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

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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-Sm (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 Sm ELISA (Cat# 708560)
- ImmuLisa™ Sm antibody Enhanced ELISA(Cat#5127)
- IBL International Sm ELISA (Cat# RE75221)

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

Sm antigen coated surface serves as the capture surface for the Anti-Sm ENA antibody assay. The sample (plasma or serum) is diluted and then incubated on the capture surface for 10 minutes, the surface is washed, and then an alkaline phosphatase(AP)-labeled anti-human IgG antibody is incubated on the surface for 10 minutes. After the detection antibody incubation, another washing cycle is performed and the alkaline phosphatase substrate is incubated on the surface for 10 minutes, and the resulting chemiluminescence is read in Relative Light Units (RLU).

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Antigen Sm	Arotec (Binding Site)	ATS02-10
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 Sm 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-Sm 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 biotin conjugated antigen surface provided better dose response compared to the unconjugated antigen. The biotin conjugated surface 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.

Control	Biotin conjugated		Unconjugated	
	Inter-Cartridge RLU			
	Mean	CV%	Mean	CV%
Positive control	218707	8	56474	32
Pooled positive samples	74710	18	57368	7
Pooled normal samples	15117	4	5801	0
Negative Control	4904	4	2472	37
Positive control/negative control	45		23	
Positive control/pooled normal	14		10	
Pooled positive /pooled normal	4.9		9.9	

[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 Sm antigen biotinylated surface was titrated at levels: 5, 2.5, 1 and 0.5µg/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 ug/mL		2.5 ug/mL		1 ug/mL		0.5 ug/mL	
Control	**Inter-Cartridge RLU**							
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Positive control	218707	8	56697	12	18644	24	7642	16
Pooled positive	74710	18	74346	4	81206	12	82870	11
Pooled normal	15117	4	8050	13	8743	3	8463	7
Negative Control	5654	22	3932	1	2955	6	4324	17
Positive control/negative control	39		14		6		2	
Positive control/pooled normal	14		7		2		1	
Pooled positive /pooled normal	4.9		9.2		9.3		9.8	

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 1:10 into 3% BSA in TBS Blocking Buffer. Signal modulation between the positive and negative control was best with both Biostab and the Theranos Dab stabilizer. The modulation between the pooled positive and pooled normal samples was highest with the Theranos AP conjugate stabilizer and hence this was chosen as the conjugate stabilizer. Table 4 summarizes the results.

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

Control	Biostab		StabilZyme		Theranos	
	Inter-Cartridge RLU					
	Mean	CV%	Mean	CV%	Mean	CV%
Positive control	300901	7	40521	45	111185	16
Pooled positive	70062	1	17602	18	35003	14
Pooled normal	16743	8	3551	19	6050	13
Negative Control	7039	4	3213	2	3547	7
Positive control/negative control	43		13		31	
Positive control/pooled normal	18		11		18	
Pooled positive /pooled normal	4.2		5.0		5.8	

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

Control	200 ng/mL		100 ng/mL		50 ng/mL		25 ng/mL	
	Inter-Cartridge RLU							
	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
Positive control	230505	21	101608	17	57868	9	24114	10
Pooled positive	60008	8	33588	15	21185	16	8200	6
Pooled normal	11098	25	6181	9	3186	5	1408	5
Negative Control	7126	28	3193	22	1563	14	774	5
Positive control/negative control	32		32		37		31	
Positive control/pooled normal	21		16		18		17	
Pooled positive /pooled normal	5.4		5.4		6.6		5.8	

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 1:5, 1:10 and 1:25 into 3% BSA in TBS blocking buffer. Modulation between positive control and negative sera was greatly improved when the diluti8on was lowered to 5 fold. This was finalized as the sample dilution. Results are summarized in Table 6.

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

Control	5x		10x		25x	
	Inter-Cartridge RLU					
	Mean	CV%	Mean	CV%	Mean	CV%
Positive control	151948	18	64156	11	28202	10
Pooled normal	2762	19	2320	12	1579	13
Negative Control	1220	11	1150	24	1059	35
Positive control/negative control	125		56		27	
Positive control/pooled normal	55		28		18	

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 10,10,10 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

	10,10,10		5,5,5		2,2,1	
Control	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Positive control	811846	15	224746	11	21639	16
Pooled normal	5129	13	2146	11	507	9
Negative Control	2208	7	785	86	315	22
Positive control/negative control	368		286		69	
Positive control/pooled normal	158		105		43	

1.7 Effect of HBR in assay diluent

The effect of adding Heterophilic blocking reagent (HBR) to the assay diluent was tested. The overall modulation between the pooled positive samples and the pooled normals decreased slwith the addition of HBR. It was decided to keep the control diluent as such without any added HBR. Table 8 summarizes the data.

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

	Control diluent (Blocking buffer)		Blocking buffer plus 200 µg/mL HBR		Blocking buffer plus 400 µg/mL HBR	
Control	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Positive Control	811846	15	766222	10	726660	17
Pooled Positive	7521	16	7692	2	6820	5
Pooled normals	1957	6	2135	2	2483	22
Negative Control	2208	7	2403	32	2227	11
Positive control/negative control	368		319		326	
Positive control/pooled normal	415		359		293	
Positive sample/neg ctrl	3.4		3.2		3.1	
Pooled positive /pooled normal	3.8		3.6		2.7	

1.8 Normal sample screen: Cut off Determination

Normal donor plasma (N=15) 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 15 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 15 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-SM assay: cut off determination

Samples	Inter-Cartridge Mean	CV%	Ab Index
M1	2761	10	0.30
M2	1607	11	0.17
M3	1562	16	0.17
M4	1910	9	0.21
M5	1922	4	0.21
M6	4792	6	0.52
M7	1682	9	0.18
M8	6324	7	0.69
M9	1662	9	0.18
M10	1874	13	0.20
M11	2076	13	0.23
M12	1393	12	0.15
M13	1890	28	0.20
M14	1691	17	0.18
M15	1704	8	0.18
MEAN	2323		
CUT OFF	9223		

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

Samples	Inter-Cartridge Mean	Inter-Cartridge CV%	Theranos Ab Index	IMMCO Result (EU/mL)	INNOVA Units	IBL Int Ratio
M1	2761	10	0.30	6	4.7	0.17
M2	1607	11	0.17	6	5.3	0.63
M3	1562	16	0.17	3	4.1	0.25
M4	1910	9	0.21	6	5.1	0.47
M5	1922	4	0.21	12	4.1	0.33
M6	4792	6	0.52	15	5.3	1.07
M7	1682	9	0.18	7	4.1	0.46
M8	6324	7	0.69	10	4.1	0.45
M9	1662	9	0.18	6	4.1	0.35
M10	1874	13	0.20	12	5.6	0.69
M11	2076	13	0.23	8	4.4	0.66
M12	1393	12	0.15	11	5.7	0.66
M13	1890	28	0.20	10	4.7	0.40
M14	1691	17	0.18	3	4.1	0.30
M15	1704	8	0.18	7	4.1	0.35

1.9 Clinical Sample Correlation

A total of 104 samples samples obtained from Lupus, Scleroderma, Sjogren's syndrome patients as well as ANA positive samples were screened on commercial Sm antibody ELISAs. Only a fraction of these samples tested positive. A clinical sample set of N=18 samples was chosen from among these and tested on the Theranos Anti-SM assay. The correlation of the results to the Theranos assay is reported in Table 12.

Table [SEQ Table * ARABIC]: N=18 Clinical samples: Theranos Anti SM assay result

Samples	Inter-Cartridge Mean	CV%	Theranos Ab Index	INNOVA Units	IBL Int. Ratio	IMMCO Result (EU/mL)
SL4	9842	15	1.07		0.52	14
SL7	17081	11	1.85		1.75	30
SL9	9210	8	1.00		1.90	26
Sjog7	5095	16	0.55	4.2	1.16	12
Sjog9	3153	2	0.34	6.5	0.97	26
Sjog10	3250	3	0.35	5.3	1.51	18
Scleroderma 1	5774	18	0.6			13
Scleroderma 2	4931	41	0.5	TBD	TBD	8
Scleroderma 3	7841	16	0.9			6
Scleroderma 4	6169	4	0.7			5
Scleroderma 5	18168	22	2.0			22
Scleroderma 6	5064	62	0.5			7
Scleroderma 7	7702	9	0.8			17
Scleroderma 8	8490	4	0.9			7
Scleroderma 9	5156	19	0.6			7
Scleroderma 10	8903	3	1.0			7
Scleroderma 11	19170	10	2.1			24
Scleroderma 14	222202	6	24.1			29

1.10 Specificity

The specificity of the Anti SM 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-SM assay demonstrates specificity only against SM antibodies as seen by the positive results shown by CDC ref sera # 1, #3, #4 and #5.

Table [SEQ Table * ARABIC]: Specificity

CDC ref sera	Human antibodies against?	Corresponding IFA pattern	Inter-Cartridge Mean	Inter-Cartridge CV%	Theranos Ab index
	Information from CDC datasheet				
#1	native DNA, Sm, Sm/RNP	Homogeneous/rim	10639	17	1.15
#2	SS-B/La, SSA 52, SSA 60	Speckled/La	6577	6	0.71
#3	RNP, Sm, Sm/RNP, SSB,SSA 60	Speckled	1282266	13	139.02
#4	U1-RNP, Sm/RNP		13031	10	1.41
#5	Sm antigen, Sm RNP		2001042	13	216.96
#6	U3-RNP	Nucleolar pattern	8335	2	0.90
#7	SS-A/Ro		7715	10	0.84
#8	Centromere B	Centromere pattern	6100	1	0.66
#9	Scl-70		5347	9	0.58
#10	Jo-1		8214	6	0.89
#12	rRNP/Ribosomal P		3057	15	0.33

1.11 HAMA and Rf Positive Sample Testing

6 HAMA positive and Rf positive sera obtained from a commercial source were tested on the Theranos Anti-Sm assay. These were also tested on 2 commercial ELISAs. The results indicate that there is excellent correlation.

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

Samples	Inter-Cartridge Mean	CV%	Theranos Ab Index	Immco Result (EU/mL)
H6	3188	7	0.35	10
H15	10551	7	1.14	31
H16	6793	56	0.74	5
H17	7373	2	0.80	10
H18	1468	10	0.16	7
H19	3700	19	0.40	15

Table [SEQ Table * ARABIC]: RF positive sample screen

Samples	Inter-Cartridge Mean	CV%	Theranos Ab Index	Immco Result (EU/mL)
RF18	6687	72	0.72	6
Rf19	7778	8	0.84	9
Rf20	3292	15	0.36	4
Rf21	5681	16	0.62	5
Rf22	5693	47	0.62	4
Rf23	9166	16	0.99	7