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ANA (Anti -Nuclear Antibodies) 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-nuclear antibodies (ANA) (IgG) in human whole blood, 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 ANA ELISA (Cat# 708750)
- Immco Diagnostics Immulisa anti-ANA Ab Screen ELISA (Cat# 1175)
- IBL International ANA-Hep2 Screen ELISA (Cat# 70151)
- US Biological ANA Screen ELISA (Cat# A229812K)

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

A mixture of an 8 anti-nuclear antigen coated surface serves as the capture surface for the ANA IgG assay. The sample (whole blood, 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 1: RNP/Sm	Arotec	ATR01-02
Antigen 2: Jo-1	Genway	10-663-45294
Antigen 3: R0-52 (SS-A)	Genway	10-663-45615
Antigen 4: Scl -70 antigen native	Genway	11-511-248357
Antigen 5: La(SSB) antigen	Arotec	ATL01
Antigen 6: CENP B antigen	Arotec	ATC02-02
Antigen 7: dsDNA (plasmid)	Fitzgerald	30R-AD006
Antigen 8: Sm	Arotec	ATS02-10
Mouse Anti-Human IgG1 Antibody	Novus Biologicals	NB100-2046
Alkaline Phosphatase Labeling Kit	Dojindo	LK13-10
Phospho Glo Substrate	KPL	
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 Capture Surface Screen [TC "Capture Surface screen" \f C \l "1"]

Two combinations of antigens were tested as capture surface. The first comprised of a mixture of 8 antigens (dsDNA, Sm, RNP/Sm, Ro-52(SSA), La (SSB), Scl70, Jo-1 and CENPB) and the second comprised of the above mixture as well as genomic DNA extracted from Hep 2 cells. The Hep 2 cell line is the substrate of choice since it has a high level of expression of several nuclear antigens. The two surfaces were tested against 11 samples of CDC reference sera which are specific for the most commonly occurring ANA antigens. The screen was performed on the Theranos system. The final concentration of the mixture on the capture surface was 5 ug/mL in carbonate bi carbonate buffer. An anti-human IgG detection antibody AP conjugate was used at a concentration of 100 ng/mL in blocking buffer. This test was also repeated with commercially available positive and negative antibody controls as well as controls that produced specific IFA patterns. The responses to the CDC reference sera were similar on both the capture surfaces. Since the second capture surface included an extra step of trypsinizing and genomic extraction from Hep 2 cells it was decided to pursue the first capture surface option. The results are summarized in Table 2 and Table 3.

Table [SEQ Table * ARABIC]: Capture Surface screen on Theranos System- CDC Reference Sera

			Capture surface (i)		Capture Surface (ii)	
CDC ref sera	**Human antibodies against?**	**Corresponding IFA pattern**	**Inter-Cartridge**		**Inter-Cartridge**	
	Information from CDC datasheet		**Mean**	**CV%**	**Mean**	**CV%**
#1	native DNA, Sm, Sm/RNP	Homogeneous/rim	41600	11	42593	11
#2	SS-B/La, SSA 52, SSA 60	Speckled/La	623542	12	738999	10
#3	RNP, Sm, Sm/RNP, SSB,SSA 60	Speckled	453841	3	458369	14
#4	U1-RNP, Sm/RNP		219522	19	347124	9
#5	Sm antigen, Sm RNP		263689	10	291984	16
#6	U3-RNP	Nucleolar pattern	3770	10	5483	13
#7	SS-A/Ro		26358	16	29222	5
#8	Centromere B	Centromere pattern	229076	12	235867	13
#9	Scl-70		161475	7	197112	10
#10	Jo-1		331606	17	352854	12
#12	rRNP/Ribosomal P		3325	8	6126	3

Table [SEQ Table * ARABIC]: Capture Surface screen on Theranos System- Positive and Negative controls

		Capture surface (i)		Capture Surface (ii)	
Source	**Standard /Control**	**Inter-Cartridge**		**Inter-Cartridge**	
	Autoantibodies against	**Mean**	**CV%**	**Mean**	**CV%**
Bio Rad	SSB	484537	10	339393	13
	Sm	94135	19	140089	18
	RNP	84077	27	95419	5
	Scl70	20472	6	22954	13
	SSA	355172	11	378427	23
	nDNA	12841	9	18880	6
Antibodies, Inc.	Homogeneous	58656	19	60247	15
	Centromere	436446	7	385401	19
	Speckled	224383	2	166959	2
	Endpoint titre	2994		3820	11
	Mitotic Spindle	6394		4634	16
	Nuceolar	5262		5490	11
	Biorad negative control	1247	8	1390	7
	Antibodies Inc. Negative Ctrl	1072		2570	7
	Blocking buffer	1044	10	1020	10

1.2 Detection antibody Screen

Three clones of anti-human IgG conjugated to AP were screened as possible detection partner for the ANA ELISA. The clone 2c11 shown below performed the best and afforded the highest S/B and modulation and was finalized as the detection antibody for this assay.

Table [SEQ Table * ARABIC]: Plasma Screening (MTP)

	Clone 2c11		Clone JDC-10		Clone H2	
Control	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Speckled	588332	7	2949645	2	3392949	1
Centromere	172789	11	613101	5	1251840	14
Homogeneous	65078	6	114128	4	730743	13
Negative Control	3412	9	74784	19	101448	5
S/B	172		39		33	

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

The mixture of 8 antigens was titrated at 3 levels: 5, 2.5 and 1 µg/mL. The coating condition at 5 µg/mL was picked as the final condition for the capture surface since it afforded the best modulation between the positive and negative control samples.

Table [SEQ Table * ARABIC]: Capture Surface Titration

	5 µg/ml		2.5 µg/ml		1 µg/ml	
Control	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Speckled	497681	12	497759	9	333270	9
Centromere	313074	10	314313	13	320075	14
Homogeneous	57250	10	60310	17	53324	15
Negative Control	3192	9	3435	4	3757	7
S/B	156		145		89	

1.4 Effect of Alkaline phosphatase conjugate stabilizer[TC "Effect of alkaline phosphatase conjugate stabilizer " \f C \l "1"]

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 1:25 into 3% BSA in TBS Blocking Buffer . Signal modulation as best with Biostab.

Table [SEQ Table * ARABIC]: Effect of AP conjugate stabilizers

Control	In house		StabilZyme		Biostab	
	Inter-Cartridge RLU					
	Mean	CV%	Mean	CV%	Mean	CV%
Speckled	392998	21	1608676	8	371948	14
Homogeneous	84984	2	747658	10	60514	10
Negative Control	3412	9	78451	6	2234	17
S/B	115		21		167	

1.5 Detection Antibody titration

The AP conjugated detection antibody was titrated in Biostab. The best modulation between the positive and negative control was achieved with 100 ng/mL of the anti-IgG Dab.

Table [SEQ Table * ARABIC]: Detection conjugate titration

Control	100 ng/ml		50 ng/ml		10 ng/mL		5 ng/mL	
	Inter-Cartridge RLU							
	Mean	CV%	Mean	CV%	Mean	CV%	Mean	CV%
Speckled	574942	2	371948	14	71629	12	35399	6
Homogeneous	94471	3	60514	10	11924	11	5634	5
Negative Control	2660	2	2234	17	595	40	339	13
S/B	216		167		120		104	

1.6 Effect of sample dilution

The effect of sample dilution was tested with final sample dilution factors of 1:25, 1:10 and 1:50 into 3% BSA in TBS blocking buffer. Modulation between pooled positive and negative sera was best at 1:25 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 8.

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

	25x		10x		50x	
Control	**Inter-Cartridge RLU**					
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
Speckled	574942	2	838037	9	499786	10
Homogeneous	94471	3	237692	3	60513	11
Negative Control	2660	2	4205	19	3844	15
S/B	216		199		130	

1.7 Effect of changing reagent incubation times

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 between the pooled ANA positive clinical sampels and the pooled normal plasma samples improved slightly with the 2,2,1 incubation times. The 2,2,1 minute incubation protocol was chosen as the final condition.

Table [SEQ Table * ARABIC]: Effect of reagent incubation times

	10,10,10		5,5,5		2,2,1			
Control	**Mean**	**CV%**	**Mean**	**CV%**	**Mean** Tip 1	**CV%**	**Mean** Tip 2	**CV%**
Speckled	288942	7	105738	1	14451	12	20481	6
Homogeneous	84854	8	24678	13	2961	18	3519	30
Negative Control	2326	15	905	16	378	6	554	27
Pooled donor plasma	42046	2	12811	5	1692	22	1808	8
Ctrls mod Positive ctrl/pooled plasma	124 / 6.9		117 / 8.3		38 / 8.5		37 / 11.3	

1.8 Effect of post sample wash

The effect of a post sample wash was tested with the capture surface coated at 5 µg/mL of the mixture of 8 Ana, a 1:25 sample dilution, and the detection antibody at 100 ng/mL in Biostab. The assay was optimized with a post sample wash since this afforded the best modulation between the positive control and pooled positive samples.

Table [SEQ Table * ARABIC]: Effect of post sample wash

	One Post sample wash				**No post sample wash**			
Control	**Inter-Cartridge**				**Inter-Cartridge**			
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
	Tip 1		Tip 2		Tip 1		Tip 2	
Speckled	35275	14	36858	9	40941	2	37124	19
Homogeneous	3437	20	3458	17	4408	6	4958	4
Pooled donor plasma	3156	16	3034	24	5236	10	5001	27
Negative Control	389	5	453	9	559	11	589	4
Ctrls mod	**91**		**81**		**73**		**63**	
Positive ctrl/pooled plasma	**11.2**		**12.1**		**7.8**		**7.4**	

1.9 Effect of assay diluent

Different commercially available blockers were tested as diluents for the assay and data was compared to the control diluent which is blocking buffer in TBS. The control condition produced the best modulation all other diluents produced a much diminished response. Blocking buffer in TBS was finalized as the diluent.

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

	Control: Blocking buffer diluent				**Blocking buffer + 400µg/ml HBR**				**Starting Block**				**Sea Block**			
Control	**Inter-Cartridge**				**Inter-Cartridge**				**Inter-Cartridge**				**Inter-Cartridge**			
	Mean	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**	**Mean**	**CV%**
	Tip 1		Tip 2		Tip 1		Tip 2		Tip 1		Tip 2		Tip 1		Tip 2	
Speckled	35275	14	36858	9	11660	9	13385	11	2733	38	3222	30	3565	13	3855	6
Homogeneous	3437	20	3458	17	2344	6	2905	13	650	16	735	22	818	6	847	7
Pooled plasma	3156	16	3034	24	1848	29	1978	20	441	10	501	16	810	8	805	11
Negative Control	389	5	453	9	489	13	525	14	521	7	507	14	578	33	684	12
Ctrls mod	**91**		**81**		**24**		**26**		**5**		**6**		**6**		**6**	
Pos ctrl/pooled plasma	**11.2**		**12.1**		**6.3**		**6.8**		**6.2**		**6.4**		**4.4**		**4.8**	

1.10 Clinical Sample Correlation Cut off Determination

ANA antibody positive sera (N=60) and normal sera (N=35) were obtained and tested in the commercial ELISA kits and in the Theranos System.

The Theranos cutoff value was determined by taking the mean RLU of the normal samples plus 2 times the standard deviation of the 60 normal samples. The sample RLU divided by the cutoff value yields the Antibody Index.

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

Out of the 60 normals tested 1 was spositive on the Theranos assay and two others were borderline.

There was in general good agreement among the Theranos Ana assay and the Immco Diagnostics Ana ELISa which is an FDA approved bench top ELISA.

Table [SEQ Table * ARABIC]: Normal donor sample screen on Theranos vs. IBL International ANA ELISA

Sample ID	Matrix	Inter-Cartridge				Theranos Ab Index	IBL Ab index
		Mean Tip 1	CV%	Mean Tip 2	CV%		
M1	Serum	2665	18	2918	12	0.55	0.45
M2	Serum	2660	2	3153	10	0.59	0.53
M3	Serum	3731	6	3546	8	0.67	0.32
M4	Serum	3758	13	3552	18	0.67	0.43
M5	Serum	3841	1	3978	4	0.75	0.49
M6	Serum	2382	7	2456	5	0.46	0.43
M7	Serum	2220	11	2321	19	0.44	0.67
M8	Serum	2391	9	2560	6	0.48	0.62
M9	Serum	4522	3	4510	12	0.85	0.43
M10	Serum	5289	6	5200	5	0.98	0.30
M11	Serum	2010	8	1943	13	0.37	0.31
M12	Serum	2053	6	2068	9	0.39	0.47
M13	Serum	2152	9	2364	20	0.45	0.29
M15	Serum	2144	8	2293	6	0.43	0.30
M16	Serum	7983	4	8321	6	1.57	0.51
M17	Serum	2262	12	2282	13	0.43	0.40
M18	Serum	2764	6	3036	9	0.57	0.48
M21	Serum	3008	16	3070	8	0.58	0.29
M23	Serum	4350	1	4431	3	0.83	0.27
M24	Serum	2309	6	2218	5	0.42	0.42
F1	Serum	3419	5	3441	2	0.65	0.58
F2	Serum	3168	14	3227	20	0.61	0.52
F3	Serum	3715	9	3998	7	0.75	0.41
F4	Serum	4057	16	3785	19	0.71	0.20
F5	Serum	2061	3	2011	20	0.38	0.15
F6	Serum	4086	1	4145	12	0.78	0.49
F7	Serum	5207	8	5037	12	0.95	0.20
F8	Serum	2691	10	2983	15	0.56	0.40
F9	Serum	3760	11	3309	13	0.62	2.00
F10	Serum	1938	11	2238	4	0.42	0.30

Table [SEQ Table * ARABIC]: Normal donor sample screen on Theranos vs. IBL International ANA ELISA

Sample ID	Matrix	Inter-Cartridge				Theranos Ab Index	IBL Ab index
		Mean Tip 1	CV%	Mean Tip 2	CV%		
F11	Serum	2669	5	2431	13	0.46	0.29
F12	Serum	3757	9	3361	20	0.63	0.69
F13	Serum	1899	17	1965	22	0.37	0.46
F14	Serum	3343	11	3269	6	0.62	0.33
F15	Serum	2517	11	2546	14	0.48	0.63
F16	Serum	2905	13	2775	13	0.52	0.39
F17	Serum	4470	3	4003	5	0.75	0.30
F18	Serum	3904	1	3864	7	0.73	0.60
F19	Serum	4126	7	4016	10	0.76	0.77
F20	Serum	4595	14	4532	9	0.85	0.41
Li-Hep 1	Li-Heparin plasma	3849	14	3812	14	0.72	0.55
Li-Hep 2	Li-Heparin plasma	3333	12	3372	12	0.63	0.46
Li-Hep 3	Li-Heparin plasma	2829	3	2822	8	0.53	0.32
Li-Hep 4	Li-Heparin plasma	2344	2	2193	7	0.41	0.52
Li-Hep 5	Li-Heparin plasma	1287	9	1308	5	0.25	0.33
Li-Hep 6	Li-Heparin plasma	1990	1	1853	7	0.35	0.42
Li-Hep 7	Li-Heparin plasma	1859	5	1884	13	0.35	0.29
Li-Hep 8	Li-Heparin plasma	2160	11	2199	12	0.41	0.24
Li-Hep 9	Li-Heparin plasma	1925	14	1914	5	0.36	0.21
Li-Hep 10	Li-Heparin plasma	2080	17	2228	5	0.42	0.59
BR43	Serum	2646	11	2630	7	0.50	0.64
BR44	Serum	2121	11	2400	4	0.45	0.20
BR45	Serum	2425	3	2481	7	0.47	0.18
BR46	Serum	2396	9	2103	11	0.40	0.22
BR49	Serum	2271	9	2075	13	0.39	0.20
BR50	Serum	3403	7	3541	1	0.67	0.27
BR51	Serum	2937	11	2820	4	0.53	0.28
BR52	Serum	4065	6	3936	3	0.74	0.23

Table [SEQ Table * ARABIC]: Clinical Samples on Theranos vs. Commercial ANA ELISAs

Sample ID	Theranos Ab Index	Immco Diagnostics ANA value	USBIOL Ab index	Innova Ab index	IBL Ab Index	ProMeddx Nephelometry ratio
A1	0.77	39.1	0.53	12.2	0.82	1:160
A2	0.73	23.6	0.73	10.4	0.49	1:320
A3	1.78	36.6	0.90	31.2	0.76	1:320
A4	2.36	34.3	1.07	21.5	0.72	1:160
A5	0.62	12.8	0.52	13.3	0.27	1:160
A6	1.14	39.9	1.26	13.3	0.83	1:160
A7	1.64	27.0	0.58	8.4	0.56	1:320
A8	1.89	22.5	0.69	8.4	0.47	1:320
A9	2.18	143.8	2.41	39.1	3.00	1:1280
A10	6.14	32.8	2.13	21.2	0.69	1:1280
A11	1.68	74.1	2.50	74.5	1.55	1:1280
A12	19.53	157.3	2.78	106.2	3.29	1:1280
A13	1.51	32.8	2.75	20.5	0.69	1:160
A14	3.16	31.8	1.85	19.2	0.66	1:160
A15	1.92	24.4	1.01	13.8	0.51	1:320
A16	1.85	58.5	5.36	39.4	1.22	1:1280
A17	5.57	153.8	3.81	99.8	3.21	1:1280
A18	1.45	33.4	1.03	25.1	0.70	1:640
A19	7.98	99.6	2.83	81.5	2.08	1:640
A20	1.87	51.4	0.86	21.3	1.07	1:640
A21	1.06	50.0	0.83	11.9	1.04	1:640
A22	1.38	31.7	1.16	23.2	0.66	1:640
A23	0.95	53.8	1.00	14.0	1.12	1:320
A24	1.57	38.1	1.41	13.7	0.80	1:1280
A25	1.65	55.6	2.55	17.8	1.16	1:640
A26	0.87	43.2			0.90	1:640
A27	1.69	28.2			0.59	1:640
A28	1.85	37.9			0.79	1:640
A29	0.92	23.4			0.49	1:160
A30	3.19	87.4			1.83	1:160
A32	1.68	48.9			1.02	1:160
A33	1.23	45.3			0.95	1:1280
A34	2.10	50.0			1.04	1:1280
A35	0.85	31.0			0.65	1:320
A36	0.54	40.0			0.84	1:160

1.11 Specificity

The specificity of the Ana assay towards samples containing antibodies specific for other disorders was tested. Rf positive, HAMA positive, Lyme disease positive, inactivated control samples positive for Syphilis, anti-thyroid peroxidase and thyroglobulin positive samples and anti –HIV 1 and 2 positive controls were tested. Of the 40 samples tested only the anti-HIV control samples tested positive for the ANA assay. Based on this data samples that are tested positive for HIV 1 and 2 antibodies must be assumed to be positive for ANA antibodies as well.

Table [SEQ Table * ARABIC]: Cross reactivity testing for ANA assay

Sample Info	Ab Index
Lyme disease positive #4	0.37
Lyme disease positive #25	0.34
Lyme disease positive #26	0.31
Lyme disease positive #28	0.40
Lyme disease positive #29	0.79
Lyme disease positive #30	0.29
HAMA positive #2	0.78
HAMA positive # 3	0.09
HAMA positive # 4	0.69
HAMA positive # 6	1.01
HAMA positive # 8	0.40
HAMA positive # 9	0.37
RF positive #334 (result: 206 IU/mL)	0.44
RF positive #618 (result: 133 IU/mL)	0.15
RF positive #612 (result: 161 IU/mL)	0.25
RF positive #625 (result: 122 IU/mL)	0.20
RF positive #647 (result: 207 IU/mL)	0.39
RF positive #629 (result: 104 IU/mL)	0.15

Table [SEQ Table * ARABIC]: Cross reactivity testing for ANA assay(continued)

Sample Info	Ab Index
Syphilis Low control (bioreclamation)	0.40
Syphilis Negative control (bioreclamation)	0.47
Syphilis High control (ASI)	0.23
Syphilis Low control (ASI)	0.99
Syphilis Negative control (ASI)	0.33
Syphilis positive sample	0.23
Anti-TPO/Tg #1	0.23
Anti-TPO/Tg #2	0.33
Anti-TPO/Tg #3	0.48
Anti-TPO/Tg #4	0.28
Anti-TPO/Tg #5	0.20
Anti-TPO/Tg #6	0.70
Anti HIV 1- A	1.46
Anti HIV 1- B	1.27
Anti HIV 1- C	1.18
Anti HIV 1- E	1.29
Anti HIV 1- O	1.50
Anti HIV 2	1.13
Anti-HCV control	0.51