theranos	LDT Validation Report	Theranos Bicarbonate Assay	Rev:
		CL-RPT-14049	1
Description	Validation Report for Modified Siemens Assay of Bicarbonate in Lithium Heparin Plasma		
Originator: Curtis Schneider		Date: 09/24/2013	

Validation of Modified Siemens Bicarbonate Assay

Author(s):

Signature:	Date: 4/18/14
Name: Paul Patel, Ph.D.	Title: Team Lead, General Chemistry

Reviewer(s):

Signature:	Date:
Name:	Title:
Signature:	Date: 4/23/2014
Name: Daniel Young, Ph.D.	Title: Vice President

Approver(s):

Signature:	Date: 4/21/2014
Name: Adam Rosendorff, M.D.	Title: Laboratory Director

9/19/14

Sunil S. Dhawan M.D.

Bicarbonate Plasma Assay

I. Overview

Bicarbonate comprises the second largest fraction of the total anion pool in plasma. Included in this fraction are the bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions (carbon dioxide in physical solutions), as well as carbamino compounds. At the physiological pH of blood, the concentration of bicarbonate is 1000-times greater than that of carbonate. The carbamino compounds are also present in such low quantities that they are generally not monitored specifically.

II. Method Principle

PEPC catalyzes the first reaction which generates oxaloacetate. In the presence of MDH, the NADH analog is oxidizied by oxaloacetate to nictinamine adenine dinucleotide (NAD+) analog. The oxidation of NADH analogue is measured by the decreased absorbance at 410/478 nm, which is proproational to the amount of CO_2 in the sample.

Reaction Equation

$$\text{Phosphoenolpyruvate} + HCO_3^- \xrightarrow{\text{PEPC} + Mg^{++}} \text{Oxaloacetate} + H_2PO_4^-$$

$$\text{Oxaloacetate} + \text{NADH analog} + H^+ \xrightarrow{\text{MDH}} \text{Malate} + \text{NAD}^+ \text{analog}$$

III. 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 measurand 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.

IV. 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 1.64 mM, 2.19 mM and 2.2 mM respectively.

Limit of blank

CLSI guideline EP17-A section 4.3.1

Level	Number of samples	N	Mean	SD
Blank	1	20	1.03	0.38

Alpha	5%
Parametric LoB	1.64

Limit of detection

CLSI guideline EP17-A section 4.3.2

Level	Number of samples	N	Pooled SD
Low	1	20	0.33

Beta	5%
Parametric LoD	2.19

Limit of quantitation

CLSI guideline EP17-A section 5.1

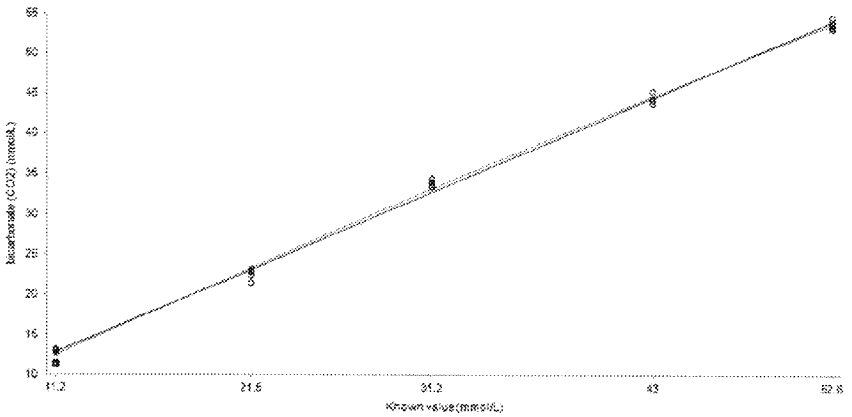
Level	Number of samples	N
Low	1	20
Bias	-0.21	
Pooled imprecision	0.33	
95% total error	-0.85	
Allowable error	1.0	
LoQ	2.19	

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 Bicarbonate in plasma. This method is linear from 12.6 – 53.7 mmol/L within the 10% allowable non-linearity in this interval.



Level	Mean	Linear fit	Nonlinear fit (2nd order polynomial)	Nonlinearity	Allowable nonlinearity
1	12.64	12.81	12.40	-0.40	1.12
2	22.54	23.08	23.28	0.20	2.15
3	33.80	32.76	33.17	0.41	3.12
4	44.46	44.53	44.71	0.18	4.30
5	53.74	54.10	53.72	-0.39	5.26

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 mg/dL) and triglycerides (395 mg/dL) on plasma samples spiked with the interferents and then compared with un-spiked controls. Bicarbonate concentration at which the interference testing was performed was at 23 mM. 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 95% to 99% (see table below).

Table 1. Interference Testing For Bicarbonate

Analyte (mM)	% Recovery		
	Interferent		
	Bilirubin (12 mg/dL)	Hemoglobin (100 mg/dL)	Triglycerides (395 mg/dL)
Bicarbonate (23)	95	99	99

No significant interference was observed.

Precision

Level = L1

Number of observations	78
Number of runs	39
Number of runs excluded	1
Number of days	20
% of days with 1 run	5%
Runs per day	2
Replicates per run	2

CLSI guideline EP05-A2 section 10.4 recommends a minimum of 40 runs

Mean	16.14

	SD	95% CI	CV
Repeatability	0.67	0.55 to 0.86	4.2%
Between-run	0.00		0.0%
Between-day	0.74		4.6%
Within-laboratory	1.00	0.82 to 1.28	6.2%

Level = L2

Number of observations	78
Number of runs	39
Number of runs excluded	1
Number of days	20
% of days with 1 run	5%
Runs per day	2
Replicates per run	2

CLSI guideline EP05-A2 section 10.4 recommends a minimum of 40 runs

Mean	21.74

	SD	95% CI	CV
Repeatability	0.41	0.34 to 0.53	1.9%
Between-run	0.00		0.0%
Between-day	0.13		0.6%
Within-laboratory	0.43	0.37 to 0.51	2.0%

Level = L3

Number of observations	80
Number of runs	40
Number of days	20
Runs per day	2
Replicates per run	2
Mean	27.45

	SD	95% CI	CV
Repeatability	0.61	0.50 to 0.78	2.2%
Between-run	0.00		0.0%
Between-day	1.76		6.4%
Within-laboratory	1.87	1.44 to 2.64	6.8%

Where CV's are reported as zeros in the precision summary output this is most likely a consequence of rounding values in StatisPro.

V. *Clinical Validation*

a. Method Comparison with Predicate (Accuracy/Comparability)

To test the accuracy of the assay on the Theranos System, forty one (41) unique patient samples were screened on the predicate method (Siemens, Advia) and on the Theranos method. Using the predicate method twenty eight (28) values were within the reference range (20 – 34 mmol/L), six (6) were below the reference range, and seven (7) 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 Bicarbonate is 10%. 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)	**6.0 (16.1)**	**20 (21.7)**	**33 (27.5)**
Precision (%)	**4.6**	**0.6**	**6.4**
Allowable Bias (%)	**5.4**	**9.4**	**3.6**

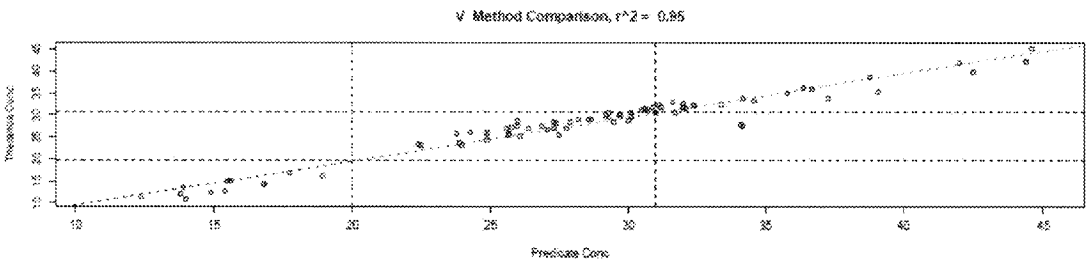


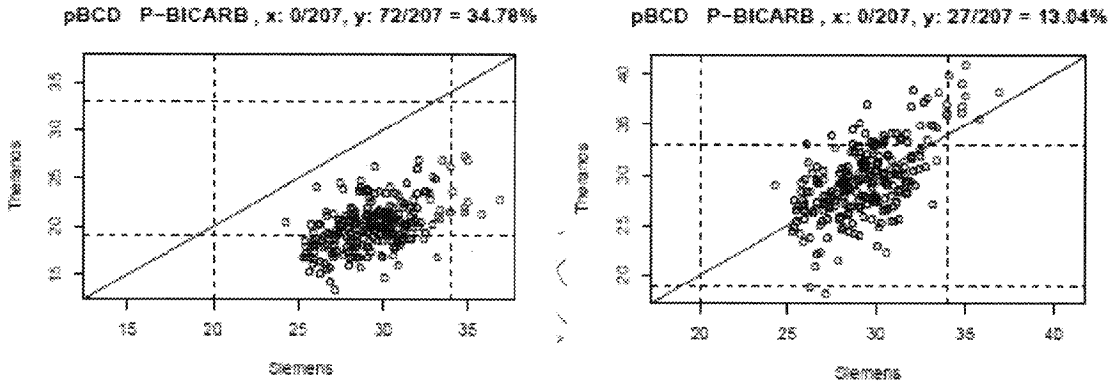
Figure 1. Graph showing Theranos method versus Predicate Method (Siemens Advia). Simple linear regression was used to establish a slope, intercept and an r2. The slope, intercept and clinical correlation were determined to be 0.99, -0.43 and 0.95 respectively.

b. Transference and Verification of Reference Interval (Venous)

Not determined. Established predicate reference range 20 – 34 mmol/L will be used for lithium venous plasma samples.

c. Venous versus Finger Stick Comparison

A comparison of matched venous versus finger stick samples from two hundred and seven (207) healthy subjects showed a negative bias with the finger stick samples.



A) B)

Figure 2. Plots of matched venous versus finger stick bicarbonate results without A) bias correction and B) with bias correction.

To normalize finger stick to venous a correction factor needs to be applied to finger stick results. The following correction is used:

Corrected Finger Stick value = (Finger stick result – Intercept)/ Slope

(where, slope = 0.533, intercept = 7.77)

d. Establishment of Reference Interval with Finger Stick Samples

A new reference range was established for bicarbonate in lithium heparin according to CLSI guideline C28-A3 by testing finger stick samples from 219 healthy subjects.

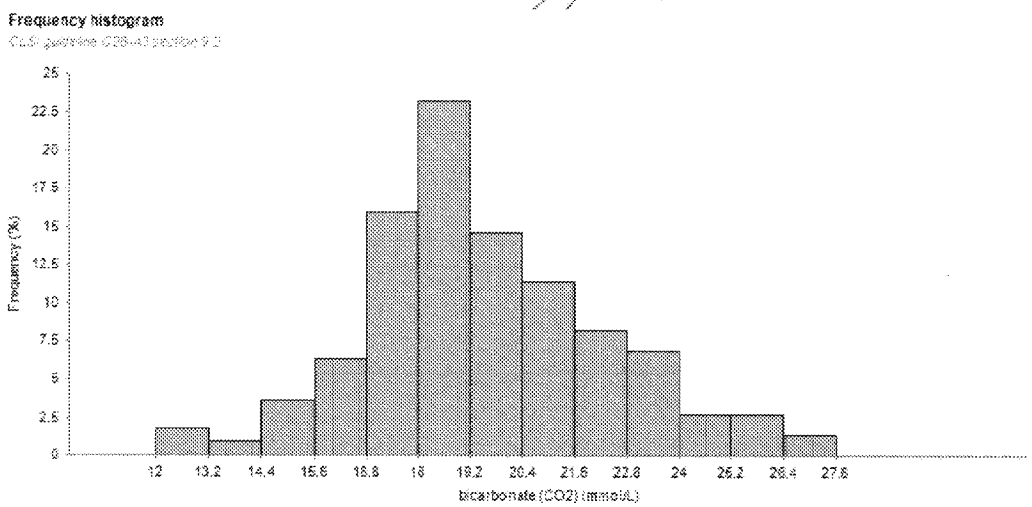
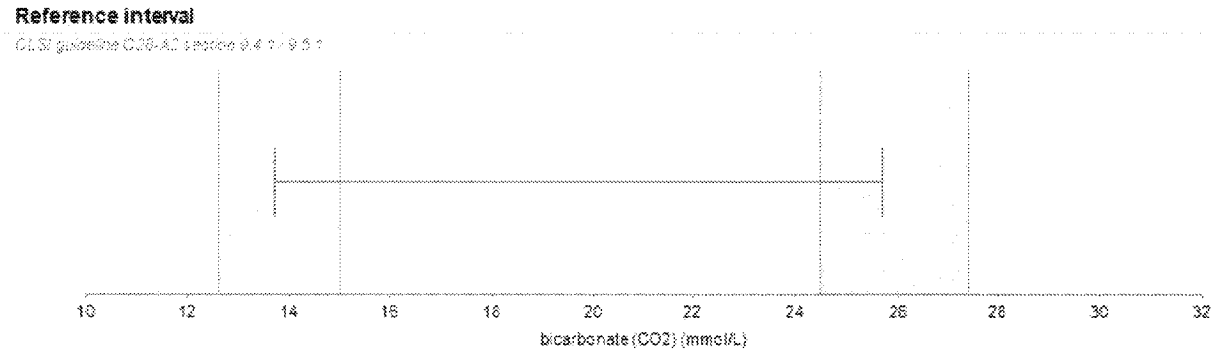


Figure 3. Frequency histogram showing distribution of finger stick values for reference range establishment.



Computation method	95% reference interval	Lower reference limit 90% CI	Upper reference limit 90% CI
Nonparametric	13.69 to 25.70	12.62 to 15.01	24.50 to 27.40

For bicarbonate in lithium heparin plasma the established reference range determined in finger stick samples is: 13.7 – 25.7 mmol/L.

VI. Stability

a. Reagents

On-board Reagent Stability

System	Stability
ADVIA 1200	14 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° - 8°C. Do not freeze reagents.
For complete details, refer to the Methods Introduction section of the system-specific Operator's Guide.

b. Samples

Plasma samples for Bicarbonate analysis are stable sealed for 2 weeks at 2-8 °C.

c. Calibrators

The Siemens CO_2 Calibrator/Diluent should be stored at 2-8 °C. Unopened Calibrator/Diluent is stable until the expiration date on the vial label. Opened Calibrator/Diluent is stable for 30 days.