theranos™
redefining healthcare

JO-1 (histidyl-tRNA synthetase), Extractable Nuclear Antigen (ENA) Antibody (IgG) Qualitative
Assay Development Report

Theranos, Inc

September 2012

Prepared by: Nahal Gharaati

TABLE OF CONTENTS

[TOC \o "1-3" \h \z \u]**LIST OF TABLES**

[TOC \h \z \c "Table"]

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-JO-1 (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 JO-1 ELISA (Cat# 708585)
- Immco diagnostics ImmuLisa™ JO-1 antibody ELISA (Cat# 5151)
- IBL International JO-1 Antibody ELISA (Cat# RE70131)

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

JO-1 antigen coated surface serves as the capture surface for the Anti-JO-1 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	Genway	GWB-AB09C6
Mouse Anti-Human IgG1 Antibody	Novus Biologicals	NB100-2046
Alkaline Phosphatase Labeling Kit	Dojindo	LK13-10
Theranos In-House Substrate	N/A	N/A
Theranos AP Conjugate Stabilizer	N/A	N/A
Low-Cross Buffer	CANDOR Bioscience	100 500
Blocking Buffer (3% BSA in TBS, 0.05% Sodium Azide)	N/A	N/A

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 JO-1 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-JO-1 antibodies and an autoimmune negative control sample obtained from a commercial source. Six 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 Blocking Buffer. The response for the directly coated antigen clearly is the better choice to further optimize. The results are summarized in Table 2.

Table [SEQ Table * ARABIC]: Effect of Capture Antigen Surface on assay response.

	JO-1 Antigen Direct Coated Surface		JO-1 Biotin Conjugated JO-1 Antigen	
Controls	Inter-Cartridge RLU Mean	CV%	Inter-Cartridge RLU Mean	CV%
Negative (Biorad Ctrl)	445	13	2978	40
Positive (Immunovision, Jo-1)	357971	19	19127	6
Pooled Negatives	1039	10	2660	20
Pooled Positives	207729	11	4174	12
Positive ctrl/Negative ctrl	804		6	
Positive ctrl/Pooled normal	345		7	

[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 JO-1 antigen coated surface was titrated at levels: 5, 2.5 and 1 µg/mL. Table 3 summarizes the results. 1 µg/mL provides good enough modulation between the positive and pooled normal clinical samples as well as lowering the negative background in both. This was finalized as the capture antigen surface concentration.

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

[SHAPE * MERGEFORMAT]

1.3 Effect of Detection Conjugate Stabilizer

Two commercial and two in house formulated alkaline phosphatase stabilizers were tested as detection antibody diluents, with the anti-human IgG DAb at 100 ng/mL. Signal modulation was best with the Theranos In-house detection antibody stabilizer. Table 4 summarizes the results.

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

	Blocking Buffer (3% BSA, TBS, 0.05% Sodium Azide)		Theranos In-house AP Antibody Conj. Stabilizing Buffer	
Controls	Inter-Cartridge RLU Mean	CV%	Inter-Cartridge RLU Mean	CV%
Negative (Biorad Ctrl)	451	3	437	35
Positive (Immunovision, Jo-1)	140733	6	157452	9
Positive ctrl/Negative ctrl	312		360	

	Biostab AP Conj. Stabilizer		Stabilzyme AP Conj. Stabilizer	
Controls	Inter-Cartridge RLU Mean	CV%	Inter-Cartridge RLU Mean	CV%
Negative (Biorad Ctrl)	3312	21	1062	44
Positive (Immunovision, Jo-1)	321425	5	48614	8
Positive ctrl/Negative ctrl	97		46	

1.4 Detection Conjugate Titration

The AP conjugated detection antibody was titrated in the Theranos detection conjugate stabilizer. The best modulation between the positive and negative control was achieved with 100 ng/mL of the anti-IgG Dab. This concentration was chosen for the rest of this assay's development.

Table [SEQ Table * ARABIC]: Detection Conjugate Titration

	200 ng/mL		100 ng/mL	
Controls	Inter-Cartridge RLU Mean	CV%	Inter-Cartridge RLU Mean	CV%
Negative (Biorad Ctrl)	1055	23	484	14
Positive (Immunovision, Jo-1)	342983	8	187229	6
Positive ctrl/Negative ctrl	325		387	

	50 ng/mL		25 ng/mL	
Controls	Inter-Cartridge RLU Mean	CV%	Inter-Cartridge RLU Mean	CV%
Negative (Biorad Ctrl)	400	9	237	13
Positive (Immunovision, Jo-1)	89612	14	43778	3
Positive ctrl/Negative ctrl	224		185	

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 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. Therefore, the 50X Sample Dilution Protocol is the one we will continue with for this assay. Results are summarized in Table 6.

Table [SEQ Table * ARABIC]: Effect of Sample Dilution

	25 X		50 X		100 X	
Controls	Inter-Cartridge RLU Mean	CV%	Inter-Cartridge RLU Mean	CV%	Inter-Cartridge RLU Mean	CV%
Negative (Biorad Ctrl)	616	17	648	15	685	19
Negatives (Pooled)	937	20	710	6	577	30
Positive (Immunovision, Jo-1)	162151	6	153782	16	85615	8
Positives (Pooled)	48490	15	51789	12	27787	21
Positive control/negative control	263		237		125	
Positive control/pooled normal	173		217		148	
Pooled positive /pooled normal	52		73		48	

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 the 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 the 10, 10, 10 minute incubation protocol and this was chosen as the final conditions for this JO-1 assay.

.

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

	10x10x10		5x5x5		2x2x1	
Controls	Inter-Cartridge RLU Mean	CV%	Inter-Cartridge RLU Mean	CV%	Inter-Cartridge RLU Mean	CV%
Negative (Biorad Ctrl)	588	6	548	25	248	12
Negatives (Pooled)	702	28	468	13	288	8
Positive (Immunovision, Jo-1)	142241	5	50894	23	6313	2
Positives (Pooled)	45599	21	17090	13	2102	3
Positive control/negative control	242		93		25	
Positive control/pooled normal	203		109		22	
Pooled positive /pooled normal	65		37		7	

1.7 Effect of Testing Various Blocking Buffers

The effect of testing various blocking buffers was necessary since Rf and HAMA samples tested were resulting in higher RLUs and possibly giving non-specific binding. Since these samples were confirmed to be negative on all 3 kits tested for JO-1 antibody, various blockers were tried to lower or eliminate any non-specificity that was occurring. A total of 8 different blockers were tested and the best, which was Low Cross Buffer, was chosen as seen in the chart below. In these blocking buffer tests, same Rf #1 and HAMA #1 samples were used throughout to see any change in response. Table 8 summarizes the data.

Table [SEQ Table * ARABIC]: Effect of Testing Various Blocking Buffers

Test Hama #1		
Control	Inter-Cartridge Mean	CV%
Starting Block (Ctrl)	2833	20
Tru Block (400ug/mL)	13571	31
Super Block	10939	14
Surmodics	1610	11
Sea Block	2680	13
Low-Cross Buffer	893	32
Blocking Buffer	13470	14
Blocker Casein in TBS	8328	11

Test Rf #1		
Control	Inter-Cartridge Mean	CV%
Starting Block (Ctrl)	1230	30
Tru Block (400ug/mL)	2525	5
Super Block	2255	30
Surmodics	715	33
Sea Block	1248	23
Low-Cross Buffer	662	7
Blocking Buffer	2667	15
Blocker Casein in TBS	1517	16

1.8 Normal Sample Screen: Cut-off Determination

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

Ab index	Sample RLU/Cut off
Ab index > 1.1	Positive
Ab index > 0.9 < 1.1	Equivocal/Borderline
Ab index < 0.9	Negative

Table 9: Normal Sample Screen: Theranos vs. 3 Commercial Anti-JO-1 ELISAs

Samples	Inter-Cartridge Mean	CV%	Theranos Ab Index 10*STDEV	INOVA kit Units	IMMCO kit Result (EU/mL)	IBL International Ratio
Normals (#1)	478	10	0.20	2.1	3.7	0.13
Normals (#2)	766	15	0.32	0.3	6.3	0.15
Normals (#3)	590	8	0.25	0.1	2.6	0.05
Normals (#4)	1058	23	0.44	0.0	4.9	0.15
Normals (#5)	411	16	0.17	0.1	4.3	0.07
Normals (#6)	396	31	0.16	0.0	3.7	0.06
Normals (#7)	549	20	0.23	0.1	7.7	0.12
Normals (#8)	533	21	0.22	3.2	9.0	0.17
Normals (#9)	469	57	0.20	0.3	5.2	0.14
Normals (#10)	542	24	0.23	1.2	5.6	0.14
Normals (#11)	762	37	0.32	0.1	3.0	0.06
Normals (#12)	680	33	0.28	0.4	2.0	0.06
Normals (#13)	464	20	0.19	3.1	3.7	0.20
Normals (#14)	384	17	0.16	0.9	10.5	0.10
Normals (#15)	541	21	0.23	0.7	5.4	0.19
Normals (#16)	528	19	0.22	0.5	3.5	0.06
Normals (#17)	859	27	0.36	0.1	1.1	0.05
Normals (#18)	519	14	0.22	4.2	9.4	0.15
Normals (#19)	869	10	0.36	0.3	6.8	0.11
Normals (#20)	491	11	0.20	0.3	1.1	0.11
MEAN	594					
CUT OFF	2402	10*STDEV				

1.9 Clinical Sample Correlation

N=39 samples obtained from myositis, dermato-myositis and poly-myositis patients were tested on the Theranos Anti-JO-1 assay. The same samples were run on three commercial Anti-JO-1 ELISAs and the correlation of the results to the Theranos assay is reported in Table 10 below. Excellent correlation was seen for all samples. The Cl15 and Cl25 both resulted 1 out of 3 predicate tests to give low positive and matched closely with the Theranos test as well.

Table 10: Clinical Sample Screen: Theranos vs. 3 Commercial Anti-JO-1 ELISAs

				10*STDEV
	Innova	Immco	IBL Int.	Theranos
Sample	Units	Result (EU/mL)	Ratio	Ab Index
Cl01	0	6	0.1	0.6
Cl02	1	7	0.1	0.7
Cl03	32	126	4.4	5.7
Cl04	127	177	6.6	18.1
Cl05	111	169	6.6	9.6
Cl06	5	8	0.1	0.7
Cl07	19	4	0.1	0.0
Cl08	3	10	0.4	0.6
Cl09	8	13	0.3	0.7
Cl10	1	14	0.2	0.7
Cl11	2	12	0.3	0.0
Cl12	1	7	0.1	0.0
Cl13	0	5	0.1	0.0
Cl14	1	3	0.1	0.0
Cl15	1	20	0.2	0.3
Cl16	0	8	0.1	0.0
Cl17	165	175	6.2	16.6
Cl18	1	8	0.2	0.0
Cl19	50	138	4.4	8.1
Cl20	9	10	0.2	0.0
Cl21	107	175	6.3	8.9
Cl22	8	8	0.1	0.0
Cl23	3	10	0.4	0.0
Cl24	5	14	0.3	0.5
Cl25	23	2	0.1	0.4
Cl35	107	201	7.9	2.5
Cl36	69	158	6.5	2.3
Cl37	110	203	8.1	2.6
Cl38	108	208	8.2	2.7
Cl39	109	201	8.2	3.0

1.10 Specificity

The specificity of the Anti JO-1 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-JO-1 assay demonstrates specificity only against JO-1 antibodies as seen by the positive results shown by CDC reference sera # 10.

Table [SEQ Table * ARABIC]: Specificity

Sample ID	Information from CDC datasheet		Mean	CV%	Ab index
#1	native DNA, Sm, Sm/RNP	Homogeneous/rim	855	48	0.36
#2	SS-B/La, SSA 52, SSA 60	Speckled/La	549	17	0.23
#3	RNP, Sm, Sm/RNP, SSB,SSA 60	Speckled	584	23	0.24
#4	U1-RNP, Sm/RNP		742	23	0.31
#5	Sm antigen, Sm RNP (Biorad Ctrl)		497	27	0.21
#6	U3-RNP	Nucleolar pattern	639	27	0.27
#7	SS-A/Ro		753	51	0.31
#8	Centromere B	Centromere pattern	684	21	0.28
#9	Scl-70		775	22	0.32
#10	Jo-1		112330	16	46.77
#12	rRNP/Ribosomal P		886	21	0.37

1.11 Rf and HAMA Positive Sample Testing

6 Rf positive and 6 HAMA positive sera obtained from a commercial source were tested on the Theranos Anti-Jo-1 assay. The same samples were also tested on the 3 commercial Anti-JO-1 ELISA kits and all results were negative. Out of the 12 samples tested, all were in agreement in each test for Anti-JO-1 antibodies.

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

Samples	Inter-Cartridge		Theranos	INOVA	IMMCO	IBL
	Mean	CV%	Ab	kit	kit	International
			10*STDEV	Units	Result (EU/mL)	Ratio
Rf - 1	458	10	0.19	0.88	8.00	0.25
Rf - 2	1323	12	0.55	1.53	5.58	0.38
Rf - 3	675	8	0.28	1.35	9.43	0.31
Rf - 4	1975	36	0.82	1.22	8.99	0.54
Rf - 38	824	26	0.34	2.25	10.57	0.36
Rf - 39	577	20	0.24	5.29	8.54	0.33
Hama - 1	1398	1	0.58	3.00	9.43	0.51
Hama - 2	756	26	0.31	1.75	7.21	0.33
Hama - 3	1051	3	0.44	3.79	8.20	0.43
Hama - 4	1026	48	0.43	4.04	18.72	0.57
Hama - 5	997	48	0.42	1.72	8.25	0.35
Hama - 6	1017	22	0.42	1.69	13.83	0.43