proxy and provided to the IRL. The IRL initiates chain of custody and release the PT samples to the analyst designated by the Laboratory Supervisor. PT samples are analyzed and reported within 2 weeks of receipt. Samples are analyzed in accordance with the documented method. Analytical results are reviewed and submitted by the analyst to the LRN-C Quality Assurance Program. The PT results are evaluated by the LRN-C Quality Assurance Program and a summary report of the PT Challenge is submitted to the Laboratory Supervisor.

The results of the ERB Internal PT Challenge are provided to the Laboratory Supervisor within 45 days after the end of the challenge. A corrective action report is required for any PT failures (<80% of the Z-scores per analyte are acceptable).

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

### Internal Standard Response

If the area counts of the internal standard fall below 8592 counts for OP- BChE1 materials, this indicates that the instrument sensitivity, or analyte recovery, has fallen below acceptable limits. The internal standard threshold can change for subsequent lots. The following are some possible causes

for loss of sensitivity:

1) Dirty source: Clean the source, including the source cone and heated capillary.
2) Improperly prepared samples: Prepare an additional set of QC samples to determine if the samples are the cause of the difficulty.
3) Leaks or injection errors in HPLC: Check the HPLC for leaks and resubmit the sample for injection.
4) Injection error (bubble in sample)

**QC Sample Outside of Acceptable Criteria**

If the concentration of one or more of the QC materials falls outside the 3σ confidence limits, the cause must be determined. The QC sample may be re- injected one time, to ascertain if the error is an injection error. If the QC sample is still outside of 3σ, the batch results will not be reported for that analyte, and all batch samples must be re-prepared. An attempt should be made to determine the cause of the QC failure prior to preparing the patient samples again.

## 12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Care is required to prevent contamination of QC materials, standards, and samples. This method is an isotope-dilution mass spectrometry method. This type of analysis has been shown to be reliable for quantitative analysis of small molecules in a matrix. However, even with sample preparation through bead extraction and protein precipitation, unknown interferences may present themselves during analysis of patient samples. Monitoring of the confirmation ion ratio (CIR or CR), S/N of the confirmation ion, peak shape, and retention time will assist in identifying interfering peaks. If one of more of these values are outside of the limits, the patient sample should be prepared again. If the CR is outside the limits again, patient sample results should not be reported.

### A. Possible Interferences

Individual human plasma, serum, and blood samples (50 blood, 60 serum, and 60 plasma) were screened for possible interferences. There have been no specific interferences identified.

### B. Limitations of the Method

Extended exposure to heat has been shown to degrade the BChE protein in serum or plasma. Care must be taken to avoid prolonged exposure of the samples to these conditions. Routine, daily bench

analysis will not compromise the samples.

## 13. REFERENCE RANGES (NORMAL VALUES)

Endogenous BChE was analyzed in 170 individual samples (50 blood, 60 serum, and 60 plasma) from Tennessee Blood Services (Memphis, TN). The average BChE nonapeptide concentration was the following in each matrix: 59.8 ±15.0 ng/mL in plasma; 58.9 ± 13.5 ng/mL in serum; and 25.9 ± 6.4 ng/mL in whole blood.

## 14. CRITICAL CALL RESULTS ("PANIC VALUES")

Biomarker detection significantly below average BChE level in the general population may be assumed to be consistent with organophosphorus nerve agent exposure and reviewed in the context of available epidemiological information by the Division Toxicologist.

## 15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Samples may reach and maintain ambient temperature while preparing the sample for analysis. If the measurement is delayed until the next day, prepared samples can be kept at room temperature or colder.

### A. Stability

The stability of the BChE protein and nonpeptide were evaluated in plasma matrix (using QCs) and peptide solutions (using calibrators). The stability of the QCs and calibrators was assessed using the following: 1) freeze-thaw cycles; 2) storage at room temperature (bench-top); 3) long-term storage at -70 °C; and 4) storage of the processed sample at 4 °C. Stability experiment data available is summarized in *Appendix A, Method Performance*.

#### 1) Freeze-thaw Cycle Stability

Freeze-thaw stability was assessed for the BChE plasma protein and nonpeptide using QCs and calibrators, respectively. QCs and calibrators were frozen (to -70 °C) and then thawed (to room temperature) for 3 cycles. After the 3 freeze-thaw cycles, samples were processed and analyzed. Results were compared to samples that were not freeze-thaw cycled.

#### 2) Bench-top Stability

Bench-top stability was assessed for the BChE plasma protein and nonpeptide using QCs and calibrators, respectively. Unprocessed QCs and calibrators were stored at room temperature for 24 hours. After 24 hours, the samples were processed and analyzed, and results were compared to initial measurement values ($t_0$).

**3) Processed Sample Stability**

Processed sample stability (ready for instrument analysis) was assessed for the BChE plasma protein and nonpeptide using QCs and calibrators, respectively. The QCs and calibrators were processed and stored on the multisampler at 4 °C for 24 hours. After 24 hours, the samples were analyzed, and results were compared to initial measurement values ($t_0$).

**4) Long-term Stability**

Long-term stability was assessed for the BChE plasma protein and nonpeptide using QCs and calibrators, respectively. QCs and calibrators were stored at -70 °C for 1 month. After 1 month, the samples were analyzed, and results were compared to initial measurement values ($t_0$).

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

None.

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

The concentration of unknowns should be reported to 3 significant figures.

Data is processed within Analyst or MultiQuant and exported to Excel, generating files containing the unknown and QC concentrations, retention times, standard curves, and other run information. Data from the Excel files is transferred to a relational database called STARLIMS which tracks the study from beginning to the end. Among the features of STARLIMS are the following: QC Review, Sample Review, QC Characterization, and SAS Analysis. The data, QC results, and a cover letter are routed through the appropriate channels for approval (i.e.,

supervisor, Branch Chief, Division Director). After approval at the division level, the report will be sent to the contact person who requested the analyses.

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

The IRL is responsible for receiving, accessioning, aliquoting, and distributing samples for analysis to ERB laboratories. The IRL initiates a chain of custody (CoC) that tracks sample aliquots physically. Any remaining sample after analysis should be returned to the IRL. A copy of the closed out CoC should be attached to the relevant batch in STARLIMS.

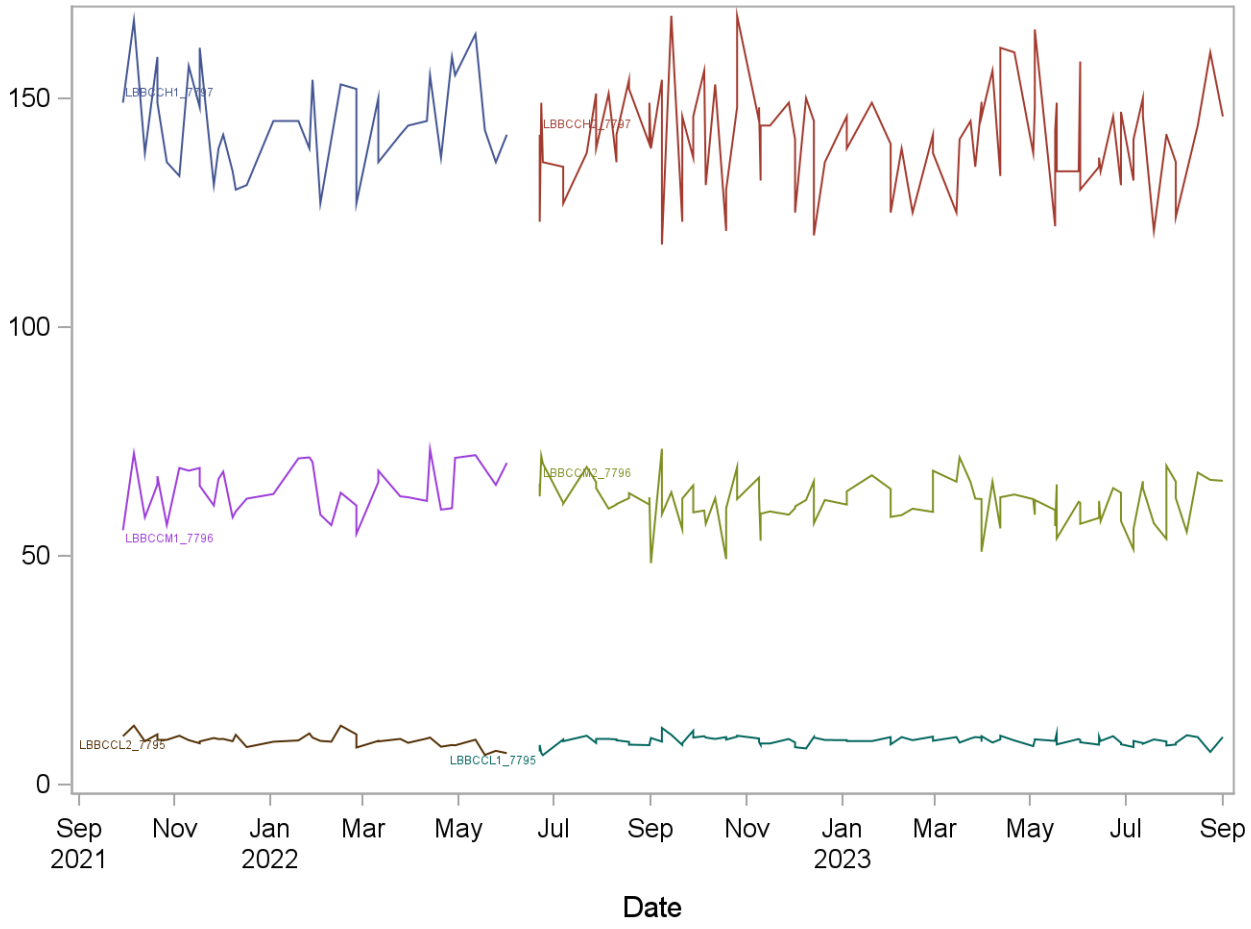
Records are maintained for 2 years, including QA/QC data. Data is stored on the acquisition instrument and is backed up to the local storage as well as CDC encrypted storage. Exported quant data is also loaded into STARLIMS.

## 19. SUMMARY STATISTICS AND QC GRAPHS

Please see the following pages

## August 2021-August 2023 Summary Statistics and QC Chart
## LBXCH (Butyrylcholinesterase Con (ng/mL))

| Lot | N | Start Date | End Date | MEAN | Standard Deviation | Coefficient of Variation |
|---|---|---|---|---|---|---|
| LBBCCH1_7797 | 38 | 29SEP21 | 01JUN22 | 144.605 | 10.523 | 7.3 |
| LBBCCH2_7797 | 92 | 22JUN22 | 01SEP23 | 141.228 | 11.085 | 7.8 |
| LBBCCL1_7795 | 92 | 22JUN22 | 01SEP23 | 9.596 | 0.972 | 10.1 |
| LBBCCL2_7795 | 38 | 29SEP21 | 01JUN22 | 9.675 | 1.318 | 13.6 |
| LBBCCM1_7796 | 38 | 29SEP21 | 01JUN22 | 64.784 | 5.297 | 8.2 |
| LBBCCM2_7796 | 92 | 22JUN22 | 01SEP23 | 61.743 | 4.944 | 8.0 |



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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 it can be 80-120%

**Method name:** BChE Concentration
**Method #:** 2512
**Date(s) Performed:** 4/14/2021 (day 1) and 5/3/2021 (day 2)
**Matrix:** Plasma (matrix #1 is OPNA-inhibited plasma and matrix #2 is OPNA-inhibited serum)
**Units:** ng/mL
**Analyte:** BChE

| BChE | | Matrix Sample 1 | | | | | Matrix Sample 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Measured | | | | | Measured concentration | | | |
| | Replicate | Spike concentration | Day 1 | Day 2 | Mean | Recovery (%) | Spike concentration | Day 1 | Day 2 | Mean | Recovery (%) |
| Sample | 1 | | 0 | 0 | | | | 0 | 0 | | |
| | 2 | 0.00 | 0 | 0 | 0.0 | | 0.00 | 0 | 0 | 0.0 | |
| | 3 | | 0 | 0 | | | | 0 | 0 | | |
| Sample + Spike 1 | 1 | | 8.45 | 9.50 | | | | 9.03 | 9.80 | | |
| | 2 | 10.00 | 8.51 | 9.40 | 9.2 | 91.6 | 10.00 | 9.35 | 9.10 | 9.4 | 94.2 |
| | 3 | | 9.41 | 9.70 | | | | 9.15 | 10.10 | | |
| Sample + Spike 2 | 1 | | 46.43 | 43.40 | | | | 48.79 | 48.50 | | |
| | 2 | 50.00 | 48.73 | 46.60 | 46.7 | 93.5 | 50.00 | 51.96 | 45.20 | 47.9 | 95.8 |
| | 3 | | 47.47 | 47.80 | | | | 45.49 | 47.40 | | |
| Sample + Spike 3 | 1 | | 146.19 | 144.50 | | | | 144.61 | 138.60 | | |
| | 2 | 150.00 | 138.80 | 148.70 | 143.4 | 95.6 | 150.00 | 138.73 | 141.70 | 141.5 | 94.3 |
| | 3 | | 143.68 | 138.40 | | | | 141.66 | 143.60 | | |

| Mean recovery (%) | SD (%) |
|---|---|
| 94.2 | 1.5 |

**Table A2.** Method Precision

## Precision

Evaluate two QC reference materials. For each QC material, make 20 measurements in 10 different analytical runs (2 analyses/run). Total relative standard deviation should be ≤ 15% (CV ≤ 15%)

Method name: **BChE Concentration**
Method #: **2512**
Matrix: **Plasma**
Units: **ng/mL**
Analyte: **BChE**

**Quality material 1: QCL**

| Run | Result 1 | Result 2 | Mean | SS 1 | SS 2 |
|---|---|---|---|---|---|
| 1 (4/6/2021) | 9.73 | 9.44 | 9.59 | 0.021 | 0.021 |
| 2 (4/7/2021) | 11.69 | 8.90 | 10.30 | 1.946 | 1.946 |
| 3 (4/14/2021) | 11.06 | 7.66 | 9.36 | 2.890 | 2.890 |
| 4 (5/3/2021) | 9.50 | 12.00 | 10.75 | 1.563 | 1.563 |
| 5 (5/10/2021) | 10.73 | 10.03 | 10.38 | 0.123 | 0.123 |
| 6 (5/11/2021) | 8.50 | 10.79 | 9.65 | 1.311 | 1.311 |
| 7 (5/12/2021) | 11.20 | 9.11 | 10.16 | 1.092 | 1.092 |
| 8 (5/13/2021) | 8.29 | 9.07 | 8.68 | 0.152 | 0.152 |
| 9 (6/13/2021) | 11.09 | 10.81 | 10.95 | 0.020 | 0.020 |
| 10 (6/24/2021) | 7.77 | 10.14 | 8.96 | 1.404 | 1.404 |
| | | Grand mean | 9.88 | | |

| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev (%) |
|---|---|---|---|---|
| Within Run | 21.04205 | 2.10421 | 1.45059 | 14.69 |
| Between Run | | | 0.75334 | 7.63 |
| Total | | | 1.27265 | **12.89** |

**Quality material 2: QCH**

| Run | Result 1 | Result 2 | Mean | SS 1 | SS 2 |
|---|---|---|---|---|---|
| 1 (4/6/2021) | 153.36 | 149.44 | 151.40 | 3.842 | 3.842 |
| 2 (4/7/2021) | 178.19 | 164.11 | 171.15 | 49.562 | 49.562 |
| 3 (4/14/2021) | 151.37 | 136.08 | 143.73 | 58.446 | 58.446 |
| 4 (5/3/2021) | 144.40 | 142.60 | 143.50 | 0.810 | 0.810 |
| 5 (5/10/2021) | 145.70 | 142.76 | 144.23 | 2.161 | 2.161 |
| 6 (5/11/2021) | 157.81 | 154.86 | 156.34 | 2.176 | 2.176 |
| 7 (5/12/2021) | 163.50 | 108.78 | 136.14 | 748.570 | 748.570 |
| 8 (5/13/2021) | 161.95 | 136.70 | 149.33 | 159.391 | 159.391 |
| 9 (6/13/2021) | 139.65 | 153.27 | 146.46 | 46.3761 | 46.376 |
| 10 (6/24/2021) | 144.94 | 152.30 | 148.62 | 13.542 | 13.542 |
| | | Grand mean | 149.09 | | |

| | Sum squares | Mean Sq Error | Std Dev | Rel Std Dev |
|---|---|---|---|---|
| Within Run | 2169.74895 | 216.97490 | 14.73007 | 9.88 |
| Between Run | | | 9.44585 | 6.34 |
| Total | | | 14.06099 | **9.43** |

**Table A3.** Stability of BChE plasma protein using quality control samples

## Stability

**The initial measurement can be from the same day for all stability experiments.**

**Freeze and thaw stability**

Description: The sample was frozen at -70 °C and then thawed (3 times)

**Bench-top stability**

Description: Unprepared samples were stored at RT for 24 hours

**Processed sample stability**

Description: Processed samples (ready for instrument analysis) stored at RT for 48 hours

**Long-term stability**

Description: Sample stored at -70 °C for 1 month

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

| | |
|---|---|
| Method name: | **BChE Concentration** |
| Method #: | **2512** |
| Matrix: | **Plasma** |
| Units: | **ng/mL** |
| Analyte: | **BChE** |

**QCL**

| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability | Long-term stability |
|---|---|---|---|---|---|
| Replicate 1 | 8.88 | 11.13 | 6.63 | 11.30 | 9.89 |
| Replicate 2 | 9.48 | 9.40 | 8.12 | 9.60 | 10.37 |
| Replicate 3 | 9.85 | 9.58 | 8.74 | 10.18 | 10.40 |
| Mean | 9.40 | 10.04 | 7.83 | 10.36 | 10.22 |
| **% difference from initial measurement** | -- | **6.7** | **-16.7** | **10.2** | **8.7** |

**QCH**

| | Initial measurement | Three freeze-thaw cycles | Bench-top stability | Processed sample stability | Long-term stability |
|---|---|---|---|---|---|
| Replicate 1 | 134.32 | 136.72 | 156.17 | 125.90 | 159.72 |
| Replicate 2 | 159.96 | 160.15 | 132.77 | 137.25 | 146.06 |
| Replicate 3 | 142.38 | 143.58 | 154.86 | 141.79 | 139.51 |
| Mean | 145.55 | 146.82 | 147.93 | 134.98 | 148.43 |
| **% difference from initial measurement** | -- | **0.9** | **1.6** | **-7.3** | **2.0** |

**Table A4.** Stability of BChE nonapeptide using calibrators

## Stability

**The initial measurement can be from the same day for all stability experiments.**

**Freeze and thaw stability**

Description: The sample was frozen at -70 °C and then thawed (3 times)

**Bench-top stability**

Description: Unprepared samples were stored at RT for 24 hours

**Processed sample stability**

Description: Processed samples (ready for instrument analysis) stored at RT for 48 hours

**Long-term stability**

Description: Sample stored at -70 °C for 1 month

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

| | |
|---|---|
| Method name: | **BChE Concentration** |
| Method #: | **2512** |
| Matrix: | **0.6% formic acid in HPLC-grade water** |
| Units: | **ng/mL** |
| Analyte: | **BChE** |

| **Low Calibrator - 10 ng/mL** | **Initial measurement** | **Three freeze-thaw cycles** | **Bench-top stability** | **Processed sample stability** | **Long-term stability** |
|---|---|---|---|---|---|
| Replicate 1 | 9.15 | 8.25 | 10.38 | 9.25 | 10.78 |
| Replicate 2 | 8.76 | 9.46 | 9.09 | 9.95 | 10.25 |
| Replicate 3 | 10.22 | 10.03 | 8.69 | 10.25 | 10.09 |
| Mean | 9.38 | 9.25 | 9.39 | 9.82 | 10.37 |
| **% difference from initial measurement** | -- | **-1.4** | **0.1** | **4.7** | **10.6** |

| **High Calibrator - 150 ng/mL** | **Initial measurement** | **Three freeze-thaw cycles** | **Bench-top stability** | **Processed sample stability** | **Long-term stability** |
|---|---|---|---|---|---|
| Replicate 1 | 147.56 | 138.26 | 151.17 | 153.59 | 138.76 |
| Replicate 2 | 148.32 | 139.71 | 132.98 | 148.41 | 151.15 |
| Replicate 3 | 152.50 | 156.26 | 141.01 | 140.50 | 157.08 |
| Mean | 149.46 | 144.75 | 141.72 | 147.50 | 149.00 |
| **% difference from initial measurement** | -- | **-3.2** | **-5.2** | **-1.3** | **-0.3** |

**Table A5.** Method limit of detection, specificity, and fit for intended use

**LOD, specificity and fit for intended use**

**Method name:** BChE Concentration
**Method #:** 2512
**Matrix:** Serum, plasma, and blood
**Units:** ng/mL
**Analyte:** BChE

| 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 |
|---|---|---|---|
| BChE | 0.340 ng/mL (plasma) | Yes | Yes/No |

## Appendix B: Method Ruggedness

**Table B1.** Ruggedness from BChE1-QCL and BChE1

**Ruggedness**
**Purpose:** To evaluate 5 different parameters for ruggedness
**Method:** Evaluate low and high level QCs at regular method parameters and ±20% for each parameter. Some suggested parameters are digestion concentration, digestion time, digestion
**Description:** Tested the following parameters; enzyme concentration, digestion time, digestion temp, digestion volume, and heating plate type. The low and high level QC (n=4) was run at each condition, plus ±20% the methods parameters.
**Analyst:** Brooke Pantazides
**Date(s) Performed:** 4/1/2021 (all parameters except those notes on 4/7/21) and 4/7/2021 (44C repeat and enzyme lots) and 5/3/2021 (to confirm pepsin lot P7000 results)
**Plate/File name:** J210401-Q-2512, J210407-Q-2512 and J210503-Q-2512
**Results:** All parameters are stable, EXCEPT enzyme lot P7000 will cause a decrease in BChE conc

| BChE | QCL | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CONTROL (From Method Parameters) | Enzyme Conc | | Digestion Time | | Digestion Temp | | Bead Volume | | Heating Plate | |
| | | -20% 1.6 mg/mL Enzyme | +20% 2.4 mg/mL Enzyme | -20% 24 min Digestion | +20% 36 min Digestion | -20% 30°C Digestion | +20% 44°C Digestion | -20% 60 µL Volume | +20% 90 µL Volume | Type 1 Thermomixer (Tonya bench) | Type 2 Thermomixer (Steph bench) |
| Replicate 1 | 11.1 | 11.3 | 10.1 | 11.4 | 9.6 | 10.2 | 13.5 | 11.2 | 11.2 | 10.8 | 9.8 |
| Replicate 2 | 10.7 | 10.7 | 10.1 | 10.6 | 9.7 | 11.9 | 10.9 | 12.2 | 10.8 | 9.3 | 9.4 |
| Replicate 3 | 11.1 | 11.5 | 9.9 | 11.0 | 9.4 | 10.3 | 11.4 | 10.9 | 11.1 | 10.1 | 9.7 |
| Replicate 4 | 11.4 | 10.5 | 12.1 | 11.7 | 9.8 | 9.8 | 11.3 | 11.5 | 11.8 | 9.9 | 9.6 |
| Mean | 11.1 | 11.0 | 10.6 | 11.2 | 9.6 | 10.6 | 11.8 | 11.5 | 11.2 | 10.0 | 9.6 |
| % difference from initial measurement | -- | -0.7% | -4.7% | 0.9% | -13.1% | -4.7% | 6.4% | 3.4% | 1.4% | -9.5% | -13.1% |

| BChE | QCH | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CONTROL (From Method Parameters) | Enzyme Conc | | Digestion Time | | Digestion Temp | | Bead Volume | | Heating Plate | |
| | | -20% 1.6 mg/mL Enzyme | +20% 2.4 mg/mL Enzyme | -20% 24 min Digestion | +20% 36 min Digestion | -20% 30°C Digestion | +20% 44°C Digestion | -20% 60 µL Volume | +20% 90 µL Volume | Type 1 Thermomixer (Tonya bench) | Type 2 Thermomixer (Steph bench) |
| Replicate 1 | 150.7 | 155.0 | 140.2 | 150.3 | 147.3 | 135.5 | 166.2 | 137.5 | 143.9 | 134.0 | 142.0 |
| Replicate 2 | 151.1 | 145.4 | 145.8 | 155.6 | 148.6 | 130.6 | 141.0 | 151.9 | 141.5 | 139.0 | 148.2 |
| Replicate 3 | 161.4 | 159.9 | 139.3 | 166.5 | 148.2 | 146.4 | 147.8 | 149.6 | 144.1 | 138.9 | 148.6 |
| Replicate 4 | 151.7 | 151.7 | 146.5 | 154.7 | 142.0 | 140.0 | 148.1 | 149.2 | 162.5 | 147.6 | 147.3 |
| Mean | 153.7 | 153.0 | 143.0 | 156.8 | 146.5 | 138.1 | 150.8 | 147.1 | 148.0 | 139.9 | 146.5 |
| % difference from initial measurement | -- | -0.5% | -7.0% | 2.0% | -4.7% | -10.1% | -1.9% | -4.3% | -3.7% | -9.0% | -4.7% |

| BChE | QCL | | | | | QCH | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | CONTROL (From Method Parameters) | Enzyme Lots | | | | CONTROL (From Method Parameters) | Enzyme Lots | | | |
| | | Lot 1 P7012 SLCB988 | Lot 2 P6887 109K3536 | Lot 3 P6887 SLBK255V | Lot 4 P7000 SLCD49 | | Lot 1 P7012 SLCB988 | Lot 2 P6887 109K3536 | Lot 3 P6887 SLBK255V | Lot 4 P7000 SLCD49 |
| Replicate 1 | 11.1 | 12.9 | 10.8 | 13.2 | 9.3 | 150.7 | 159.4 | 155.3 | 157.4 | 130.9 |
| Replicate 2 | 10.7 | 11.4 | 11.0 | 10.6 | 8.5 | 151.1 | 154.6 | 145.9 | 149.3 | 119.8 |
| Replicate 3 | 11.1 | 12.0 | 12.2 | 12.1 | 8.0 | 161.4 | 142.3 | 149.5 | 147.4 | 112.9 |
| Replicate 4 | 11.4 | 11.2 | 11.8 | 13.4 | 8.1 | 151.7 | 129.8 | 156.8 | 159.3 | 116.3 |
| Mean | 11.1 | 11.8 | 11.5 | 12.3 | 8.5 | 153.7 | 146.5 | 151.9 | 153.3 | 120.0 |
| % difference from initial measurement | -- | 6.9% | 3.4% | 11.2% | -23.4% | -- | -4.7% | -1.2% | -0.2% | -21.9% |

Note: Pepsin enzyme lot P7000 was retested on 5/3/2021 to confirm results. These results are reproducible. DO NOT use pepsin (lot P7000)