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| Originator: Paul Patel | | Date: 11/15/2013 | |

# Validation of Modified Siemens Cholinesterase Assay

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# Cholinesterase Plasma Assay

## Overview

Cholinesterase refers to a family of enzymes namely pseudocholinesterase and acetylcholinesterase that catalyze the hydrolysis of acetylcholine into choline and acetic acid.
Individuals deficient in the enzyme pseudocholinesterase are extremely sensitive to the muscle relaxant succinylcholine which is often used during surgery. Pseudocholinesterase is responsible for metabolizing local anesthetic; a deficiency therefore decreases safety during surgery and increases the risk of systemic effects such as prolonged apnea and respiratory difficulties.

Cholinesterase levels in the blood can also provide an indication of liver function and insecticide poisoning. Levels are approximately half in patients with acute/chronic hepatitis, liver cirrhosis and carcinoma.

## Principles of the Procedure

Cholinesterase catalyzes the hydrolysis of butyrylthiocholine (BTC) to butyrate and Thiocholine, which reduces 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to 5-thio-2-nitrobenzoate (TNB). The rate of increase in absorbance at 410/596 nm is proportional to the cholinesterase activity in the sample.



### Reaction Equation

$$BTC + H_2O \xrightarrow{CHE} Butyrate + Thiocholine$$

$$DTNB + Thiocholine \longrightarrow TNB$$

## I. Definitions and Abbreviations

The following definitions and abbreviations are used in this document and related documents and attachments:

a. **Accuracy:** Accuracy is defined by CLSI as the closeness of agreement between a test result and an accepted reference value. Method accuracy is used in a different sense by the American Association of Pharmaceutical Scientists where it is expressed as percent relative error (%RE). Trueness, a related CLSI term, is the closeness of agreement between the average of a number of replicate measured quantity values and a reference quantity value.

b. **Analyte:** Component represented in the name of a measurable quantity. The closely related term measureand is defined as the particular quantity subject to measurement.

c. **Analytical sensitivity:** There are several alternative uses of this term. Most commonly, and for the purposes of this Validation Plan, it is used interchangeably with limit of detection. It is also used to describe the ability of an analytical method to assess small variations of the concentration of an analyte, such as the slope of the calibration curve (IUPAC).

d. **Analytical specificity:** Ability of a test or procedure to correctly identify or quantify an entity, including in the presence of interfering substance(s) or phenomena.

e. **Calibration:** Set of operations that establish, under specified conditions, the relationship between values of quantities indicated by a measuring instrument or measuring system, or values represented by a material measure or a reference material, and the corresponding values realized by standards. Under CLIA, calibration refers to the process of testing and adjusting an instrument, kit, or test system, to provide a known relationship between the measurement response and the value of the substance being measured by the test procedure (42 CFR 493.1217).

f. **Calibrator:** A substance, material, or article intended to be used to establish the measurement relationships of a diagnostic medical device.

g. **CLIA:** Clinical Laboratory Improvement Amendments of 1988. Congressional legislation that defined and requires specific quality assurance practices in clinical laboratories.

h. **CLSI:** Clinical and Laboratory Standards Institute.

i. **Coefficient of Variation:** The ratio of the standard deviation to the average, often multiplied by 100 and expressed as a percentage, abbreviated as %CV .

j. **Colorimetry:** A technique used to determine the concentration of colored compound(s) in solution.

k. **Interfering substance:** A substance or quantity thereof that is not the measurand but that affects the result of the measurement.

l. **IUPAC:** International Union of Pure and Applied Chemistry

m. **LDT:** Laboratory –developed Test.

n. **Linearity:** Linearity is the ability of a quantitative analytical method to provide results that are directly proportional to the concentrations of an analyte in test samples, within a given measuring interval. It is an important parameter to confirm when evaluating an analytical method because it verifies correct interpolation of results between points.

o. **LMR:** Lower end of the measuring range is the lowest level at which defined conditions, including all stated characteristic of the method, are met.

p. **LoB:** Limit of Blank is the highest value in a series of results on a sample that contains no analyte.

q. **LoD:** Limit of Detection is the lowest amount of analyte in a sample that can be detected with stated probability, although perhaps not quantified as an exact value.

r. **LoQ:** When used without a prefix, the Limit of Quantitation is the lowest actual concentration at which an analyte is reliably detected and at which uncertainty of the test result is less than or equal to the goal set by the manufacturer or laboratory. The term may also be used with prefixes L for lower (LLOQ) and U for upper (ULOQ), respectively. Note: LoB < LoD ≤ LoQ.

s. **Matrix:** All components of a material system, except the analyte. A specimen matrix is the biological milieu in which an analyte exists (e.g., plasma, serum, urine, or other body fluids).

t. **Measuring Interval (reportable range; analytical measurement range or AMR):** A measuring interval consists of all numeric values between the lower and upper numeric values for which a method can produce quantitative results suitable for clinical use. Where applicable, a linearity study is frequently used to establish or verify the measuring interval that can be reported for a measurement method. Alternatively, the lower limit of the measuring interval may be assigned as the LoQ (LLOQ).

u. **Precision:** Precision is the closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions. It is usually expressed numerically in terms of standard deviation (SD) or percent Coefficient of Variation (%CV).

v. **Reference interval:** The interval between and including two reference limits. It is common practice to define a reference limit so a stated fraction of the reference values is less than or equal, or greater than or equal, to the respective upper or lower limit.

w. **SOP:** Standard Operating Procedure.

x. **Spectrophotometry:** The quantitative measurement of the transmission (or reflection) properties of a material as a function of wavelength.

y. **Testing System:** The entirety of the testing process, including instrument, sample, reagents, supplies, and procedures. Personnel are sometimes included in the definition.



## II. *Pre-clinical Validation*

### a. Analytical Measurement Range

#### i. Limits of Blank, Detection and Quantitation

The limits of blank, detection, and quantitation were determined to be 26.1 U/L, 64.0 U/L and 64.0 U/L respectively.

**Limit of blank**

*CLSI guideline EP17-A section 4.3.1*

| Level | Number of samples | N | Mean | SD |
|---|---|---|---|---|
| Blank | 1 | 20 | 10.3 | 9.6 |

| | |
|---|---|
| Alpha | 5% |
| Parametric LoB | 26.1 |

**Limit of detection**

*CLSI guideline EP17-A section 4.3.2*

| Level | Number of samples | N | Pooled SD |
|---|---|---|---|
| Low | 1 | 20 | 22.7 |

| | |
|---|---|
| Beta | 5% |
| Parametric LoD | 64.0 |

**Limit of quantitation**

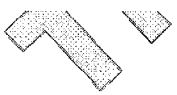
*CLSI guideline EP17-A section 5.1*

| Level | Number of samples | N |
|---|---|---|
| Low | 1 | 20 |

| | |
|---|---|
| Bias | -42.2 |
| Pooled imprecision | 22.7 |
| 95% total error | -86.8 |
| Allowable error | 181 |
| LoQ | 64.0 |

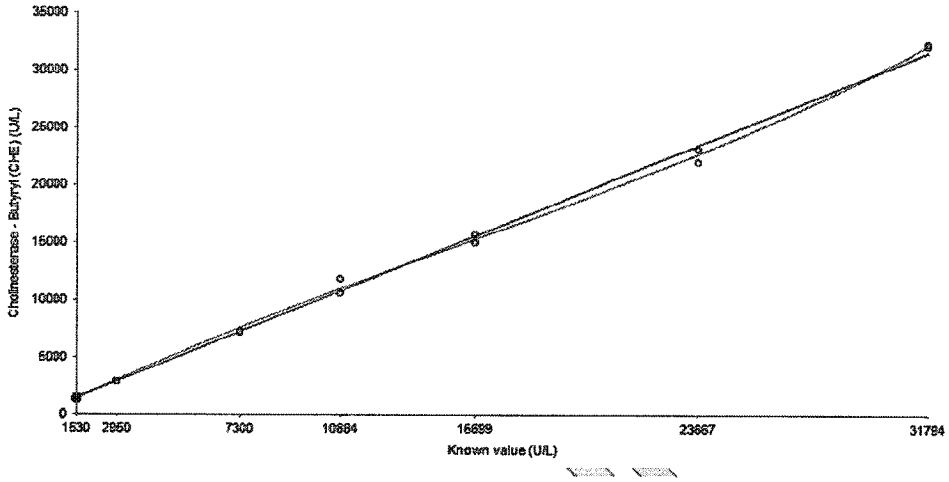
**95% total error is less than allowable error: 20%.**

**LoQ has been established.**



## ii. Linearity

The Analytical Measurement Range (AMR) including linear measurement interval has been determined for Cholinesterase in plasma. This method is linear from 1454.0 – 32305.0 U/L within the 10% allowable non-linearity in this interval.




| Level | Mean | Linear fit | Nonlinear fit (3rd order polynomial) | Nonlinearity | Allowable nonlinearity |
|---|---|---|---|---|---|
| 1 | 1454.0 | 1471.9 | 1323.2 | -148.7 | 153.0 |
| 2 | 2922.5 | 2889.9 | 2981.5 | 91.5 | 295.0 |
| 3 | 7246.0 | 7233.9 | 7582.0 | 358.1 | 730.0 |
| 4 | 11297.0 | 10812.9 | 11007.4 | 194.5 | 1088.4 |
| 5 | 15408.0 | 15621.2 | 15338.5 | -282.8 | 1569.9 |
| 6 | 22669.5 | 23578.2 | 22787.2 | -791.0 | 2366.7 |
| 7 | 32305.0 | 31693.9 | 32272.2 | 578.3 | 3179.4 |

**Nonlinearity is less than allowable nonlinearity: 10%.**
**Performance requirement verified over the measuring interval.**

## Analytical Specificity

The analytical specificity for this assay was determined by testing the effect of hemoglobin (100 mg/dL), bilirubin (11.6 mg/dL) and triglycerides (415 mg/dL) on plasma samples spiked with the interferents and then compared with un-spiked controls. Cholinesterase concentration at which the interference testing was performed at was 9144 U/L. Non-interference was defined as the mean result from testing of spiked samples within 10% of the mean of the un-spiked samples. Recoveries for the Theranos method are shown in the table below.

Table 1. Interference Testing For Cholinesterase

| Test Method | Analyte (U/L) | % Recovery Interferent | | |
|---|---|---|---|---|
| | | Bilirubin (15 mg/dL) | Hemoglobin (100 mg/dL) | Triglycerides (400 mg/dL) |
| Theranos (p-protocol) | Cholinesterase (9144) | 100.8 | 100.0 | 101.6 |

No significant interference was observed.

**Precision**

**Precision**

*CLSI guideline EP05-A2 section 10.8*

**Level = Level 1**

| | |
|---|---|
| Number of observations | 80 |
| Number of runs | 40 |
| Number of days | 20 |
| Runs per day | 2 |
| Replicates per run | 2 |
| Mean | 4954.3 |

| | SD | 95% CI | CV | Allowable Total SD |
|---|---|---|---|---|
| Repeatability | 124.1 | 101.8 to 158.7 | 2.5% | - |
| Between-run | 79.7 | | 1.6% | - |
| Between-day | 136.4 | | 2.8% | - |
| Within-laboratory | 200.9 | 165.7 to 255.1 | 4.1% | 445.9 |

**Imprecision is less than allowable total imprecision: 9% upto 15000U/L then 10%.**

**Level = Level 2**

| | |
|---|---|
| Number of observations | 80 |
| Number of runs | 40 |
| Number of days | 20 |
| Runs per day | 2 |
| Replicates per run | 2 |
| Mean | 7257.8 |

| | SD | 95% CI | CV | Allowable Total SD |
|---|---|---|---|---|
| Repeatability | 183.5 | 150.7 to 234.8 | 2.5% | - |
| Between-run | 50.1 | | 0.7% | - |
| Between-day | 147.6 | | 2.0% | - |
| Within-laboratory | 240.8 | 202.3 to 297.5 | 3.3% | 653.2 |

**Imprecision is less than allowable total imprecision: 9% upto 15000U/L then 10%.**

**Level = Level 3**

| | |
|---|---|
| Number of observations | 80 |
| Number of runs | 40 |
| Number of days | 20 |
| Runs per day | 2 |
| Replicates per run | 2 |
| Mean | 8754.0 |

| | SD | 95% CI | CV | Allowable Total SD |
|---|---|---|---|---|
| Repeatability | 302.6 | 248.4 to 387.2 | 3.5% | - |
| Between-run | 0.0 | | 0.0% | - |
| Between-day | 258.0 | | 2.9% | - |
| Within-laboratory | 397.7 | 332.6 to 494.7 | 4.5% | 787.9 |

**Imprecision is less than allowable total imprecision: 9% upto 15000U/L then 10%.**

The percent CV reported as zeros in the above precision summary are most likely a consequence of rounding the values in StatisPro.

## III. Clinical Validation

### a. Method Comparison with Predicate (Accuracy/Comparability)

To test the accuracy of the assay on the Theranos System, forty (40) unique patient samples were screened on the predicate method (Siemens, Advia) and on the Theranos method. Using the predicate method twenty four (24) values were within the reference range (4900 U/L - 11900 U/L), eight (8) were below the reference range, and eight (8) were above the reference range. Based on the results of the data examination, either a simple linear regression or alternative procedures were used to estimate expected (average) bias and the confidence interval of expected bias at the desired medical decision level(s) as per CLSI guidance EP09-A2. StatisPro was used for bias calculations. These estimates were compared with internal criteria to judge the acceptability of the Theranos method. Each sample was run in duplicate on the predicate, and the average used for comparison to the Theranos method. Some samples were stored before analysis on both methods. If the confidence interval for the predicted bias includes the defined acceptable bias or if the acceptable bias is greater than the higher limit of the confidence interval of the predicted bias, then the data do not show that the bias of the Theranos method is different from the acceptable

bias or there is a high probability (97%) that the predicated bias is acceptable, respectively. The acceptable bias at each medical decision level was determined based on the total allowable error (TEa) minus the measured precision at the level closest to that decision level. Total allowable error (TEa) was taken from American Proficiency Institute (API) peer proficiency testing criteria or CLIA proficiency testing criteria for acceptable analytical performance, as printed in the Federal Register February 28, 1992;57(40):7002-186, when available. The TEa for Cholinesterase is 9%. The table below shows the allowable bias and precision at 3 levels (values shown in parentheses) and the corresponding closest medical decision limits.

Table 2. Allowable Bias and Precision at the Medical Decision Levels

| Medical Decision Levels (U/L) | 1500 (4954) | 7000 (7257) | 20000 (8754) |
|---|---|---|---|
| Precision (%) | 2.8 | 2.0 | 2.9 |
| Allowable Bias (%) | 6.2 | 7.0 | 6.1 |

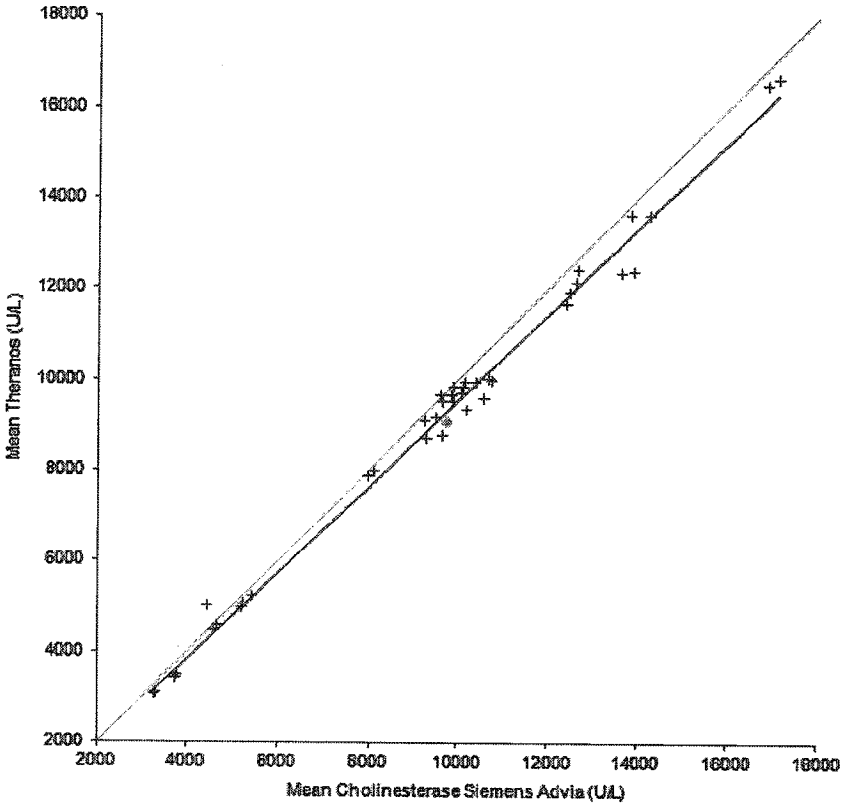



**Figure 1.** Graph showing Theranos method versus Predicate Method (Siemens Advia).

Simple linear regression was used to establish a slope, intercept and an $r^2$. The slope, intercept and clinical correlation were determined to be 0.95, 46.5 and 0.99 respectively.

**Comparability**

*CLSI guideline EP09-A2-IR section 7*

| Level ID | Value | Difference | SE | 95% CI | Allowable difference |
|---|---|---|---|---|---|
| | 1500.000000 | -30.4437886 | 127.202312267 | 7.9514083 to 227.063 | 135.0000000 |
| | 7000.000000 | -312.413219 | 63.27853736 | -40.5139210 to -184.31 | 630.0000000 |
| | 20000.00000 | -978.886419 | 160.928027304 | -674.6681793 to -653.1 | 1800.000000 |

Difference is less than allowable bias: 9% upto 10000U/L then 8%.

The difference between the two methods is not greater than the allowable difference. The performance requirement is verified.

**b. Transference and Verification of Reference Interval (Venous)**

Reference ranges were modified by applying the regression equation to the lower and upper reference limits of existing reference interval to generate a new reference range. New reference ranges were verified with venous samples using forty three (43) new normal subjects. For a reference range to pass verification, 95% of values should fall within the upper and lower reference limits and 5% or fewer values fall outside of the upper and lower reference limits. For venous verification 41 (95%) values fell within the new reference range and 2 (5%) values fell outside the new reference range. See graphs below for venous samples verification.

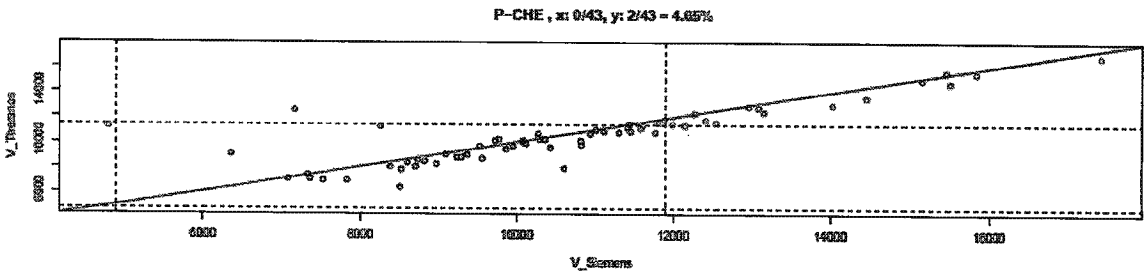



**Figure 2.** Graph showing venous sample reference range verification.

### c. Verification of Reference Interval with Finger Stick Samples

New reference ranges were also verified with venous matched finger sticks (Lithium heparin) from a total of forty three (43) new normal subjects. The finger stick samples were collected in a Theranos blood collection device (BCD) configured with two separate Lithium heparin vessels. For finger stick verification 42 values (98 %) fell within the new reference range and one value (2%) fell outside the new reference range. See graphs below for finger stick samples verification.

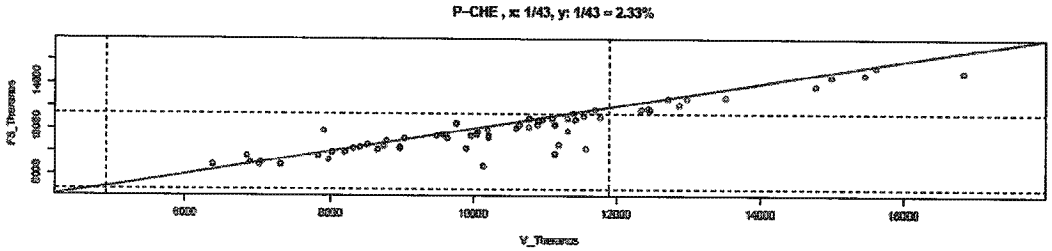



**Figure 3.** Graph showing Finger stick sample reference range verification.

The new reference range for finger stick Cholinesterase was determined to be 4702 - 11340 U/L.

## VI. Stability

### a. Reagents

On-board Reagent Stability

| System | Stability without Reagent Container Inserts | Stability with Reagent Container Inserts (R1, R2) |
|---|---|---|
| ADVIA 1200 | 42 days | 60 days |
| ADVIA 1650/1800 | 14 days | 28 days |
| ADVIA 2400 | 14 days | 28 days |

For all systems, unopened reagents are stable until the expiration date printed on the product label when stored at 2°C - 8°C. Do not freeze the reagents.

For additional details, refer to the Methods Introduction section of the system-specific Operator's Guide.

**b. Sample**

Plasma samples for acetaminophen analysis are stable for 2 weeks at 2-8 °C, or at least 90 days at -20 °C.

**c. Calibrators**

Siemens ToxAmmonia Calibrator should be stored at 2-8 °C, protected from light, and are stable until the expiration date on the vial label. Opened calibrators are stable for at least 3 days.