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Anti –SSA (Ro) Extractable Nuclear Antigen (ENA) Antibody (IgG) Qualitative Assay Development Report

Theranos, Inc

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1. ASSAY INFORMATION[TC "ASSAY INFORMATION" \F C \L "2"]

1.1 Assay Specifications[TC "Assay Specifications" \f C \l "3"]

This assay is designed to qualitatively determine anti-SSA (Ro)(ENA) antibodies (IgG) in human plasma and serum.

1.1.1 Reference Assays [TC "Reference Assays and Standards" \f C \l "3"]

The following commercial ELISA kits have been used in house as predicate methods:

- INOVA Quantalite SSA ELISA (Cat# 708575)
- ImmuLisa™ SS-A (Ro) antibody Enhanced ELISA(Cat#5128)
- IBL International SSA ELISA (Cat# RE75231)

1.1.2 Materials and Methods[TC "Materials and Methods" \f C \l "1"]

SSA antigen coated surface serves as the capture surface for the Anti-SSA ENA antibody assay. The sample (plasma or serum) is diluted and then incubated on the capture surface for 5 minutes, the surface is washed, and then an alkaline phosphatase(AP)-labeled anti-human IgG antibody is incubated on the surface for 5 minutes. After the detection antibody incubation, another washing cycle is performed and the alkaline phosphatase substrate is incubated on the surface for 5 minutes, and the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Antigen R0-52 (SS-A)	Genway	10-663-45615
Mouse Anti-Human IgG1 Antibody	Novus Biologicals	NB100-2046
Alkaline Phosphatase Labeling Kit	Dojindo	LK13-10
Phospho Glo Substrate	KPL	55-60-04
Heterophilic Blocking Reagent	Scantibodies, Inc.	3KC533
Blocking Buffer (3% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G

2. ASSAY DEVELOPMENT[TC "ASSAY OPTIMIZATION" \F C \L "2"]

1.1 Effect of Capture Antigen Conjugation on Assay Response [TC " Effect of Capture Antigen Conjugation on Assay Response " \f C \l "1"]

A biotin conjugate version and unconjugated versions of the SSA antigen were tested as capture surface. The biotin conjugate was coated on an avidin surface followed by blocking. The unconjugated antigen was coated directly followed by a blocking step. The two surfaces were tested against a positive control sample containing anti-SSA antibodies and an autoimmune negative control sample obtained from a commercial source. 5 normal donor plasma samples were pooled and used as a negative control as well. An anti-human IgG detection antibody AP conjugate was used at a concentration of 100 ng/mL in Biostab. The response to both the surfaces was good enough that either could be picked as the final capture antigen surface. The biotin conjugated antigen surface was finalized as a starting point upon which further optimizations were carried out. The results are summarized in Table 2

Table [SEQ Table * ARABIC]: Effect of capture antigen surface on assay response.

	SSA Antigen Direct Coated Surface		**Biotin Conjugated SSA Antigen**	
Control	**Inter-Cartridge RLU**			
	Mean	**CV%**	**Mean**	**CV%**
Positive	304222	15	497759	9
Negative Control	1357	20	314313	13
Pooled normals	1680	2	60310	17
Positive control/negative control	224		120	
Positive control/pooled normal	181		69	

[LINK Excel.Sheet.12 "\\\\theranos.local\\folders\\Projects\\Experiment Log\\E0700 - E0799\\E0728\\Anti SSA_assay development report.xlsx" "Test Biotin conjugation!R79C2:R88C6" \a \f 4 \h]

1.2 Capture Antigen Surface Titration [TC " Capture Antigen Surface Antigen " \f C \l "1"]

The biotinylated antigen surface was titrated at levels: 5, 2.5 and 1 µg/mL. Table 3 summarizes the results. 5 µg/mL provides the best modulation between the pooled positive and pooled normal clinical samples and was finalized as the capture antigen surface concentration.

Table [SEQ Table * ARABIC]: Capture Antigen Surface Titration

	5 µg/mL		2.5 µg/mL		1 µg/mL	
Control	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Positive Control	530169	4	430791	16	344792	11
Pooled Positive	34039	5	31109	4	20630	3
Pooled normals	5338	11	5448	22	5532	30
Negative Control	3279	10	3674	14	3873	38
Positive control/negative control	162		117		89	
Positive control/pooled normal	99		79		62	
Pooled positive /pooled normal	6.4		5.7		3.7	

1.3 Effect of Detection Conjugate Stabilizer

Two commercial and one in house formulated alkaline phosphatase stabilizers were tested as detection antibody diluents, with the anti-human IgG DAb at 100 ng/mL. The samples were diluted 150 into 3% BSA in TBS Blocking Buffer . Signal modulation was best with the Theranos Dab stabilizer. Table 4 summarizes the results.

Table [SEQ Table * ARABIC]: Effect of Detection Conjugate Stabilizer

	Biostab		StabilZyme		Theranos AP Conjugate Stabilizer	
Control	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Positive Control	674300	7	261199	8	421435	4
Pooled Positive	13492	17	4969	13	5620	22
Pooled normals	3659	9	2792	36	892	29
Negative Control	2678	13	2437	18	545	34
Positive control/negative control	252		107		773	
Positive control/pooled normal	184		94		473	
Pooled positive /pooled normal	3.7		1.8		6.3	

1.4 Detection antibody Titration

The AP conjugated detection antibody was titrated in the Theranos AP conjugate stabilizer. The best modulation between the positive and negative control was achieved with 50 ng/mL of the anti-IgG Dab.

Table [SEQ Table * ARABIC]: Detection Conjugate Titration

	25 ng/mL		50 ng/mL		100 ng/mL		200 ng/mL	
Control	**Inter-Cartridge RLU**							
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Positive Control	843607	5	324509	12	421435	4	108475	13
Pooled Positive	11791	6	3890	7	5620	22	1449	17
Pooled normals	1329	30	496	38	892	29	298	17
Negative Control	1033	11	308	4	545	34	229	10
Positive control/negative control	817		1054		773		475	
Positive control/pooled normal	635		654		473		364	
Pooled positive /pooled normal	8.9		7.8		6.3		4.9	

1.5 Effect of Sample Dilution[TC “Effect of Sample dilution" \f C \l "1"]

The effect of sample dilution was tested with final sample dilution factors of 1:25, 1:50 and 1:100 into 3% BSA in TBS blocking buffer. Modulation between pooled positive and negative sera was best at both 25 and 50 fold sample dilution. as a result of a greater reduction in the signal from negative samples compared to the reduction in signal from the positive samples. Results are summarized in Table 6.

Table [SEQ Table * ARABIC]: Effect of sample dilution

	25x		50x		100x	
Control	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Positive Control	537935	6	421435	4	144921	18
Pooled normals	664	19	892	29	411	11
Negative Control	363	22	545	34	312	18
Positive control/negative control	1483		773		465	
Positive control/pooled normal	811		473		353	

1.6 Effect of changing reagent incubation time [TC "Effect of changing reagent incubation time" \f C \l "1"]

The effect of shorter reagent incubation times was tested with sample, detection conjugate and substrate incubation times respectively of 10, 10, 10; 5, 5, 5; and 2, 2, 1 minutes. Assay modulation was best at 5,5,5 minute incubation protocol and this was chosen as the final condition.

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Table [SEQ Table * ARABIC]: Effect of Changing Reagent Incubation Time

	5x5x5		2x2x1		1x1x1	
Control	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Positive Control	270118	9	25395	13	12525	13
Pooled normals	648	6	193	10	202	7
Negative Control	463	33	254	23	173	8
Positive control/negative control	584		100		73	
Positive control/pooled normal	417		131		62	

1.7 Effect of HBR in assay diluent

The effect of adding Heterophilic blocking reagent (HBR) to the assay diluent was tested. The modulation between the pooled positive samples and the pooled normals increased slightly with the addition of HBR. The assay response remained the same overall. It was decided to include HBR in the diluent since it does not affect the overall response negatively and might help in mitigating any non-specific binding. Table 8 summarizes the data.

Table [SEQ Table * ARABIC]: Effect of HBR in assay diluent

	Control diluent (Blocking buffer)		**Blocking buffer plus 400 µg/mL HBR**	
Control	**Inter-Cartridge RLU**			
	Mean	**CV%**	**Mean**	**CV%**
Positive Control	270118	9	271400	5
Pooled Positive	3342	5	4499	5
Pooled normals	648	6	725	14
Negative Control	463	33	541	21
Positive control/negative control	584		501	
Positive control/pooled normal	417		374	
Pooled positive /pooled normal	5.2		6.2	

1.8 HBR titration in diluent

A set of normal samples, clinical samples as well as the positive and negative controls were assayed at 400 and 200 µg/mL of HBR spiked into the assay diluent, the control data had the assay diluent without HBR. Data is summarized in Table 9. 200 µg/mL of HBR was finalized as the final concentration of HB R in the assay diluent based on the increase in modulation between the pooled positive and pooled normals samples as well as the positive and negative controls.

Table [SEQ Table * ARABIC]: HBR titration in assay diluent

	[HBR], ug/mL	0		200		400	
	Sample	Mean RLU	%CV	Mean RLU	%CV	Mean RLU	%CV
Normal sera (stanford donor)	M1	746	14	718	24	957	22
	M2	665	11	565	10	616	13
	M3	598	4	540	9	563	14
	M4	728	15	573	7	681	15
	M5	1234	17	1318	12	1374	19
	M6	801	9	774	14	712	9
	Mean RLU of normals	795		748		817	
Clinical samples	SL01	556375	19	633435	16	578291	28
	SL08	6426	2	8055	25	7808	20
	SL05	5803	6	12677	34	6298	15
	Sjogren01	168518	9	108901	1	91629	10
	Sjogren03	796888	5	821409	26	869187	23
	Sjogren05	491274	16	676276	27	609807	25
	Mean RLU of positive samples	337547		376793		360503	
Biorad Controls	Positive control (BioRad)	215196	22	237346	13	193606	21
	Negative control (BioRad)	454	11	343	25	419	22
	Modulation(Mean pos/Mean normals)	424.3		503.7		441.2	
	Modulation (postive ctrl/neg ctrl)	474		692		462	
	Modulation(Mean pos/neg ctrl)	743		1099		860	

1.9 Normal sample screen: Cut off Determination

Normal donor plasma (N=20) were obtained and tested in the three commercial ELISA kits and in the Theranos System. The Theranos cutoff value was determined by taking the mean RLU of the normal samples plus 5 times the standard deviation of the 20 normal samples (Table 10). The sample RLU divided by the cutoff value yields the Antibody Index. The following criteria was applied to categorize the result as positive (red), negative (green) or borderline (yellow).

Ab Index > 1.1
Ab Index > 0.9, < 1.1
Ab Index < 0.9

Out of the 20 normals tested all were negative on the Theranos assay based on the aforementioned cutoff computation. These same samples were all negative on the 3 ELISa kits and showed excellent correlation with the Theranos result (Table 11).

Table [SEQ Table * ARABIC]: Theranos Anti-SSA assay: cut off determination

Samples	Inter-Cartridge Mean	CV%	Ab Index
M1	685	18	0.35
M2	598	10	0.31
M3	540	9	0.28
M4	573	7	0.30
M5	1318	12	0.68
M6	774	14	0.40
M7	555	13	0.29
M8	1454	18	0.75
M9	622	12	0.32
M10	783	19	0.40
M11	806	7	0.42
M12	613	24	0.32
M13	490	13	0.25
M14	752	21	0.39
M15	635	13	0.33
M16	635	11	0.33
M17	588	2	0.30
M18	686	9	0.35
M19	630	25	0.32
M20	607	15	0.31
MEAN	717		
CUT OFF	1940		

Table [SEQ Table * ARABIC]: Normal sample correlation: Theranos vs. 3 Commercial Anti-SSA ELISAs

Samples	Inter-Cartridge Mean	Inter-Cartridge CV%	Theranos Ab Index	Innova kit	Immco	IBL International
				Units	Result (EU/mL)	Ratio
M1	685	18	0.35	4.1	3	0.23
M2	598	10	0.31	4.1	3	0.26
M3	540	9	0.28	3.7	3	0.17
M4	573	7	0.30	3.4	3	0.22
M5	1318	12	0.68	3.6	3	0.26
M6	774	14	0.40	4.9	4	0.46
M7	555	13	0.29	4.6	5	0.59
M8	1454	18	0.75	4.4	4	0.27
M9	622	12	0.32	4.2	3	0.33
M10	783	19	0.40	3.6	5	0.49
M11	806	7	0.42	4.0	5	0.39
M12	613	24	0.32	4.1	2	0.31
M13	490	13	0.25	3.7	3	0.23
M14	752	21	0.39	3.6	2	0.21
M15	635	13	0.33	3.8	4	0.21
M16	635	11	0.33	4.8	3	0.23
M17	588	2	0.30	3.8	2	0.19
M18	686	9	0.35	4.0	3	0.21
M19	630	25	0.32	3.6	3	0.27
M20	607	15	0.31	3.5	3	0.26

1.10 Clinical Sample Correlation

N=25 samples obtained from 10 Lupus, 10 Scleroderma and 5 Sjogren's syndrome patients were tested on the Theranos Anti-SSA assay. The results are summarized in Table 12. The same samples were run on three commercial Anti-SSA ELISAs and the correlation of the results to the Theranos assay is reported in Table 13. Excellent correlation was seen for all 25 samples.

Table [SEQ Table * ARABIC]: N=25 Clinical samples: Theranos Anti SSA assay result

Samples	Inter-Cartridge		Theranos
	Mean	CV%	Ab index
Scleroderma 1	9693	19	5.0
Scleroderma 2	1231	13	0.6
Scleroderma 3	1077	5	0.6
Scleroderma 4	849	30	0.4
Scleroderma 5	607	3	0.3
Scleroderma 6	952	18	0.5
Scleroderma 7	679	10	0.3
Scleroderma 8	845	19	0.4
Scleroderma 9	643	9	0.3
Scleroderma 10	2185	18	1.1
SLE 1	412775	24	212.8
Sle 2	938	9	0.5
SLE 3	789	14	0.4
SLE 4	4884	13	2.5
SLE 5	610	1	0.3
SLE 6	1799	4	0.9
SLE 7	1075	12	0.6
SLE 8	3230	19	1.7
SLE 9	1254	4	0.6
SLE 10	787	10	0.4
Sjogrens 1	64349	14	33.2
Sjogrens 2	651	11	0.3
Sjogrens 3	724156	17	373.3
Sjogrens 4	28564	20	14.7
Sjogrens 5	394169	2	203.2

Table [SEQ Table * ARABIC]: Clinical Samples on Theranos vs. Commercial Anti-SSA ELISAs

	Innova	Immco	IBL Int.	Theranos
Sample	Units	Result (EU/mL)	Ratio	Ab Index
Scl01	42.1	53	0.28	5.0
SCL02	3.9	4	0.38	0.6
ScL03	8.5	9	0.16	0.6
ScL04	17.8	10	0.42	0.4
ScL05	3.8	3	0.44	0.3
ScL06	3.7	2	0.24	0.5
ScL07	4.5	2	0.23	0.3
ScL08	3.7	2	0.22	0.4
ScL09	5.1	5	0.33	0.3
ScL10	17.4	8	0.29	1.1
SL01	102.9	78	5.51	212.8
SL02	4.7	5	0.30	0.5
SL03	3.8	3	0.23	0.4
SL04	70.8	66	5.50	2.5
SL05	3.6	2	0.58	0.3
SL06	59.5	49	5.29	0.9
SL07	4.5	7	0.24	0.6
SL08	86.1	69	5.32	1.7
SL09	3.6	11	0.67	0.6
SL10	3.7	3	0.19	0.4
Sjo01	108.6	57	5.87	33.2
Sjo02	3.5	3	0.23	0.3
Sjo03	125.6	74	6.22	373.3
Sjo04	24.2	57	2.45	14.7
Sjo05	96.9	78	5.61	203.2

1.11 Specificity

The specificity of the Anti SSA assay was established by assaying a reference serum panel provided by the Centers for Disease Control (CDC). The panel comprises 11 serum samples that have been annotated with the presence of specific ENA antibodies. The results indicate that the Theranos Anti-SSA assay demonstrates specificity only against SSA antibodies as seen by the positive results shown by CDC ref sera # 2 and 7.

Table [SEQ Table * ARABIC]: Specificity

	Information from CDC datasheet		Mean	CV%	Ab index
	ENA antibodies	IFA pattern			
#1	native DNA, Sm, Sm/RNP	Homogeneous/rim	962	12	0.50
#2	SS-B/La, SSA 52, SSA 60	Speckled/La	5733	15	2.96
#3	RNP, Sm, Sm/RNP, SSB,SSA 60	Speckled	1718	23	0.89
#4	U1-RNP, Sm/RNP		1083	5	0.56
#5	Sm antigen, Sm RNP		1329	29	0.69
#6	U3-RNP	Nucleolar pattern	918	7	0.47
#7	SS-A/Ro		5113	16	2.64
#8	Centromere B	Centromere pattern	774	9	0.40
#9	Scl-70		952	20	0.49
#10	Jo-1		1643	13	0.85
#12	rRNP/Ribosomal P		1076	11	0.55

1.12 HAMA and Rf Positive Sample Testing

8 HAMA positive and 8Rf positive sera obtained from a commercial source were tested on the Theranos Anti-SSA assay. Out of the 16 samples tested, 3 were borderline and the remaining were negative for anti-SSA antibodies.

Table [SEQ Table * ARABIC]: HAMA and Rf positive sample screen

Samples	Inter-Cartridge Mean	CV%	Theranos Ab Index
HAMA positive			
H8	1626	8	0.84
H9	1344	18	0.69
H10	1702	1	0.88
H11	1681	16	0.87
H12	1638	5	0.84
H14	1469	8	0.76
H15	1786	16	0.92
H16	1900	1	1.0
Rf Positive			
RF1	932	12	0.48
Rf2	777	5	0.40
Rf3	853	22	0.44
Rf4	1903	9	0.98
Rf5	779	8	0.40
Rf6	553	27	0.29
Rf7	1222	16	0.63
Rf8	1382	24	0.7