



Anti-HAV IgM Ab 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"]

An enzyme linked immunosorbent assay (ELISA) was developed for the qualitative detection of IgM antibodies due to hepatitis A virus infections. The hepatitis A virus (HAV) is a non-enveloped, RNA virus that causes liver disease. Measurement of IgM antibodies to HAV is important for early diagnoses of HAV infections. This report describes the development and assay performance of the Theranos anti-HAV IgM antibody assay.

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

The following assays were used as predicament methods.

1. Monolisa Anti-HAV IgM EIA, Biorad, Cat. 72495

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

The anti-HAV IgM ELISA was developed using biotinylated capture antibodies on an ultravidin (UA) surface as the capture surface. Human IgM antibodies in serum or plasma bind specifically to the anti-human IgM capture antibodies for 10 minutes followed by a wash cycle. After washing, the human IgM antibodies were detected using an AP labeled mouse monoclonal to human IgM. After incubation with the detector antibody for 10 minutes, another was cycle was performed, and the alkaline phosphatase substrate was added. The resulting chemiluminescence was read in relative light units (RLU) on the Theranos analyzer.

Table [SEQ Table * ARABIC]: Materials

Name	Supplier	Catalog #
Goat F(ab') ₂ anti-human IgM	Southern Biotech	2022-01
Mouse monoclonal anti-HAV, clone MK-01	Meridian Life Science	[HYPERLINK "https://meridianlifescience.com/bioSpecs/C86318M.pdf" t " blank"]
HAV antigen	Meridian Life Science	Cat: 8505
Biorad Calibrator, Positive, Negative Controls	Biorad	72495
QCRIIAVIGMQC2-Anti-IIAV Quality Control Serum: Sample2	NIBSC	09/B572
Alkaline Phosphatase Labeling Kit (SH)	Dojindo	LK13-10
Biotin Labeling Kit (SH)	Dojindo	LK10
Phospha Glo Substrate	KPL	55-60-04
Blocking Buffer (3% BSA in TBS, 0.05% Sodium Azide)	Sigma (BSA, Fraction V, 99% Pure)	A3059-500G
Theranos AP Conjugate Stabilizer (Blocking buffer containing 0.1 mM Zn ²⁺ , and 5mM Mg ²⁺)	Theranos	
Tris Buffered Saline	Sigma	T6664-10PAK
Theranos Cartridge	Theranos	
Theranos Analyzer	Theranos	
Heterophilic Blocking Reagent (HBR)	Scantibodies	3KC533

1.1.3 Labeling of Detector Antibody

The mouse anti-human IgM clone MK-01 was labeled with alkaline phosphatase according to kit instructions (Dojindo, LK13-10).

1.1.4 Labeling of Capture Antibody

The goat F(ab')₂ anti-human IgM was labeled with biotin according to kit instructions (Dojindo, LK-10)

1.1.5 Preparation of Assay Buffer

The assay buffer was prepared by dissolving 1 packet of TBS into water and adding 10 mL of 10% azide, and 30 g of BSA to a final volume of 1000mL. The final composition of the assay buffer is 3% BSA, 50mM Tris, 138mM NaCl, 2.7mM KCl pH 8.0 in water. The assay buffer was filtered before use.

1.1.6 Preparation of Theranos AP Conjugate Stabilizer

The Theranos AP Conjugate Stabilizer was prepared by adding Zn²⁺ and Mg²⁺ to the assay buffer to final concentrations of 0.1mM Zn²⁺ and 5mM Mg²⁺.

1.1.7 Preparation of HBR Assay Diluent

Add HBR to the assay buffer for a final concentration of 500 ug/mL HBR.

2. ASSAY DEVELOPMENT

2.1 Capture Antibodies

The optimum capture surface was determined by testing three different anti-human IgM antibodies. Capture antibodies were labeled with biotin and subsequently coated on UA tips. The goat F(ab')₂ anti-human IgM (Southern Biotech, Cat. 2022-01) gave the best modulations.

Table 2: Capture Antibodies

Capture Antibody	Vendor	Sample	AVG RLU	CV	Modulation
Goat anti-hlgM	Southern Biotech, Cat. 2020-01	Biorad Calibrator	72475	19%	14.4
Goat anti-hlgM	Southern Biotech, Cat. 2020-01	Biorad Positive	19230	18%	3.8
Goat anti-hlgM	Southern Biotech, Cat. 2020-01	Virotrol	25484	12%	5.1
Goat anti-hlgM	Southern Biotech, Cat. 2020-01	WHO QC	12410	6%	2.3
Goat anti-hlgM	Southern Biotech, Cat. 2020-01	Biorad Negative	5355	14%	1.0
Goat F(ab') ₂ anti- hlgM	Southern Biotech, Cat. 2022-01	Biorad Calibrator	89016	8%	18.9
Goat F(ab') ₂ anti- hlgM	Southern Biotech, Cat. 2022-01	Biorad Positive	20819	17%	4.4
Goat F(ab') ₂ anti- hlgM	Southern Biotech, Cat. 2022-01	Virotrol	26635	14%	5.7
Goat F(ab') ₂ anti- hlgM	Southern Biotech, Cat. 2022-01	WHO QC	12709	11%	2.7
Goat F(ab') ₂ anti- hlgM	Southern Biotech, Cat. 2022-01	Biorad Negative	4706	11%	1.0
Mouse anti-hlgM	Abd Serotec, Cat. 5278-5159	Biorad Calibrator	24959	33%	17.2
Mouse anti-hlgM	Abd Serotec, Cat. 5278-5159	Biorad Positive	7386	6%	5.1
Mouse anti-hlgM	Abd Serotec, Cat. 5278-5159	Virotrol	3632	24%	2.5
Mouse anti-hlgM	Abd Serotec, Cat. 5278-5159	WHO QC	2171	11%	1.4
Mouse anti-hlgM	Abd Serotec, Cat. 5278-5159	Biorad Negative	1541	20%	1.0

2.2 Detector Antibodies

The optimum detector antibody was determined by testing 5 different detection antibodies. Detections were tested at 100 ng/mL with 50 ng/mL HAV. Detection antibodies 3 and 5 worked equally well.

Table 3: Detector antibodies

Detection Antibody	Vendor	Sample	AVG RLU	CV	Modulation
Dab-1	MyBiosource, Cat. MBS430089	Biorad Calibrator	153364	20%	11.1
Dab-1	MyBiosource, Cat. MBS430089	Biorad Positive	48673	22%	3.5
Dab-1	MyBiosource, Cat. MBS430089	Virotrol HAV IgM	59706	28%	4.3
Dab-1	MyBiosource, Cat. MBS430089	WHO QC	26930	19%	2.0
Dab-1	MyBiosource, Cat. MBS430089	Biorad Negative	13767	20%	1.0
Dab-2	MyBiosource, Cat. MBS190205	Biorad Calibrator	99768	6%	18.1
Dab-2	MyBiosource, Cat. MBS190205	Biorad Positive	24248	5%	4.4
Dab-2	MyBiosource, Cat. MBS190205	Virotrol HAV IgM	26080	18%	4.7
Dab-2	MyBiosource, Cat. MBS190205	WHO QC	13498	14%	2.4
Dab-2	MyBiosource, Cat. MBS190205	Biorad Negative	5511	3%	1.0
Dab-3	MyBiosource, Cat. MBS530054	Biorad Calibrator	141974	17%	15.5
Dab-3	MyBiosource, Cat. MBS530054	Biorad Positive	314630	16%	34.3
Dab-3	MyBiosource, Cat. MBS530054	Virotrol HAV IgM	713984	9%	77.8
Dab-3	MyBiosource, Cat. MBS530054	WHO QC	479398	12%	52.3
Dab-3	MyBiosource, Cat. MBS530054	Biorad Negative	9174	11%	1.0
Dab-4	Meridian Life Science, Cat. C86318M	Biorad Calibrator	210484	10%	20.3
Dab-4	Meridian Life Science, Cat. C86318M	Biorad Positive	47170	17%	4.5
Dab-4	Meridian Life Science, Cat. C86318M	Virotrol HAV IgM	51099	23%	4.9
Dab-4	Meridian Life Science, Cat. C86318M	WHO QC	20721	29%	2.0
Dab-4	Meridian Life Science, Cat. C86318M	Biorad Negative	10380	17%	1.0
Dab-5	MyBiosource, MBS310329	Biorad Calibrator	101793	25%	14.7
Dab-5	MyBiosource, MBS310329	Biorad Positive	140	14%	0.0
Dab-5	MyBiosource, MBS310329	Virotrol HAV IgM	680423	9%	98.0
Dab-5	MyBiosource, MBS310329	WHO QC	392758	13%	56.6
Dab-5	MyBiosource, MBS310329	Biorad Negative	6944	7%	1.0

2.3 Effect of PSW

The effect of post sample wash was tested on the assay. Compared to the co-incubation protocol, the assay performed better with a post sample wash. The WHO QC and Virotrol modulations were higher with the post sample wash. The background was similar for all protocols.

Table 4: Effect of PSW

Protocol	Sample	AVG RLU	STD	CV	Modulation
10X_coincubation	Biorad Calibrator	70024	14883	21%	18.2
10X_coincubation	Biorad Positive	17627	1628	9%	4.6
10X_coincubation	Biorad Negative	3839	345	9%	1.0
10X_coincubation	Virotrol	20078	1045	5%	5.2
10X_coincubation	WHO QC	11299	922	8%	2.9
5X_coincubation	Biorad Calibrator	94719	9671	10%	27.2
5X_coincubation	Biorad Positive	6928	585	8%	2.0
5X_coincubation	Biorad Negative	3479	348	10%	1.0
5X_coincubation	Virotrol	11602	1405	12%	3.3
5X_coincubation	WHO QC	6480	1618	25%	1.9
10X_PSW	Biorad Calibrator	18845	3881	21%	5.2
10X_PSW	Biorad Positive	33026	4054	12%	9.1
10X_PSW	Biorad Negative	3617	487	13%	1.0
10X_PSW	Virotrol	68231	16858	25%	18.9
10X_PSW	WHO QC	59881	2949	5%	16.6

2.4 Capture Surface Titration

The optimum coating concentration was determined by titrating the capture antibody (Southern Biotech, Cat. 2022-01). Acceptable modulations and background signal were achieved with 1 ug/mL of capture antibody (Table 5).

Table 5: Capture Surface Titration

Coating Concentration	Sample	AVG RLU	STD	CV	Modulation
10 ug/mL	Biorad Calibrator	155640	37489	24%	16.7
10 ug/mL	Biorad Positive	317913	50773	16%	34.2
10 ug/mL	Biorad Negative	9300	1189	13%	1.0
10 ug/mL	Virotrol	657541	107915	16%	70.7
10 ug/mL	WHO QC	466432	73459	16%	50.2
5 ug/mL	Biorad Calibrator	174198	31126	18%	17.7
5 ug/mL	Biorad Positive	320511	32064	10%	32.6
5 ug/mL	Biorad Negative	9823	1978	20%	1.0
5 ug/mL	Virotrol	654399	93964	14%	66.6
5 ug/mL	WHO QC	505384	56625	11%	51.4
1 ug/mL	Biorad Calibrator	187538	46470	25%	16.6
1 ug/mL	Biorad Positive	340464	41637	12%	30.1
1 ug/mL	Biorad Negative	11303	1157	10%	1.0
1 ug/mL	Virotrol	657622	110739	17%	58.2
1 ug/mL	WHO QC	491715	77679	16%	43.5

2.5 False Positives

Negative anti-HAV IgM clinical samples were tested to determine the rate of false positives with Dab-3 (MyBiosource, Cat. MBS530054). The rate of false positives with Dab-3 was high (60%) (Table 6) and was no longer used for assay development. Backup detection antibodies 2, 4, and 5 were also tested for false positives. Detection antibodies 2 and 4 did not appear to give false positives with samples 1 and 10 (Table 7). Subsequently, more negative samples were tested using detection antibodies 2 and 4 (Table 8). Results showed detection antibodies 2 (MyBiosource, Cat. MBS190205) and 4 (Meridian Life Science, Cat. C86318M) gave the same rate of false positives (10% positive, 10% borderline).

Table 6: False positives with Dab-3

Sample	AVG RLU	STD	CV
1	5753	788	14%
2	20837	6260	30%
3	6313	640	10%
4	9591	2087	22%
5	3196	261	8%
6	21243	1119	5%
7	11381	2276	20%
8	6679	1485	22%
9	15338	1651	11%
10	98419	11147	11%
Biorad Calibrator	177709	38890	22%
Biorad Negative	7254	1149	16%
WHO QC	312218	33630	11%

Table 7: False positives with Dab-2, Dab-4, Dab-5

Detection	Sample	AVG RLU	STD	CV	Modulation
Dab-2	Biorad Calibrator	17446	3050	17%	9.3
Dab-2	Biorad Negative	1886	266	14%	1.0
Dab-2	WHO QC	36347	6149	17%	19.3
Dab-2	Negative #1	2010	262	13%	1.1
Dab-2	Negative #10	1993	229	12%	1.1
Dab-4	Biorad Calibrator	40438	5116	13%	12.7
Dab-4	Biorad Negative	3189	473	15%	1.0
Dab-4	WHO QC	75414	13628	18%	23.6
Dab-4	Negative #1	3817	805	21%	1.2
Dab-4	Negative #10	3909	809	21%	1.2
Dab-5	Biorad Calibrator	178415	16786	9%	28.6
Dab-5	Biorad Negative	6229	363	6%	1.0
Dab-5	WHO QC	386016	82505	21%	62.0
Dab-5	Negative #1	4237	695	16%	0.7
Dab-5	Negative #10	38835	7751	20%	6.2

Table 8: False positives with Dab-2 and Dab-4

	Dab-2	Dab-4
Samples	AVG RLU	AVG RLU
1	1584	3428
2	2107	4182
3	2800	5364
4	1707	3292
5	1279	2307
6	17957	41326
7	6925	7479
8	2111	3692
9	2041	4221
10	1892	3974
11	2459	4578
12	2441	4677
13	3319	8713
14	3397	7508
15	1907	3470
16	2278	2398
17	1545	3219
18	1635	3144
19	1529	2369
20	1967	4357
Biorad Calibrator	14611	32416
Biorad Positive	24958	56257
Biorad Negative	2297	3907
WHO QC	36170	66467

2.6 Effect of Blockers

Dab-2 and Dab-43 were tested with assay blockers to reduce non-specific binding and false positives. The most effective blocker was HBR. Dab-4 (Meridian Life Science, Cat. C86318M) worked best with HBR.

Table 9: Dab-2 with blockers

Sample	Blocker	AVG RLU	STD	CV
Sample #6	Assay Buffer	24595	3735	15%
Sample #7	Assay Buffer	12540	1060	8%
Calibrator	Assay Buffer	12550	2185	17%
Sample #6	1 mg/mL HBR	3543	588	17%
Sample #7	1 mg/mL HBR	2680	377	14%
Calibrator	1 mg/mL HBR	4932	1167	24%
Sample #6	Sea Block	18825	2310	12%
Sample #7	Sea Block	12309	2474	20%
Calibrator	Sea Block	12352	658	5%
Sample #6	Starting Block	28124	2770	10%
Sample #7	Starting Block	11099	1440	13%
Calibrator	Starting Block	15857	2515	16%
Sample #6	Surmodics	16279	4125	25%
Sample #7	Surmodics	6405	1237	19%
Calibrator	Surmodics	9547	1037	11%

Table 10: Dab-4 with blockers

Sample	Blocker	AVG RLU	STD	CV
Sample #6	Assay Buffer	36082	11340	31%
Sample #7	Assay Buffer	6567	1416	22%
Calibrator	Assay Buffer	26167	4564	17%
Sample #6	1 mg/mL HBR	5634	1249	22%
Sample #7	1 mg/mL HBR	3882	845	22%
Calibrator	1 mg/mL HBR	13033	2921	22%
Sample #6	Sea Block	45836	6120	13%
Sample #7	Sea Block	13401	1844	14%
Calibrator	Sea Block	21773	2076	10%
Sample #6	Starting Block	31560	5131	16%
Sample #7	Starting Block	9313	1546	17%
Calibrator	Starting Block	22445	2470	11%
Sample #6	Surmodics	24608	5649	23%
Sample #7	Surmodics	8430	1489	18%
Calibrator	Surmodics	19351	1202	6%

2.7 HBR Titration

The optimum HBR concentration for the assay was determined by titrating the HBR with Dab-4 (Table 11). Acceptable reduction in non-specific binding was achieved at 0.5 mg/mL HBR in assay buffer. Subsequently, 20 negative samples were retested with 0.5/mL HBR and the results shown. There were no obvious false positives with 0.5 mg/mL in assay buffer (Table 12).

Table 11: HBR Titration

Sample	HBR Concentration	AVG RLU	STD	CV
Sample #6	1 mg/mL	7398	1280	17%
Sample #7	1 mg/mL	3886	484	12%
Biorad Calibrator	1 mg/mL	12656	739	6%
Sample #6	0.5 mg/m L	9428	757	8%
Sample #7	0.5 mg/m L	5457	1297	24%
Biorad Calibrator	0.5 mg/m L	20729	3121	15%
Sample #6	0.25 mg/m L	10657	1560	15%
Sample #7	0.25 mg/m L	4721	591	13%
Biorad Calibrator	0.25 mg/m L	17311	1791	10%
Sample #6	0.125 mg/mL	13569	1784	13%
Sample #7	0.125 mg/mL	5587	662	12%
Biorad Calibrator	0.125 mg/mL	17589	4041	23%
Sample #6	0 mg/mL	45273	8430	19%
Sample #7	0 mg/mL	11063	1528	14%
Biorad Calibrator	0 mg/mL	38072	6591	17%

Table 12: Negative samples with 0.5mg/mL HBR

Sample	AVG RLU	CV	S/CO
1	2862	11%	0.45
2	3155	25%	0.50
3	2944	15%	0.46
4	2371	12%	0.37
5	1945	6%	0.31
6	8117	17%	1.27
7	3553	11%	0.56
8	3249	18%	0.51
9	2767	17%	0.43
10	2316	13%	0.36
11	2314	10%	0.36
12	2743	11%	0.43
13	3333	9%	0.52
14	2736	14%	0.43
15	3485	34%	0.55
16	1990	13%	0.31
17	2739	20%	0.43
18	2891	22%	0.45
19	2944	9%	0.46
20	3496	12%	0.55
Biorad Calibrator	25482	19%	4.00
Biorad Positive	37562	18%	5.90
Biorad Negative	3925	4%	0.62
WHO QC	53420	26%	8.39

2.8 Effect of Detector Stabilizers

The effect of detector stabilizers was tested to determine best conditions for Dab-4 (Meridian Life Science, C86318M). The Theranos AP conjugate stabilizer gave the best modulations with Dab-4.

Table 13: Effect of detector stabilizers

Detector Stabilizer	Sample	AVG RLU	STD	CV	Modulation
Assay Buffer	Biorad Calibrator	24591	6396	26%	8.1
Assay Buffer	Biorad Positive	14563	2243	15%	4.8
Assay Buffer	Biorad Negative	3042	443	15%	1.0
Assay Buffer	WHO QC	37125	5262	14%	12.2
Theranos AP Conjugate Stabilizer	Biorad Calibrator	20937	3013	14%	5.35
Theranos AP Conjugate Stabilizer	Biorad Positive	26024	3570	14%	6.65
Theranos AP Conjugate Stabilizer	Biorad Negative	3913	963	25%	1.00
Theranos AP Conjugate Stabilizer	WHO QC	42209	12386	29%	10.79
Biostab	Biorad Calibrator	42880	2924	7%	2.64
Biostab	Biorad Positive	31572	7331	23%	1.95
Biostab	Biorad Negative	16229	3407	21%	1.00
Biostab	WHO QC	46041	7847	17%	2.84
Stabilzyme	Biorad Calibrator	8830	1886	21%	2.18
Stabilzyme	Biorad Positive	6520	1155	18%	1.61
Stabilzyme	Biorad Negative	4054	971	24%	1.00
Stabilzyme	WHO QC	13532	3813	28%	3.34

2.9 Detection Antibody Titration

The optimum Dab-4 concentration was determined by titrating the detection antibody and HAV antigen. Best modulations and low background were achieved with 50 ng/mL of Dab-4 and 25 ng/mL of HAV antigen (Table 14).

Table 14: Dab-4 Titration

Dab-4	HAV	Sample	AVG	STD	CV	Modulation
100 ng/mL	100 ng/mL	Biorad Calibrator	34444	6427	19%	5.07
100 ng/mL	100 ng/mL	Biorad Positive	69228	14990	22%	10.20
100 ng/mL	100 ng/mL	Biorad Negative	6787	769	11%	1.00
100 ng/mL	100 ng/mL	WHO QC	168029	36416	22%	24.76
100 ng/mL	50 ng/mL	Biorad Calibrator	15424	3848	25%	4.98
100 ng/mL	50 ng/mL	Biorad Positive	21113	3267	15%	6.82
100 ng/mL	50 ng/mL	Biorad Negative	3094	491	16%	1.00
100 ng/mL	50 ng/mL	WHO QC	52784	10750	20%	17.06
100 ng/mL	25 ng/mL	Biorad Calibrator	10689	2753	26%	5.02
100 ng/mL	25 ng/mL	Biorad Positive	20521	5346	26%	9.63
100 ng/mL	25 ng/mL	Biorad Negative	2131	235	11%	1.00
100 ng/mL	25 ng/mL	WHO QC	31438	6189	20%	14.75
50 ng/mL	100 ng/mL	Biorad Calibrator	25189	7374	29%	7.01
50 ng/mL	100 ng/mL	Biorad Positive	37083	6764	18%	10.32
50 ng/mL	100 ng/mL	Biorad Negative	3593	430	12%	1.00
50 ng/mL	100 ng/mL	WHO QC	60561	15427	25%	16.85
50 ng/mL	50 ng/mL	Biorad Calibrator	9657	1061	11%	4.92
50 ng/mL	50 ng/mL	Biorad Positive	19027	4552	24%	9.68
50 ng/mL	50 ng/mL	Biorad Negative	1965	241	12%	1.00
50 ng/mL	50 ng/mL	WHO QC	36686	5530	15%	18.67
50 ng/mL	25 ng/mL	Biorad Calibrator	5030	874	17%	4.23
50 ng/mL	25 ng/mL	Biorad Positive	14601	4171	29%	12.27
50 ng/mL	25 ng/mL	Biorad Negative	1190	108	9%	1.00
50 ng/mL	25 ng/mL	WHO QC	47607	1471	3%	40.02
50 ng/mL	100 ng/mL	Biorad Calibrator	11146	3336	30%	3.93
50 ng/mL	100 ng/mL	Biorad Positive	18477	2473	13%	6.52
50 ng/mL	100 ng/mL	Biorad Negative	2836	237	8%	1.00
50 ng/mL	100 ng/mL	WHO QC	38316	7489	20%	26.67
25 ng/mL	50 ng/mL	Biorad Calibrator	7429	1209	16%	5.17
25 ng/mL	50 ng/mL	Biorad Positive	9804	3229	33%	6.82
25 ng/mL	50 ng/mL	Biorad Negative	1436	258	18%	1.00
25 ng/mL	50 ng/mL	WHO QC	15981	3968	25%	11.13
25 ng/mL	25 ng/mL	Biorad Calibrator	2450	692	28%	3.53
25 ng/mL	25 ng/mL	Biorad Positive	6466	1716	27%	9.32
25 ng/mL	25 ng/mL	Biorad Negative	694	77	11%	1.00
25 ng/mL	25 ng/mL	WHO QC	9376	702	7%	13.51

2.10 Effect of Incubation Times

The optimum incubation time was determined by testing 3 different incubation times. The 10-10-10 incubation time gave the highest modulations (Table 15).

Table 15: Effect of incubation times

Incubation Times	Sample	AVG RLU	STD	CV	Modulation
10-10-10	Biorad Calibrator	8519	853	10%	5.4
10-10-10	Biorad Positive	20363	4995	25%	13.0
10-10-10	Biorad Negative	1568	112	7%	1.0
10-10-10	WHO QC	27062	6482	24%	17.3
5-5-5	Biorad Calibrator	2262	469	21%	4.0
5-5-5	Biorad Positive	3145	268	9%	5.5
5-5-5	Biorad Negative	568	43	7%	1.0
5-5-5	WHO QC	4281	1271	30%	7.5
2-2-1	Biorad Calibrator	408	100	24%	1.7
2-2-1	Biorad Positive	1042	296	28%	4.4
2-2-1	Biorad Negative	240	39	16%	1.0
2-2-1	WHO QC	2415	647	27%	10.1

2.11 Effect of Sample Dilutions

The optimum sample dilution was determined by testing 4 different dilutions. The 10x sample dilution gave acceptable assay performance.

Table 16: Effect of sample dilutions

Sample Dilution	Sample	AVG RLU	STD	CV	Modulation
5x	Biorad Calibrator	9517	2072	22%	7.2
5x	Biorad Positive	7005	1680	24%	5.3
5x	Biorad Negative	1325	209	16%	1.0
5x	WHO QC	15017	2608	17%	11.3
10x	Biorad Calibrator	6579	1215	18%	4.0
10x	Biorad Positive	10338	1793	17%	6.3
10x	Biorad Negative	1650	269	16%	1.0
10x	WHO QC	24033	4774	20%	14.6
25x	Biorad Calibrator	5108	1129	22%	3.0
25x	Biorad Positive	7522	1030	14%	4.4
25x	Biorad Negative	1697	207	12%	1.0
25x	WHO QC	25885	1802	7%	15.3
50x	Biorad Calibrator	4406	1012	23%	2.4
50x	Biorad Positive	4313	1344	31%	2.4
50x	Biorad Negative	1820	337	19%	1.0
50x	WHO QC	13744	2723	20%	7.6

2.12 Effect of Coating Buffers

The optimum coating buffer was determined by testing 3 different coating buffers. Assay buffer containing 3% BSA was acceptable for coating.

Table 17: Effect of Coating Buffers

Coating Buffer	Sample	AVG RLU	STD	CV	Modulation
3% BSA	Biorad Calibrator	17199	3240	19%	7.9
3% BSA	Biorad Positive	19120	1821	10%	8.8
3% BSA	Biorad Negative	2174	345	16%	1.0
3% BSA	WHO QC	37336	4571	12%	17.2
Starting Block	Biorad Calibrator	16118	2845	18%	8.1
Starting Block	Biorad Positive	17712	2851	16%	8.9
Starting Block	Biorad Negative	1983	158	8%	1.0
Starting Block	WHO QC	38629	5074	13%	19.5
Super Block	Biorad Calibrator	15030	3355	22%	6.4
Super Block	Biorad Positive	17819	2298	13%	7.6
Super Block	Biorad Negative	2348	373	16%	1.0
Super Block	WHO QC	32372	1888	6%	13.8

2.13 Cutoff Determination

The assay cutoff was determined using the formula $\text{Cutoff} = \text{AVG RLU (negative samples)} + 5 * \text{STD}$. All samples were confirmed negative on the Biorad reference kit. The proposed cutoff RLU was 10,732.

Table 18: Cutoff Determination

Sample	RLU	Sample	RLU
1	2497	26	2054
2	2280	27	1770
3	2183	28	3033
4	2199	29	2783
5	2194	30	1681
6	2772	31	2324
7	2191	32	3441
8	3436	33	8892
9	2831	34	1912
10	2788	35	5710
11	1625	36	2372
12	2034	37	2256
13	2276	38	1654
14	1329	39	8785
15	1521	40	3965
16	5326	41	2811
17	1983	42	2703
18	1703	43	3028
19	1508	44	2359
20	1219	45	2206
21	1967		
22	2076		
23	1830		
24	2341		
25	1589		
AVG RLU	2699		
Cutoff	10732		

2.14 Specificity

Assay specificity was tested using various disease samples. The samples were tested on the Biorad reference test and on the Theranos analyzer. The assay was specific and did not cross react with any of the disease samples. The RLU cutoff was 10,732 (section 2.12).

Table 19: Specificity

Disease	Biorad			Theranos		
	AVG OD	S/CO	Result	AVG RLU	S/CO	Result
HIV QC1	0.07	0.23	Negative	1967	0.19	Negative
Total anti-HBc QC1	0.06	0.20	Negative	2076	0.20	Negative
Anti-HSV QC1	0.07	0.23	Negative	1830	0.17	Negative
Anti-Rubella QC1	0.07	0.21	Negative	2341	0.22	Negative
Anti-CMV QC1	0.06	0.20	Negative	1589	0.15	Negative
Anti-Toxoplasma QC1	0.06	0.18	Negative	2054	0.19	Negative
Anti-VZV QC 1	0.06	0.19	Negative	1770	0.17	Negative
Anti-EBV	0.06	0.16	Negative	3033	0.29	Negative
Virotrol RPR Panel Syphilis	0.06	0.20	Negative	2783	0.26	Negative
Virotrol Mumz (includes Mumps, Measles, VZV)	0.07	0.21	Negative	1681	0.16	Negative
Rheumatoid	0.05	0.16	Negative	2324	0.22	Negative
Rheumatoid	0.06	0.17	Negative	3441	0.33	Negative
Rheumatoid	0.05	0.17	Negative	8892	0.84	Negative
Rheumatoid	0.06	0.18	Negative	1912	0.18	Negative
Rheumatoid	0.20	0.63	Negative	5710	0.54	Negative
HAMA	0.08	0.25	Negative	2372	0.22	Negative
HAMA	0.06	0.18	Negative	2256	0.21	Negative
HAMA	0.05	0.17	Negative	1654	0.16	Negative
HAMA	0.16	0.51	Negative	8785	0.83	Negative
HAMA	0.10	0.32	Negative	3965	0.37	Negative
ANA	0.07	0.21	Negative	2811	0.27	Negative
ANA	0.06	0.20	Negative	2703	0.26	Negative
ANA	0.08	0.24	Negative	3028	0.29	Negative
ANA	0.06	0.20	Negative	2359	0.22	Negative
ANA	0.08	0.25	Negative	2206	0.21	Negative

2.15 Clinical Correlation

The assay clinical correlation was tested by testing 20 clinical samples and comparing the Theranos results to reference results. The correlation was good with the exception of 1 outlier (sample 20).

Table 20: Clinical Correlation

Sample	Theranos		Siemens Advia Centaur		Abbott Architect		DiaSorin-ETI-HA-IGMK Plus		DiaSorin Liaison		Ortho Vitros		Roche Elecsys		Biorad Kit	
	S/CO	Result	S/CO	Result	S/CO	Result	S/CO	Result	S/CO	Result	S/CO	Result	S/CO	Result	S/CO	Result
1	0.4	Negative	0.3	Negative	0.8	Negative	0.1	Negative	0.3	Negative	0.6	Negative	0.3	Negative	0.49	Negative
2	0.3	Negative	0.2	Negative	0.4	Negative	0.1	Negative	0.2	Negative	0.5	Negative	0.3	Negative	0.24	Negative
3	0.3	Negative	0.2	Negative	0.5	Negative	0.0	Negative	<0.1	Negative	0.5	Negative	0.3	Negative	0.28	Negative
4	0.2	Negative	0.2	Negative	0.3	Negative	0.1	Negative	<0.1	Negative	0.3	Negative	0.2	Negative	0.14	Negative
5	6.7	Positive	> 7.0	Positive	2.8	Positive	6.1	Positive	> 10.0	Positive	7.5	Positive	8.6	Positive	5.11	Positive
6	4.5	Positive	4.1	Positive	1.1	Positive	3.8	Positive	5.9	Positive	2.9	Positive	2.5	Positive	4.36	Positive
7	4.4	Positive	4.0	Positive	1	Positive	3.5	Positive	6.3	Positive	3.1	Positive	2.4	Positive	4.63	Positive
8	0.4	Negative	0.3	Negative	0.7	Negative	0.1	Negative	0.3	Negative	0.4	Negative	0.3	Negative	0.49	Negative
9	0.2	Negative	0.2	Negative	0.4	Negative	0.1	Negative	<0.1	Negative	0.3	Negative	0.2	Negative	0.19	Negative
10	0.3	Negative	0.4	Negative	0.4	Negative	0.1	Negative	0.1	Negative	0.2	Negative	0.3	Negative	0.26	Negative
11	8.0	Positive	> 7.0	Positive	3.6	Positive	6.5	Positive	> 10.0	Positive	10.1	Positive	15.0	Positive	4.19	Positive
12	0.2	Negative	0.2	Negative	0.4	Negative	0.0	Negative	<0.1	Negative	0.3	Negative	0.2	Negative	0.14	Negative
13	0.8	Negative	0.3	Negative	0.7	Negative	0.1	Negative	0.2	Negative	0.5	Negative	0.3	Negative	0.74	Negative
14	2.6	Positive	2.5	Positive	0.6	Negative	1.3	Positive	2.8	Positive	1.2	Positive	1.3	Positive	3.62	Positive
15	5.8	Positive	5.9	Positive	1.5	Positive	5.2	Positive	>10.0	Positive	5.7	Positive	5.8	Positive	5.01	Positive
16	0.5	Negative	0.4	Negative	0.4	Negative	0.1	Negative	0.3	Negative	0.4	Negative	0.4	Negative	0.42	Negative
17	9.6	Positive	6.5	Positive	2.1	Positive	5.3	Positive	> 10.0	Positive	6.1	Positive	6.4	Positive	5.29	Positive
18	0.2	Negative	0.2	Negative	0.5	Negative	0.0	Negative	<0.1	Negative	0.4	Negative	0.3	Negative	0.14	Negative
19	6.8	Positive	3.4	Positive	1.7	Positive	3.0	Positive	5.2	Positive	2.0	Positive	3.4	Positive	4.22	Positive
20	1.8	Positive	0.2	Negative	0.6	Negative	0.1	Negative	0.2	Negative	0.4	Negative	0.3	Negative	0.33	Negative
21	0.5	Negative	0.3	Negative	0.4	Negative	0.1	Negative	0.2	Negative	0.3	Negative	0.3	Negative	0.32	Negative

3.0 Assay Summary

The Theranos anti-HAV IgM antibody assay development has been completed. Below is the summary table of assay conditions.

Table 21: Summary Table

Surface	UA @ 20 ug/mL
Capture Antibody	Goat anti-human IgM (Southern Biotech, 2022-1, Coat at 1 ug/mL)
Detection Antibody	Mouse Monoclonal (Meridian Life Science, Cat. C86318M), Working concentration @ 50 ng/mL
HAV Antigen	Meridian Life Science, Cat. 8505, Working concentration @ 25 ng/mL
Cutoff	Lot specific and will vary depending on samples tested.
Assay Diluent	500 ug/mL HBR in assay buffer
Wash Buffer	1X Enzo
Protocol	Generic2_10x_PSW_syn_5724