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Apolipoprotein A1 (ApoA1) Assay Validation Report		

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1 ASSAY BACKGROUND

Apolipoprotein A-1 is a distinct, lipid-binding protein accounting for approximately 65% of the total protein of high density lipoprotein (HDL). Apolipoprotein A-1 provides the necessary structural component for HDL formation and functions as a cofactor for the enzyme lecithin cholesterol acyltransferase (LCAT) in the esterification of plasma free cholesterol to cholesteryl esters.

Studies show a correlation between the development of coronary arteriosclerosis and abnormal concentrations of lipoproteins. In arteriosclerosis, the inner layer of the arterial wall thickens due to the accumulation of cellular material and deposits of several substances, particularly lipids. Since apolipoproteins are unique markers for the identification and differentiation of lipoproteins, they are the most probable determinants of the structural integrity and functional specificity of lipoproteins.

Measurement of apolipoprotein A-1 in serum of patients with significant arteriosclerotic alterations shows apolipoprotein A-1 levels significantly lower than a normal patient population. Decreased apolipoprotein A-1 can indicate a risk factor even in the absence of other indications, such as a normal apolipoprotein B. Reduced apolipoprotein A-1 values also occur with dyslipoproteinemias, insulin-requiring diabetes, and liver diseases including acute hepatitis and hepatic cirrhosis.

Numerous studies suggest that serum apolipoprotein A-1 and apolipoprotein B levels indicate the severity and extent of coronary artery stenosis better than serum total cholesterol and triglyceride. In addition, studies indicate that patients with arteriosclerosis are better distinguished from patients without the disease by the finding of increased plasma apolipoprotein B levels than by the findings of decreased HDL-C and increased LDL-C levels. Measuring both apolipoprotein A-1 and apolipoprotein B and expressing the data as a ratio has been shown to be more effective than apolipoprotein A-1 or apolipoprotein B alone.

2 REGULATION AND GUIDANCE

The qualification/validation of the ELISA assays on the Theranos device will be in accordance with C.F.R. Ch IV, § 493.1253 "Standard: Establishment and verification of performance specifications" and outlined in CLSI guideline C28A3.

3 PRINCIPLE OF THE PROCEDURE

3.1 The Apolipoprotein A-1 (APO A1) method is a polyethylene glycol (PEG) enhanced turbidometric assay. A sample containing human apolipoprotein A-I and specific antiserum forms an insoluble complex that can be measured turbidometrically.

3.2 Plasma samples were diluted 1:3.125 fold in saline prior to analysis.

4 CALIBRATION

4.1 In 42 CFR Part 493.1255, it is required to perform calibration procedures with at least the frequency recommended by the manufacturer, or using criteria specified by the laboratory, or when calibration verification fails to meet acceptable limits.

4.1.1 The term "calibration verification," as used in CLIA, includes:

4.1.1.1 Confirming that a calibration meets the method manufacturer's specifications

4.1.1.2 Verifying that the calibration is suitable for the entire measuring interval (or "reportable range," which is the CLIA term)

4.2 Calibrators were diluted 1:3.125 and verified on the ADVIA system

4.2.1 This dilution factor is within the acceptable limits of the ADVIA internal calibration test.

4.3 For the purposes of this Validation Plan, calibration was carried out with every new lot of reagents.

4.3.1 Each level was tested in replicates of 3 and the average was used to create a standard curve for testing.

4.3.2 The calibration was verified using quality controls.

5 QUALITY CONTROL

5.1 Two to four level quality control samples, as appropriate to the assay, were analyzed with each calibration and before each test during the validation.

5.1.1 Low = 84.2 mg/dL

5.1.2 Mid= 182mg/dL

5.1.3 High = 286 mg/dL

5.2 The QC levels are not included when generating the calibration curve.

6 PRECISION

6.1 Precision was evaluated according to CLSI standard EP5-A2, Evaluation of Precision Performance of Quantitative Measurement Methods.

6.2 A total of 20 runs were performed over 10 days with 2 runs per day and 2 replicates per run for a total of 40 data points. The following tables indicate the between-run, between-day and within-laboratory precision at 3 levels (see 5.1.1-5.1.3) The following data describes the results obtained:

Table I: Precision at 3 decision limits

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Precision

CLSI guideline EP05-A2 section 10.8

Level = L1

Number of observations	32
Number of runs	16
Number of days	8
Runs per day	2
Replicates per run	2

CLSI guideline EP05-A2 section 10.4 recommends a minimum of 40 runs, with 2 replicates per run.

Mean	98.4

	SD	95% CI	CV	Allowable Total SD
Repeatability	1.0	0.7 to 1.5	1.0%	-
Between-run	1.0		1.0%	-
Between-day	1.6		1.6%	-
Within-laboratory	2.1	1.5 to 3.4	2.1%	19.7

Imprecision is less than allowable total imprecision: 20%.

Level = L2

Number of observations	32
Number of runs	16
Number of days	8
Runs per day	2
Replicates per run	2

CLSI guideline EP05-A2 section 10.4 recommends a minimum of 40 runs, with 2 replicates per run.

Mean	191.7

	SD	95% CI	CV	Allowable Total SD
Repeatability	1.4	1.0 to 2.1	0.7%	-
Between-run	1.2		0.6%	-
Between-day	3.6		1.9%	-
Within-laboratory	4.0	2.8 to 7.2	2.1%	38.3

Imprecision is less than allowable total imprecision: 20%.

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Level = L3

Number of observations	32
Number of runs	16
Number of days	8
Runs per day	2
Replicates per run	2

CLSI guideline EP05-A2 section 10.4 recommends a minimum of 40 runs, with 2 replicates per run.

Mean	284.2

	SD	95% CI	CV	Allowable Total SD
Repeatability	1.9	1.4 to 2.8	0.7%	-
Between-run	3.2		1.1%	-
Between-day	5.2		1.8%	-
Within-laboratory	6.4	4.5 to 11.1	2.3%	56.8

Imprecision is less than allowable total imprecision: 20%.

6.3 Acceptance criteria:

Total allowable error (TAE %) of 20%, was selected as the acceptance criteria for this assay following CLIA proficiency guidelines as printed in the Federal Register February 28, 1992;57(40):7002-186, and the American Proficiency Institute Peer Data for 2013 CHEMISTRY / IMMUNOLOGY / IMMUNOHEM -1ST EVENT. Allowable bias was calculated as the residual error budget after precision values (CV %) were subtracted from TAE (%). Values in brackets indicate the closest/corresponding API levels obtained from 2013 proficiency data.

Table II:Total Allowable Error (%)

	Level 1	Level 2	Level 3
TAE%	20	20	20
CV (%)	2.1	2.1	2.3
Allowable Bias (%)	16.9	16.9	16.7

7 BIAS ESTIMATION: ANTICOAGULANT COMPARISON

7.1 Since the predicate method has been validated for EDTA-plasma, an anticoagulant comparison is not required.

8 METHOD COMPAIRSON SIEMENS VS THERANOS METHODS

8.1 Because the ADVIA system allows users to extend the reportable range, a pre-dilution of sample is valid, and the volume of sample obtained from a fingerstick in sufficient for testing on the system. To verify the comparability of fingerstick blood to venous blood, 20 unique patients donated 2 venous tubes of blood and 2-4 fingerstick samples in EDTA. Each sample of venous blood was tested and the 2 results were used as replicate tests. Fingerstick samples were pooled and tested in replicates of 2. All samples should be within the reference range, and were also subject to the reference range criteria.

8.2 Calculated concentrations are based on the mean of 2 replicate tests.

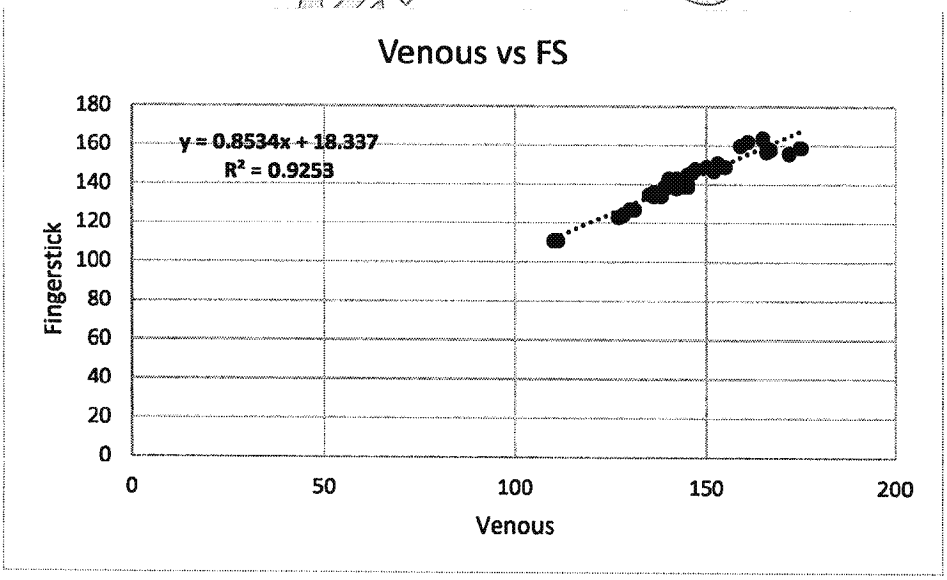
8.3 Acceptance Criteria:

8.3.1 All samples must have %CV within 20%

8.3.2 Scatter plots should have a slope of 1 +/- 0.15 and R^2 greater than 0.9

8.3.3 Regression equation for BCD versus matched venous samples is shown below and passes acceptance criteria.

Figure 1: Venous vs FS method comparison



9 REFERENCE RANGE VERIFICATION

9.1 20 unique venous and matched capillary tube and nanotainer (CTN) samples were collected and assayed in duplicate using the predicate and Theranos methods, and the average value was calculated. All samples were collected in EDTA. Values obtained from venous blood that fell out of the Siemens published reference range, as well as the corresponding values from matched CTN samples, were excluded from analysis. Of the 20 values obtained, all 20 were included for analysis, and all 20 (100%) fell within the Siemens published reference range, 76-214 mg/dL which is inclusive of male and female reference ranges, (79-169 mg/dL (M) and 76-214 mg/dL (F) The RR is therefore verified (CLSI guidance C28-A3c).

9.2 The raw data is as follows:

Donor	Rep	EDTA Plasma	Average
1	1	143	143
	2	143	
2	1	149	149.5
	2	150	
3	1	177	178
	2	179	
4	1	128	128.5
	2	129	
5	1	124	124
	2	124	
6	1	116	115.5
	2	115	
7	1	163	163.5
	2	164	
8	1	136	135.5
	2	135	

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9	1	134	134
	2	134	
10	1	124	124.5
	2	125	
11	1	126	126.5
	2	127	
12	1	141	141.5
	2	142	
13	1	139	139
	2	139	
14	1	122	122.5
	2	123	
15	1	134	135
	2	136	
16	1	120	121
	2	122	
17	1	126	126.5
	2	127	
18	1	170	170.5
	2	171	
19	1	134	133
	2	132	
20	1	135	135
	2	135	

9.3 The reference range verification raw data is as follows:

10 REFERENCES

10.1 Code of Federal Regulations, Title 42, Chapter IV, Subchapter G, Part 493, Subpart K, Sections 493.1217, 493.1253, and 493.1255.

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10.4 R (version 2.13.1). The R Foundation for Statistical Computing, 07/08/2011.

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10.6 Dexter-Immunoassay (version1.0), Theranos, Inc., 2009.

10.7 EP10-A3, Preliminary Evaluation of Quantitative Clinical Laboratory Measurement Procedures; Approved Guideline—Third Edition, 2006, Clinical and Laboratory Standards Institute, Wayne, PA.

10.8 EP15-A2, User Verification of Performance for Precision and Trueness; Approved Guideline—Second Edition. 2005, Clinical and Laboratory Standards Institute, Wayne, PA.

10.9 EP09-A2-IR, Method Comparison and Bias Estimation Using Patient Samples; Approved Guideline—Second Edition (Interim Revision), 2010, Clinical and Laboratory Standards Institute, Wayne, PA.

10.10 EP05-A2, Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline—Second Edition, 2004, Clinical and Laboratory Standards Institute, Wayne, PA.

10.11 EP06-A, Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline, 2003, Clinical and Laboratory Standards Institute, Wayne, PA.

10.12 EP7-A2, Interference Testing in Clinical Chemistry; Approved Guideline—Second Edition, 2004, Clinical and Laboratory Standards Institute, Wayne, PA.

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