theranos	LDT Validation Report	Theranos Ethanol Assay	Rev:
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Description	Validation Report for Modified Siemens Assay of Ethanol in Lithium Heparin Plasma		
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Validation of Modified Siemens Ethanol Assay

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Ethanol Plasma Assay

Overview

Ethanol is the single most important substance of abuse in the United States. It is the active agent in beer, wine, vodka, whiskey, rum, and other liquors.

Ethanol acts on cerebral functions as a depressant similar to general anesthetics. This depression causes most of the typical symptoms such as impaired thought, clouded judgment, and changed behavior. As the level of alcohol increases, the degree of impairment becomes progressively increased.

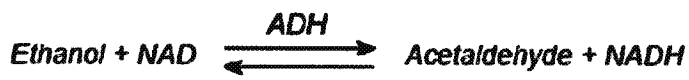
In most jurisdictions in the United States, the level of prima facie evidence of being under the influence of alcohol for purposes of driving a motor vehicle is a blood ethanol concentration 80 mg/dL (0.08 g/dL; 0.08%; 800 mcg/dL).

In the context of medical/clinical assessment, serum is submitted for analysis. On average, the serum concentration of the alcohols is 1.2-fold higher than blood. The serum would contain approximately 0.10 g/dL of ethanol in a blood specimen that contains 0.08 g/dL ethanol.

I. Method Principle

Alcohol dehydrogenase catalyzes the oxidation of ethyl alcohol to acetaldehyde. During this reaction, NAD is reduced to NADH. The increase in absorbance at 340 nm is proportional to the concentration of alcohol in the specimen.

Reaction Equation



II. 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.

III. 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 1.1 mg/dL, 2.6 mg/dL and 9.5 mg/dL respectively.

Limit of blank

CLSI guideline EP17-A section 4.3.1

Level	Number of samples	N	Mean	SD
Blank	1	20	0.37	0.45

Alpha	5%
Parametric LoB	1.11

Limit of detection

CLSI guideline EP17-A section 4.3.2

Level	Number of samples	N	Pooled SD
Low	1	20	0.91

Beta	5%
Parametric LoD	2.62

Limit of quantitation

CLSI guideline EP17-A section 5.1

Level	Number of samples	N
Low	1	20

Bias	-0.46
Pooled imprecision	0.91
95% total error	-2.24
Allowable error	2.0

The lower limit of quantitation has been established at 9.5 mg/dL (9.5% CV and 95.5% recovery).

ii. Linearity

The Analytical Measurement Range (AMR) including linear measurement interval has been determined by Siemens. Refer to the Analytical Range section of the manufacturer product information insert for additional details.

b. Analytical Specificity

The analytical specificity for this assay was determined by testing the effect of hemoglobin (100 mg/dL), bilirubin (10 mg/dL) and triglycerides (400 mg/dL) on plasma samples spiked with the interferents and then compared with un-spiked controls. Ethanol concentration at which the interference testing was performed at was 72 mg/dL. Non-interference was defined as the mean result from testing of spiked samples within 10% of the mean of the un-spiked samples. Recoveries were within 98.9% to 101.8% (see table below).

Table 1. Interference Testing For Ethanol.

Analyte (mg/dL)	% Recovery		
	Interferent		
	Bilirubin (10 mg/dL)	Hemoglobin (100 mg/dL)	Triglycerides (400 mg/dL)
Ethanol	99.1	101.8	98.9

No significant interference was observed.

c. Precision

Level = Level 1

Number of observations	80
Number of runs	40
Number of days	20
Runs per day	2
Replicates per run	2
Mean	39.68

	SD	95% CI	CV	Allowable Total SD
Repeatability	0.87	0.71 to 1.11	2.2%	-
Between-run	0.58		1.5%	-
Between-day	2.45		6.2%	-
Within-laboratory	2.67	2.08 to 3.72	6.7%	9.92

Imprecision is less than allowable total imprecision: 25% upto 100mg/dL then 15%.

Level = Level 2

Number of observations	80
Number of runs	40
Number of days	20
Runs per day	2
Replicates per run	2
Mean	96.83

	SD	95% CI	CV	Allowable Total SD
Repeatability	2.81	2.30 to 3.59	2.9%	-
Between-run	0.26		0.3%	-
Between-day	5.19		5.4%	-
Within-laboratory	5.91	4.67 to 8.03	6.1%	24.21

Imprecision is less than allowable total imprecision: 25% upto 100mg/dL then 15%.

Level = Level 3

Number of observations	80
Number of runs	40
Number of days	20
Runs per day	2
Replicates per run	2
Mean	248.71

	SD	95% CI	CV	Allowable Total SD
Repeatability	11.30	9.28 to 14.46	4.5%	-
Between-run	4.42		1.8%	-
Between-day	16.34		6.6%	-
Within-laboratory	20.36	16.42 to 26.80	8.2%	37.31

Imprecision is less than allowable total imprecision: 25% upto 100mg/dL then 15%.

IV. Clinical Validation

a. Method Comparison with Predicate (Accuracy/Comparability)

To test the accuracy of the assay on the Theranos System, forty four (44) unique patient samples were screened on the predicate method (Siemens, Advia) and on the Theranos method. Four samples were excluded as outliers (mean absolute difference greater than 4). Using the predicate method thirty three (33) values were below the toxic level (<100 mg/dL) and seven (7) were above the intoxication level. 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 Ethanol is 25%. 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 (mg/dL)	**50 (40)**	**100 (97)**	**250 (249)**
Precision (%)	**6.2**	**5.4**	**6.6**
Allowable Bias (%)	**18.8**	**19.6**	**18.4**

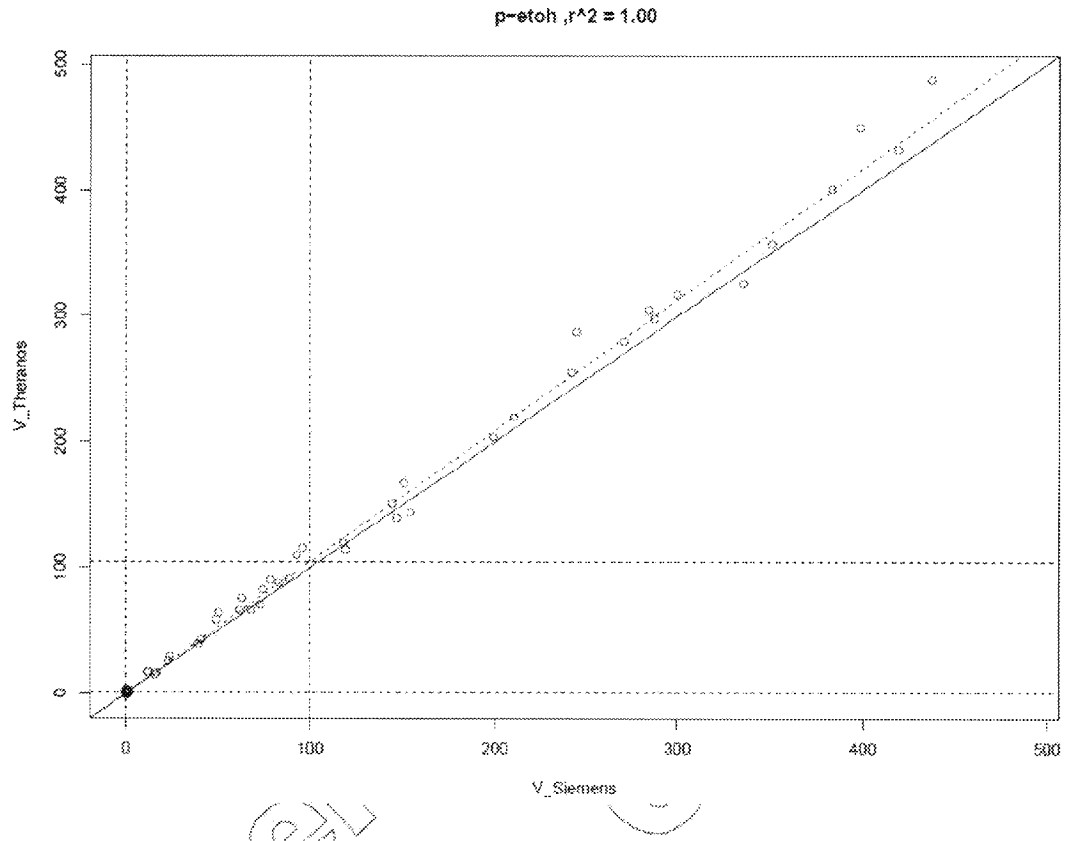


Figure 1. Graph showing Theranos method versus Predicate Method (Siemens Advia)

Simple linear regression was used to establish a slope, intercept and an r^2. The slope, intercept and clinical correlation were determined to be 1.04, 0.808 and 1.00 respectively.

Comparability

CLSI guideline EP09-A2-IR section 7

Level ID	Value	Difference	SE	95% CI	Allowable difference
	50.000000	3.2388753	0.96255940	1.2963519 to 5.1813988	9.4000000
	100.000000	5.9924560	0.97478075	4.0254303 to 7.9594818	18.4000000
	250.000000	14.2531983	1.74204615	0.7376068 to 17.768789	46.0000000

Difference is less than allowable bias: 18.8% upto 100mg/dL then 18.4%.

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 interval for this analyte has been replaced by toxic levels therefore verifying venous sample reference ranges is not required for Ethanol. Fifty seven (57) new normal venous samples were tested, 57 (100%) reported values were below the toxic level >105 mg/dL.

c. Verification of Reference Interval with Finger Stick Samples

Verifying finger stick sample reference ranges is not required for Ethanol. Fifty (58) new venous matched finger sticks samples were also tested, all 58 (100%) reported values below the toxic level >105 mg/dL.

The new level for finger stick Ethanol toxicity was determined to be > 105 mg/dL.

VI. Stability

a. Reagents

On-board Reagent Stability

System	Stability
ADVIA 1200	30 days
ADVIA 1650/1800	30 days
ADVIA 2400	30 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. Avoid prolonged exposure above 32°C. Ethanol reagents are sensitive to the presence of the same system of reactions containing ethanol. Care should be taken to avoid ethanol migration through the air from such reagents.

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

b. Sample

Plasma samples for ethanol analysis are stable for 30 mins at 2-8 °C, or at least 1 week at -80 °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.

REVISION HISTORY			
Revision Level	**Effective Date**	**Initiator**	**ECO Number**
A	11/10/2013	A. Rosendorff	CL ECO-00118
Section Number	**Description and Justification of Changes**		
All	Initial Release		