theranos	LDT Validation Report	Theranos PT Assay	Rev:
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Description	Validation Report for Theranos Laboratory Developed Test for Activated Partial Thromboplastin Time (aPTT) in EDTA Plasma		
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Theranos Activated Partial Thromboplastin Time (aPTT) Assay Validation

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Activated Partial Thromboplastin Time Plasma Assay

I. Overview

The Theranos activated partial thromboplastin time (aPTT) assay is designed for in vitro diagnostic use in the quantitative determination of how quickly human plasma clots using the TECAN liquid handling and plate reading systems. This test is used to monitor the effects of heparin therapy.

II. Method Principle

The Theranos activated partial thromboplastin time (aPTT) method uses a three reagent system to measure the activity of the intrinsic coagulation pathway (Figure 1). Reagent A, which contains the contact activator ellagic acid, is added to the sample to initiate the intrinsic clotting cascade over 3 min. Reagent B, which contains fibrinogen in buffer, is combined with Reagent C, which contains buffered calcium chloride to reverse the effects of the anticoagulant. The sample/Reagent A mixture is then combined with the Reagents B/C mixture and formation of the fibrin clot is monitored by absorbance at 500 nm.

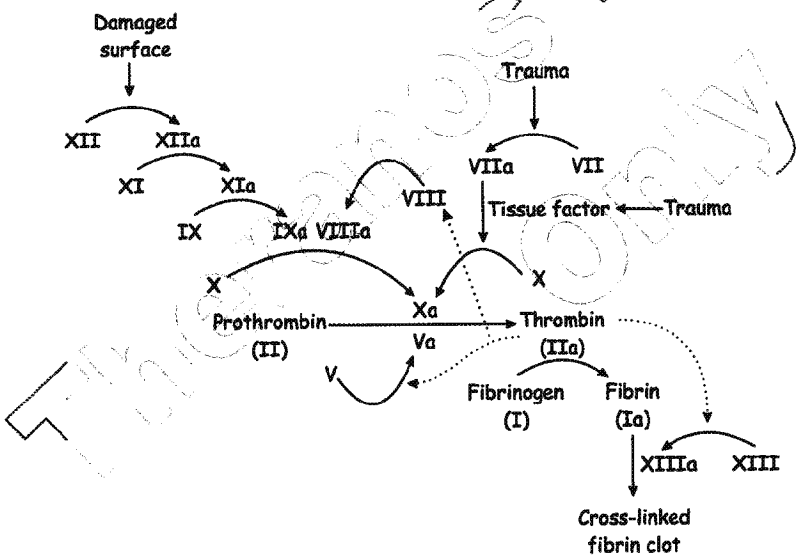

Figure 1. Scheme of the coagulation pathway.

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 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 Quantitation

The assay can quantify aPTTs within the 10 min kinetic read window, which covers an aPTT range of 15.6-97.7 seconds. See Section V.a. for information about scaling results.

ii. Linearity

The analytical measurement range including the linear measurement interval was determined for the aPTT assay. The linearity of the aPTT assay was evaluated using commercial quality control materials (BioMedica QuikCoag Control Levels 1-3). The observed values range from 23.0 – 65.5 s and all levels meet the non-linearity requirement (Figure 2 and Table 1).

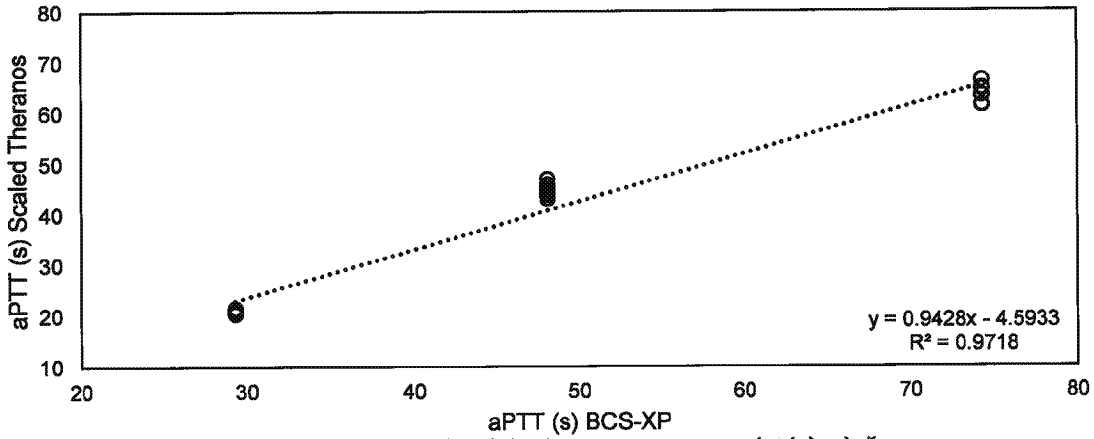

Figure 2. Linearity plot for quality control materials.

Table 1. Linearity by point for quality control materials. Nonlinearity is less than 10% for all points across the measuring interval.

Level	Mean	Linear Fit	Nonlinearity	% Difference	Allowable Nonlinearity
1	20.8	23.0	-2.2	-9.8%	2.3
2	44.6	40.8	3.9	9.5%	4.1
3	63.8	65.5	-1.6	-2.5%	6.5

The linearity of the Theranos aPTT assay was also examined by diluting a normal plasma sample. This test is linear for a normal plasma sample diluted 5 – 35X, with aPTT values from 25.3 – 73.6 seconds (Figure 3). All levels except the undiluted plasma meet the non-linearity requirement (Table 2).

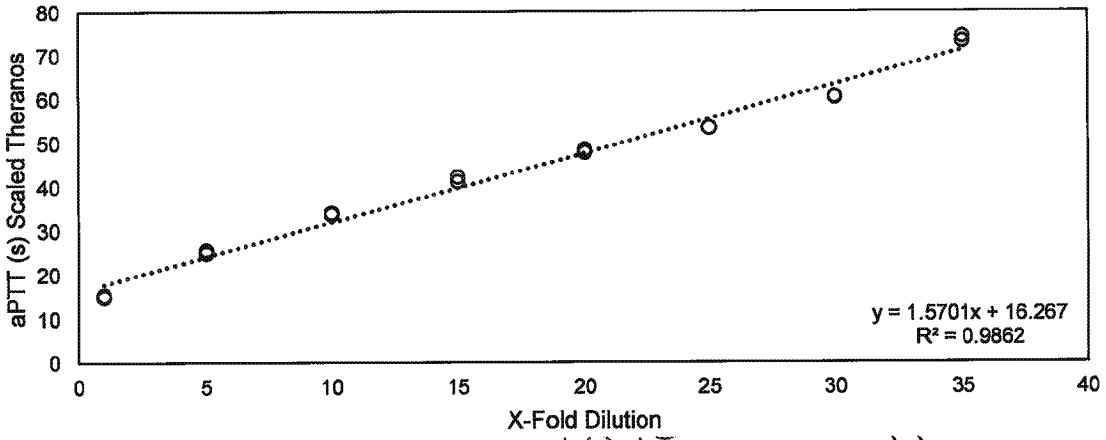

Figure 3. Linearity plot for diluted normal plasma.

Table 2. Linearity by point for diluted normal plasma. Nonlinearity is less than 10% for all points across the measuring interval, except for the lowest dilution level.

Dilution Factor	Mean	Linear Fit	Nonlinearity	% Difference	Allowable Nonlinearity
1	15.2	17.8	-2.6	-14.8%	1.8
5	25.3	24.1	1.1	4.7%	2.4
10	33.9	32.0	1.9	6.0%	3.2
15	41.7	39.8	1.9	4.7%	4.0
20	48.1	47.7	0.4	0.9%	4.8
25	53.4	55.5	-2.1	-3.8%	5.6
30	60.4	63.4	-2.9	-4.6%	6.3
35	73.6	71.2	2.4	3.3%	7.1

Last, the linearity of the aPTT assay was evaluated over the heparin therapeutic range. This test is linear for a normal plasma sample spiked with 0.1 – 1 U/mL heparin, with aPTT values from 28.2 – 76.3 seconds (Figure 4). All levels other than unspiked plasma meet the non-linearity requirement (Table 3).

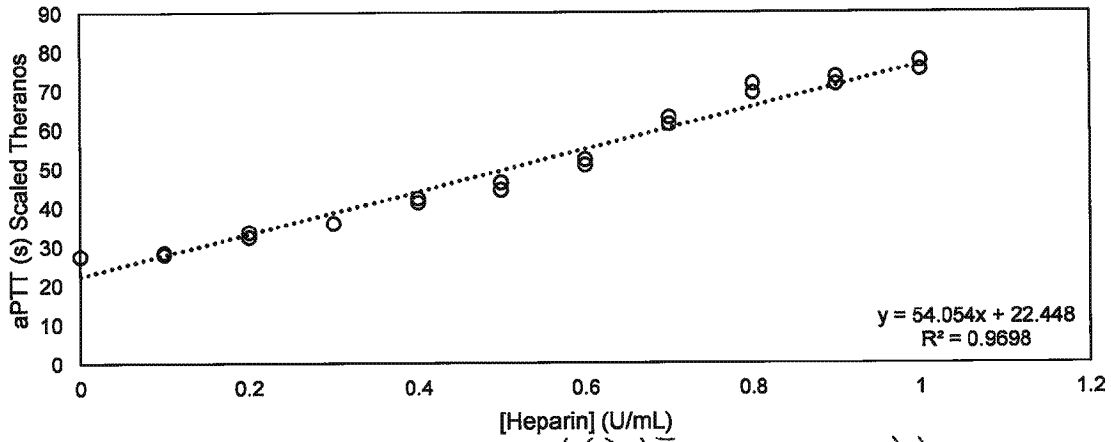

Figure 4. Linearity plot for heparin-spiked plasma.

Table 3. Linearity by point for heparin-spiked plasma. Nonlinearity is less than 10% for all points across the measuring interval, except for the unspiked plasma.

[Hep] (U/mL)	Mean	Linear Fit	Nonlinearity	% Difference	Allowable Nonlinearity
0	27.5	22.4	5.1	22.6%	2.2
0.1	28.2	27.9	0.4	1.3%	2.8
0.2	33.0	33.3	-0.3	-0.9%	3.3
0.3	35.8	38.7	-2.8	-7.3%	3.9
0.4	41.7	44.1	-2.3	-5.3%	4.4
0.5	45.4	49.5	-4.1	-8.3%	4.9
0.6	51.5	54.9	-3.4	-6.2%	5.5
0.7	62.1	60.3	1.8	3.0%	6.0
0.8	70.3	65.7	4.6	7.1%	6.6
0.9	72.3	71.1	1.2	1.7%	7.1
1.0	76.3	76.5	-0.2	-0.3%	7.7

b. Analytical Specificity

The analytical specificity of the aPTT assay was determined by testing two plasma samples spiked with 100 mg/dL hemoglobin (as hemolysate), 10 – 15 mg/dL conjugated bilirubin, and 600 mg/dL triglycerides (as interlipid). The aPTT value of each interferent-spiked sample was compared to an unspiked control to calculate the %-recovery (Table 4). Only one plasma sample displayed significant interference against 15 mg/dL bilirubin, with a recovery of 86.1%. All other recoveries were in the range of 99.0 – 104.6%.

Table 4. Interference testing summary for aPTT.

Analyte (s)	% Recovery by Interferent			
	Bilirubin (10 mg/dL)	Bilirubin (15 mg/dL)	Hemoglobin (100 mg/dL)	Triglycerides (600 mg/dL)
aPTT (28.5)	99.0	86.1	104.4	102.1
aPTT (32.1)	101.7	100.6	101.9	104.6

c. Precision

The precision of the analytical method was evaluated using commercial quality control materials (BioMedica QuikCoag Control Levels 1-3). Samples were tested over 5 days, with 10 replicates of each level per day to establish repeatability, between-run, between-day, and within-laboratory precision (Tables 5-8).

Table 5. Control level 1 precision summary for aPTT.

Number of observations	50
Number of runs	25
Number of days	5
Runs per day	5
Replicates per run	2
Mean	20.9

	SD	CV
Repeatability	0.42	2.0%
Between-run	0.2-0.5	0.9-2.4%
Between-day	0.6	2.8%
Within-laboratory	0.61	2.9%

Table 6. Control level 2 precision summary for aPTT.

Number of observations	48
Number of runs	24
Number of runs excluded	1
Number of days	5
% of days with 4 runs	20%
Runs per day	5
Replicates per run	2
Mean	46.9

	SD	CV
Repeatability	2.55	5.4%
Between-run	1.5-4.2	3.2-9.0%
Between-day	2.6	5.6%
Within-laboratory	2.56	5.5%

Table 7. Control level 3 precision summary for aPTT.

Number of observations	50
Number of runs	25
Number of days	5
Runs per day	5
Replicates per run	2
Mean	70.8

	SD	CV
Repeatability	4.64	6.6%
Between-run	3.5-6.0	4.9-8.5%
Between-day	4.6	6.5%
Within-laboratory	4.59	6.5%

V. *Clinical Validation*

a. Method Comparison with Predicate

The accuracy of the Theranos aPTT assay was evaluated by testing 156 patient samples using both the Theranos and predicate (Siemens BCS-XP) methods. According to the BCS-XP results, 138 samples were within the reference interval, 8 samples fell below the reference interval, and 10 samples were above the reference interval. The regression equation obtained by plotting the results from each method (Figure 5) was used to scale the Theranos aPTT values to the predicate time scale (Figure 6).

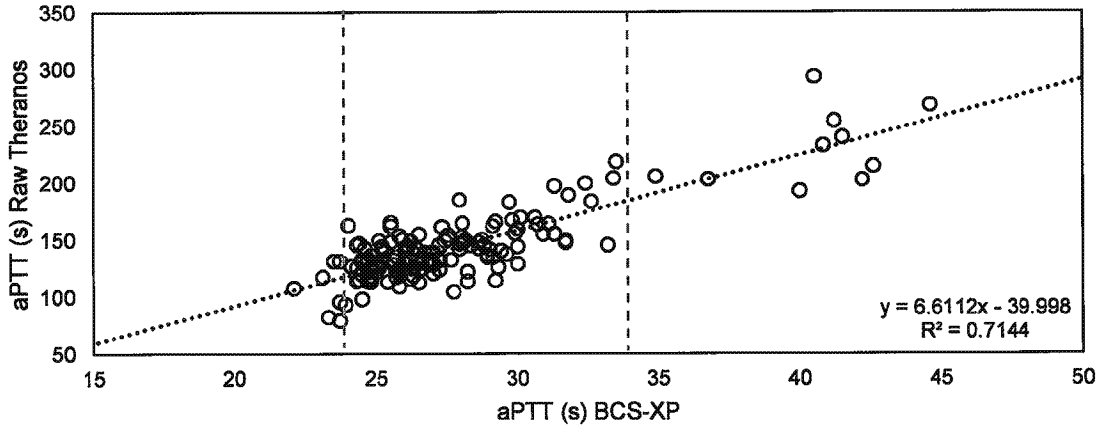

Figure 5. Correlation plot of aPTT values obtained using the Theranos method and the predicate method (Siemens BCS-XP).

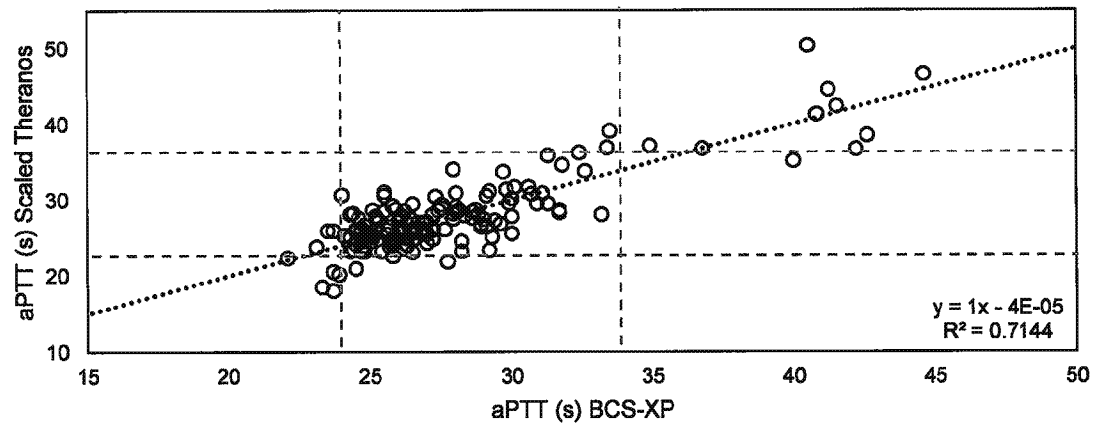

Figure 6. Correlation plot of scaled aPTT values obtained using the Theranos method and the predicate method (Siemens BCS-XP).

b. Establishing the Theranos Reference Interval (Venous)

The 138 patient samples that returned normal aPTT values by the BCS-XP method were used to establish the reference interval for the Theranos aPTT assay. Reference intervals were established from the scaled Theranos data using Gaussian (21.0 – 33.3 s) and percentile (22.8 – 36.0 s, 95%) methods (Figure 7). The data was analyzed using the Kalmogorov-Smirnov and D'Agostino & Pearson omnibus normality tests to determine which reference interval is more valid. The data did not pass either normality test, thus the percentile reference interval was chosen as the Theranos aPTT reference interval.

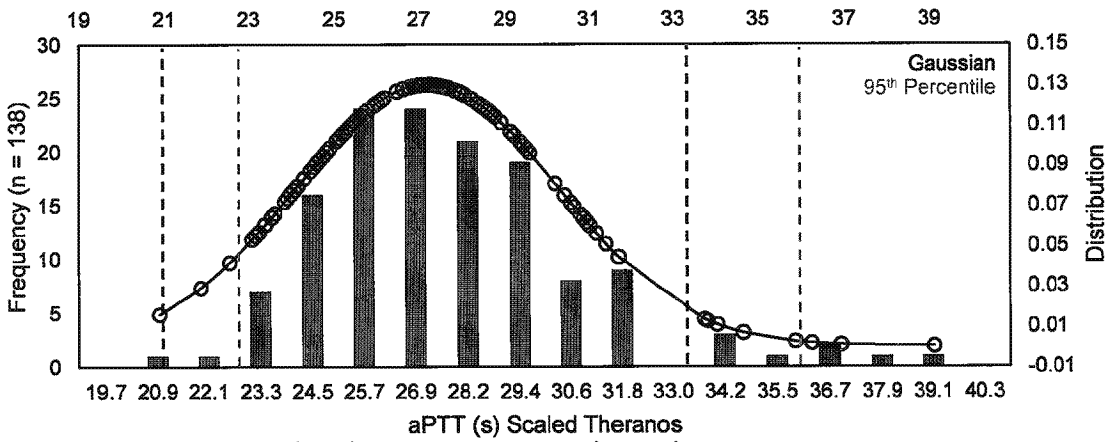

Figure 7. Distribution and frequency of normal samples in the scaled Theranos time-scale used to determine the Theranos reference interval.

c. Verification of Reference Interval with Venous Samples

This new reference interval was verified using matched EDTA and citrate venous samples from 43 patients. Samples were analyzed using the Theranos aPTT assay and compared against the BCS-XP aPTT values for the matched citrate pairs. One of the 43 samples (2.3%) was excluded from analysis because it fell below the predicate reference range. The remaining 42 samples (97.7%) fell within the reference range indicated for the BCS-XP. The Theranos reference interval must encompass 95% of the 42 samples that are within the BCS-XP reference interval. After testing, 40 (95.2%) of the venous samples examined were within the Theranos reference interval and 2 (4.8%) fell below the range (Figure 8).

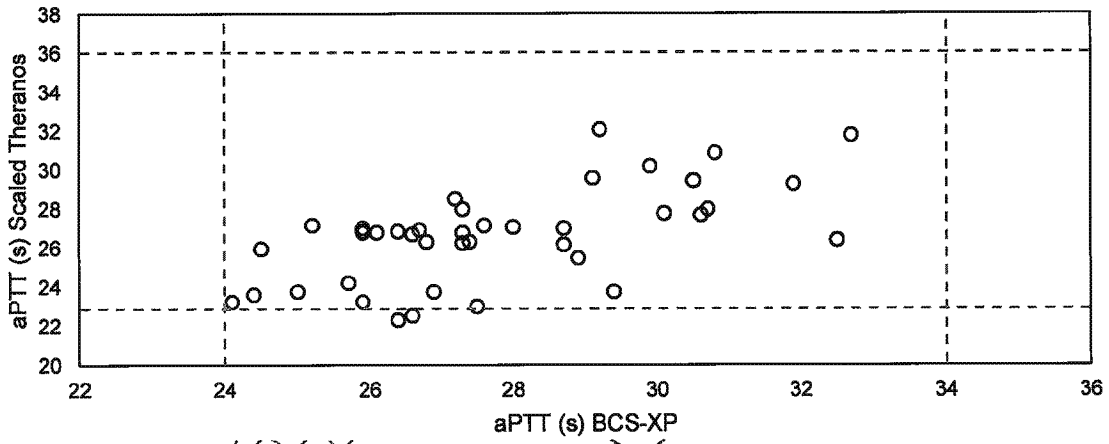

Figure 8. Plot of venous reference interval verification.

d. Verification of Reference Interval with Finger Stick Samples

The new reference interval was also verified using matched EDTA finger stick and citrate venous samples from 44 patients. Finger stick samples were collected in passive capillary transfer nanotainer (pCTN) devices, with EDTA coating both capillary channels. Samples were analyzed using the Theranos aPTT assay and compared against the BCS-XP aPTT values for the matched venous citrate pairs. One of the 44 samples (2.3%) was excluded from analysis because it fell below the predicate reference range. The remaining 43 samples (97.7%) fell within the reference range indicated for the BCS-XP. The Theranos reference interval must encompass 95% of the 43 samples that are within the BCS-XP reference interval. After testing, 42 (97.7%) of the finger stick samples examined were within the Theranos reference interval and 1 (2.3%) fell below the range (Figure 9).

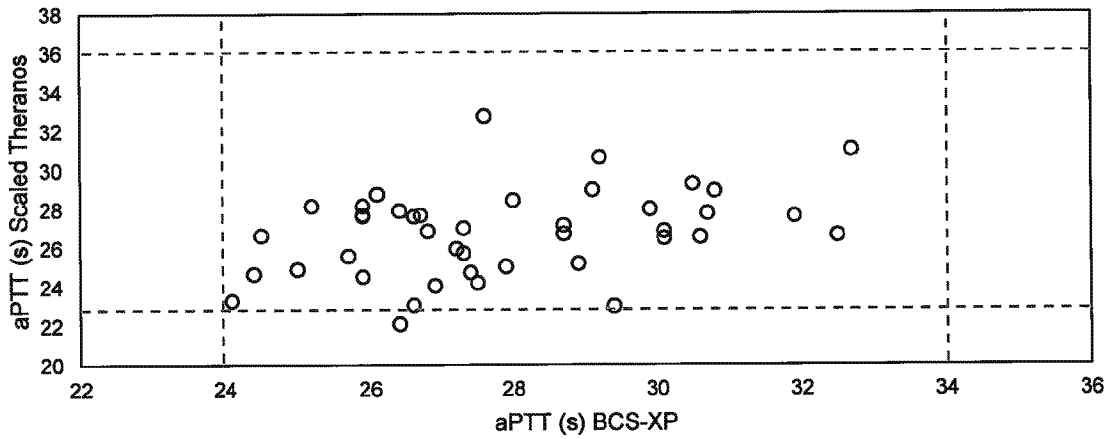

Figure 9. Plots of finger stick reference interval verification.

VI. Reagents and Sample Stability

a. Reagent Stability

Reagent storage conditions and stability were determined throughout assay development (Table 8). Unaltered, commercial reagent A is stable until the expiration date on the bottle when stored under the manufacturer's recommended conditions (2 – 8 °C). Reagent B is stored as frozen aliquots, with sufficient volume to perform 6 assay runs. Once thawed, reagent B may be stored at 2 – 8 °C for up to 24 h. Reagent C and the assay buffer are stable at room temperature for up to 1 year, unless contamination is observed.

On-board stability refers to the stability of 100-120 µL of each reagent loaded into 96-well strip plates. Reagents should be discarded and replaced after 2 h at room temperature, due to concerns about evaporation and enzyme stability.

Table 8. Reagent details and stability information.

Reagent	Composition	P/N	Amount	Stability	On-board stability
A	QuikCoag aPTT (ellagic acid) reagent	03-00405	10 x 10 mL	date on vial; 2-8 °C	2 h
B	3 mg/mL fibrinogen in buffer	05-00170	200 x 1 mL	1 yr; -20 °C	2 h
C	15 mM CaCl2 in buffer	05-00171	100 mL	1 yr; 18-25 °C	2 h
Buffer	20 mM HEPES buffered saline, pH 7.4	03-00404	100 mL	1 yr; 18-25 °C	3 h

b. Sample Stability

Samples to be tested for aPTT are stable at 2 – 8 °C for up to 4 h, as determined by CLSI guidelines and confirmed by our own studies. If a sample cannot be tested within 4 h of collection, the sample must be centrifuged and the plasma frozen. Once frozen, the sample may be stored at -18 – -25 °C for up to 2 weeks. Once thawed, the sample should be tested within 2 h.

c. Quality Control Material Stability

The commercial quality control materials (BioMedica QuikCoag Control Levels 1-3) are received as lyophilized cakes and stored at 2 – 8 °C until the expiration dates printed on the labels, per the manufacturer's instructions. Once reconstituted, the manufacturer indicates 6 h stability at 2 – 8 °C. To minimize waste of these materials, each control was reconstituted then aliquoted for use with the Theranos assay system, frozen in liquid N_2, and stored at -18 – -25 °C (stability studies ongoing). Once thawed, the control materials should be stored at 2 – 8 °C and used within 6 h, per the manufacturer's instructions.