theranos	LDT Validation Report	Theranos Alkaline Phosphatase (ALP) Assay	Rev:
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Description	Validation Report for Modified Siemens Assay of Alkaline Phosphatase (ALP) in Lithium Heparin Plasma		
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Validation of Modified Siemens Alkaline Phosphatase (ALP) Assay

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Alkaline Phosphatase (ALP) Plasma Assay

I. Overview

Alkaline phosphatase (ALP) is present in a number of tissues including liver, bone, intestine, and placenta. Serum ALP is of interest in the diagnosis of 2 main groups of conditions-hepatobiliary disease and bone disease associated with increased osteoblastic activity.

A rise in ALP activity occurs with all forms of cholestasis, particularly with obstructive jaundice. The response of the liver to any form of biliary tree obstruction is to synthesize more ALP. The main site of new enzyme synthesis is the hepatocytes adjacent to the biliary canaliculi.

ALP also is elevated in disorders of the skeletal system that involve osteoblast hyperactivity and bone remodeling, such as Paget's disease, hyperparathyroidism, rickets and osteomalacia, fractures, and malignant tumors. A considerable rise in alkaline phosphatase activity caused by increased osteoblast activity following accelerated bone growth is sometimes seen in children and juveniles.

II. Method Principle

Alkaline phosphatase hydrolyzes the p-nitrophenyl substrate to form p-nitrophenol. The reaction is followed the colorimetric measurement of the rate of formation of the p-nitrophenol at the 410/478 nm, which is proportional to the alkaline phosphatase activity. A 2-amino-2-methyl-1-propanol (AMP) buffer is used to maintain the reaction pH at the 10.3-10.4. Magnesium and zinc ions are added to the AMP buffer to activate and stabilize the enzyme.

Reaction Equation

$$PNPP + AMP \xrightarrow{ALP,\ Mg^{2+},\ Zn^{2+}} P\text{-}Nitrophenol + P\text{-}AMP$$

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 0.4 U/L, 2.1 U/L and 17.1 U/L (85.5% recovery) respectively.

Limit of blank

CLSI guideline EP17-A section 4.3.1

Level	Number of samples	N	Mean	SD
Blank	1	20	0.1	0.2
Alpha	5%			
Parametric LoB	0.4			

Limit of detection

CLSI guideline EP17-A section 4.3.2

Level	Number of samples	N	Pooled SD
Low	1	20	1.0
Beta	5%		
Parametric LoD	2.1		

Limit of quantitation

CLSI guideline EP17-A section 5.1

Level	Number of samples	N
Low	1	20
Bias	-2.9	
Pooled imprecision	1.0	
95% total error	-4.9	
Allowable error	-	
LoQ	2.1	

Level	Sample	n	Assigned value	Mean	Median	SD	CV
Blank	1	20	0	0.1	0.0	0.2	447.2%
Low	1	20	20	17.1	17.0	1.0	6.0%

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 has been determined by Siemens. Refer to the Interference section of the manufacturer product information insert for additional details.

c. Precision

Level = L1

Number of observations	80
Number of runs	40
Number of days	20
Runs per day	2
Replicates per run	2
Mean	31.2

	SD	95% CI	CV
Repeatability	0.9	0.7 to 1.1	2.8%
Between-run	0.0		0.0%
Between-day	0.5		1.5%
Within-laboratory	1.0	0.9 to 1.2	3.2%

Level = L2

Number of observations	80
Number of runs	40
Number of days	20
Runs per day	2
Replicates per run	2
Mean	167.4

	SD	95% CI	CV
Repeatability	16.2	13.3 to 20.7	9.7%
Between-run	2.7		1.6%
Between-day	2.0		1.2%
Within-laboratory	16.6	14.3 to 19.6	9.9%

Level = L3

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	307.3

	SD	95% CI	CV
Repeatability	4.2	3.5 to 5.4	1.4%
Between-run	0.9		0.3%
Between-day	1.9		0.6%
Within-laboratory	4.7	4.1 to 5.7	1.5%

V. Clinical Validation

a. Method Comparison with Predicate (Accuracy/Comparability)

To test the accuracy of the assay on the Theranos System, 39 unique patient samples were screened on the predicate method (Siemens, Advia) and on the Theranos method. Using the predicate method twenty (24) values were within the reference range (45 – 129 U/L), five (5) were below the reference range, and ten (10) 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 ALP is 30%. The table below shows the allowable bias and precision at 2 levels (values shown in parentheses) and the corresponding closest medical decision limits.

Table 1. Allowable Bias and Precision at the Medical Decision Levels

Medical Decision Levels (U/L)	**50.0 (31.2)**	**150.0 (167.4)**
Precision (%)	**1.5**	**1.2**
Allowable Bias (%)	**28.5**	**28.8**

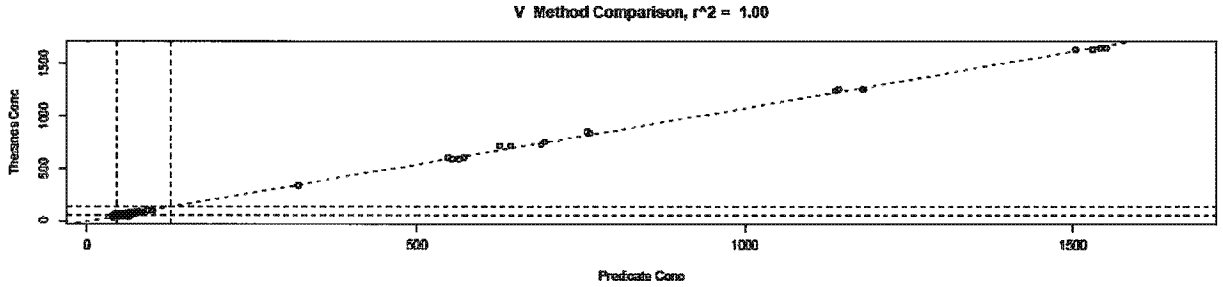

Figure 1. Graph showing Theranos method versus Predicate Method (Siemens Advia). Simple linear regression was used to establish a slope, intercept and an r2. The slope, intercept and clinical correlation were determined to be 1.08, -4.72 and 1.00 respectively.

Comparability

CLSI guideline EP09-A2-IR section 7

Level ID	Value	Difference	SE	95% CI	Allowable difference
	50	-0.8	1.98	-4.8 to 3.2	13.5
	150	7.5	1.82	3.8 to 11.2	40.5
	400	28.2	1.79	24.6 to 31.9	108.0

Difference is less than allowable bias: 27%.

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 eight (48) 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 48 (100%) values fell within the new reference range and 0 (0%) values fell outside the new reference range. See graphs below for venous samples verification.

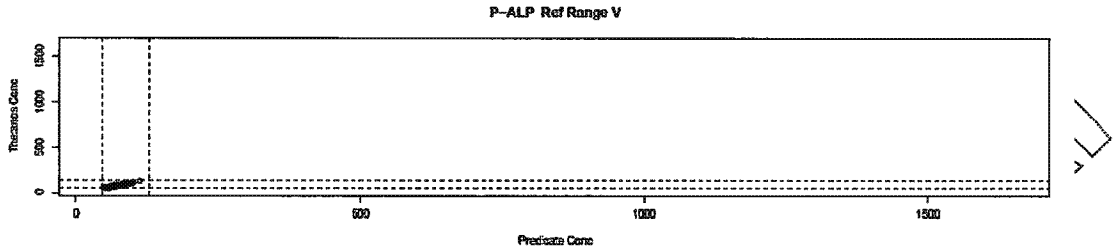

Figure 2. Graph showing venous sample reference range verification.

c. Verification of Reference Interval with Finger Stick Samples

New reference ranges were also verified with venous matched finger sticks from forty five (45) new normal subjects. For finger stick verification 44 (97.8%) values fell within the new reference range and 1 (2.2%) values fell outside the new reference range. See graphs below for finger stick samples verification.

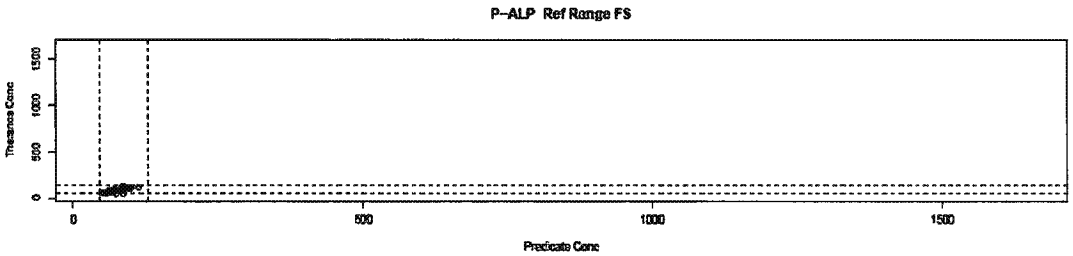

Figure 3. Graph showing Finger stick sample reference range verification.

Theranos.Analyte	Anti-coagulant	Existing Reference Range (RR) (U/L)		New Reference Range (U/L)	
		Reference Range (low)	Reference Range (High)	Transfered RR (low)	Transfered RR (high)
Alkaline Phosphatase	Lithium Heparin	35	120	44.0	135.0

The new reference range for finger stick Alkaline Phosphatase was determined to be 44 – 135 U/L.

VI. Stability

a. Reagents

On-board Reagent Stability

System	Stability
ADVIA 1200	7 days
ADVIA 1650/1800	10 days
ADVIA 2400	10 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. Sample

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

c. Calibrators

Siemens Chemistry Calibrators 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 48 hours, except for total and direct, which are stable for 8 hours.

REVISION HISTORY

Revision Level	Effective Date	Initiator	ECO Number
A	11/06/2013	A. Rosendorff	CL ECO-00117

Section Number	Description and Justification of Changes
All	Initial Release
