ACCELERATED EMERGENCY USE AUTHORIZATION (EUA) SUMMARY
AntiCoV-ID™ IgG ELISA Kit (Akston Biosciences)

Rx ONLY. For *In vitro* Diagnostic Use
For use under Emergency Use Authorization (EUA) only
www.anticov-id.com

VALIDATION OF THE AntiCoV-ID™ IgG ELISA

Validation of the AntiCoV-ID™ IgG ELISA was performed at Akston Biosciences' Quality Control Laboratory under a written method validation protocol approved by Akston's Quality System. The validation procedure included testing of large number of normal negative serum and plasma samples collected prior to the onset of COVID-19 outbreak (before November 2019) and N=35 positive samples collected from patients who had prior COVID-19 infection to evaluate Positive/Negative cutoff criteria, Sensitivity (Positive Percent Agreement), Specificity (Negative Percent Agreement), Clinical Agreement (Positive Predictive Value and Negative Predictive Value), potential cross-reactivity issues, antibody class specificity (IgG vs. IgM reactivity), and matrix equivalency of the assay kit. In addition, general analytical assay parameters such as Accuracy, Precision, Limit of quantitation, Spike-recovery, and Short-term and Freeze-thaw sample stability were also evaluated.

PERFORMANCE EVALUATION DATA SUMMARY– FULL REPORT

1) Data Analysis and Interpretation of Results

The AntiCoV-ID™ IgG ELISA includes two calibrator standard strips precoated with human IgG to generate a standard curve using a 4-PL curve fit model using an appropriate software (e.g. SoftMaxPro or Gen 5) on the plate reader computer. The quantity of SARS-CoV-2 spike protein receptor binding domain (RBD) antibodies in patient serum or plasma samples can be determined by interpolating on the standard curve and antibody titers can be expressed in µg/mL human IgG units.

A positive/negative cutoff value was established during assay validation after analyzing N=130 normal donor negative serum samples at 1:100 dilution. After removing one outlier using an outlier test (Grubb's Test), the cutoff value was established as Mean + 3 standard deviation (Mean+3SD) of N=129 negative serum samples. In order to minimize the possibility of slight variability between different lots of the ELISA kits, the negative serum samples were tested in three consecutive lots of test kits by 3 analysts independently over multiple days to calculate the average positive/negative cutoff value. The analysis was performed with samples in duplicate wells (using the average values) and separately in single wells (two sets of single well values were analyzed separately) to demonstrate that the AntiCoV-ID™ IgG ELISA can be run in duplicate well format (38 samples can be assayed per kit) or in single well format (76 samples can be performed per kit). Both the duplicate well assay format and the single well assay format showed similar performance in all evaluated assay parameters and demonstrated excellent clinical agreement when analyzing COVID 19-negative or COVID 19-positive serum samples (Table 1A, 1B, and Figure 1).

The positive/negative cutoff value was established as **2.8 µg/mL** during the validation analysis. When this 2.8 µg/mL positive/negative cutoff value was applied to screen all the negative serum samples (N=130 including the outlier for this analysis), only 2 negative serum samples gave values above the 2.8 µg/mL positive/negative cutoff level, indicating a false positive rate of

1.5–2.5% (Table 1).

Serum samples with assayed values at or above the 2.8 µg/mL cutoff value should be considered POSITIVE. Serum samples with assayed values below this cutoff should be considered NEGATIVE.

Table 1A. Testing of Negative donor serum samples in three (3) different lots of AntiCoV-ID™ IgG ELISA Kits to determine Positive/Negative Cutoff Value - **duplicate well analysis**.

	Duplicate Well Analysis	ELISA Kit Lot 1 (Lot # 016461)	ELISA Kit Lot 2 (Lot # 016467)	ELISA Kit Lot 3 (Lot # 016468)	Average of 3 Lots
# of negative sera analyzed		130	80	80	
Outlier removed		1	1	1	
Avg assay value (µg/mL)		0.43	0.35	0.56	
Std Dev (SD) (µg/mL)		0.78	0.73	0.89	
Cutoff (Avg+3SD) (µg/mL)		2.77	2.53	3.23	**2.8**
# of negatives above cutoff		2	2	2	2
% False Positives		1.5%	2.5%	2.5%	2.2%
Negative Percent Agreement		98.5%	97.5%	97.5%	

Table 1B. Testing of Negative donor serum samples in three (3) different lots of AntiCoV-ID™ IgG ELISA Kits to determine Positive/Negative Cutoff Value - **single well analysis**.

	Single Well Analysis	ELISA Kit Lot 1 (Lot # 016461)	ELISA Kit Lot 2 (Lot # 016467)	ELISA Kit Lot 3 (Lot # 016468)	Average of 3 Lots
# of negative sera analyzed		130	80	80	
Outlier removed	Set 1	1	1	1	
	Set 2	1	1	1	
Avg assay value (µg/ml)	Set 1	0.43	0.34	0.56	
	Set 2	0.38	0.36	0.54	
Std Dev (SD) (µg/ml)	Set 1	0.85	0.72	0.91	
	Set 2	0.75	0.74	0.88	
Cutoff (Avg+3SD) (µg/ml)	Set 1	2.98	2.51	3.30	**2.9**
	Set 2	2.62	2.57	3.19	**2.8**
# of negatives above cutoff	Set 1	2	2	2	
	Set 2	2	2	2	
% False Positives	Set 1	1.5%	2.5%	2.5%	2.2%
	Set 2	1.5%	2.5%	2.5%	2.2%
Negative Percent Agreement	Set 1	98.5%	97.5%	97.5%	
	Set 2	98.5%	97.5%	97.5%	

2) Sensitivity, Specificity, and Clinical Agreement

Large number of negative serum (N=130) and plasma (N=30) samples from normal donors (collected prior to beginning of COVID-19 outbreak), and N=35 patient samples from Covid-19+ donors collected approximately >14 days after the clinical infection were tested in parallel in three assay kit lots to determine the sensitivity and specificity of the assay.

Sensitivity (Positive Percent Agreement) was determined as 97.1% across all three kit lots tested. 34 serum samples of 35 COVID-19+ serum samples tested were detected above the cutoff in all three lots.

Specificity (Negative Percent Agreement) was determined as 98.5% (N=130 samples) in Lot 1 and as 97.5% (N=80 samples) in Lot 2 and Lot 3. Only two negative serum samples gave values above the cutoff values in testing of three separate lots, suggestive of low levels of pre-existing antibodies in those samples giving signals in the assay.

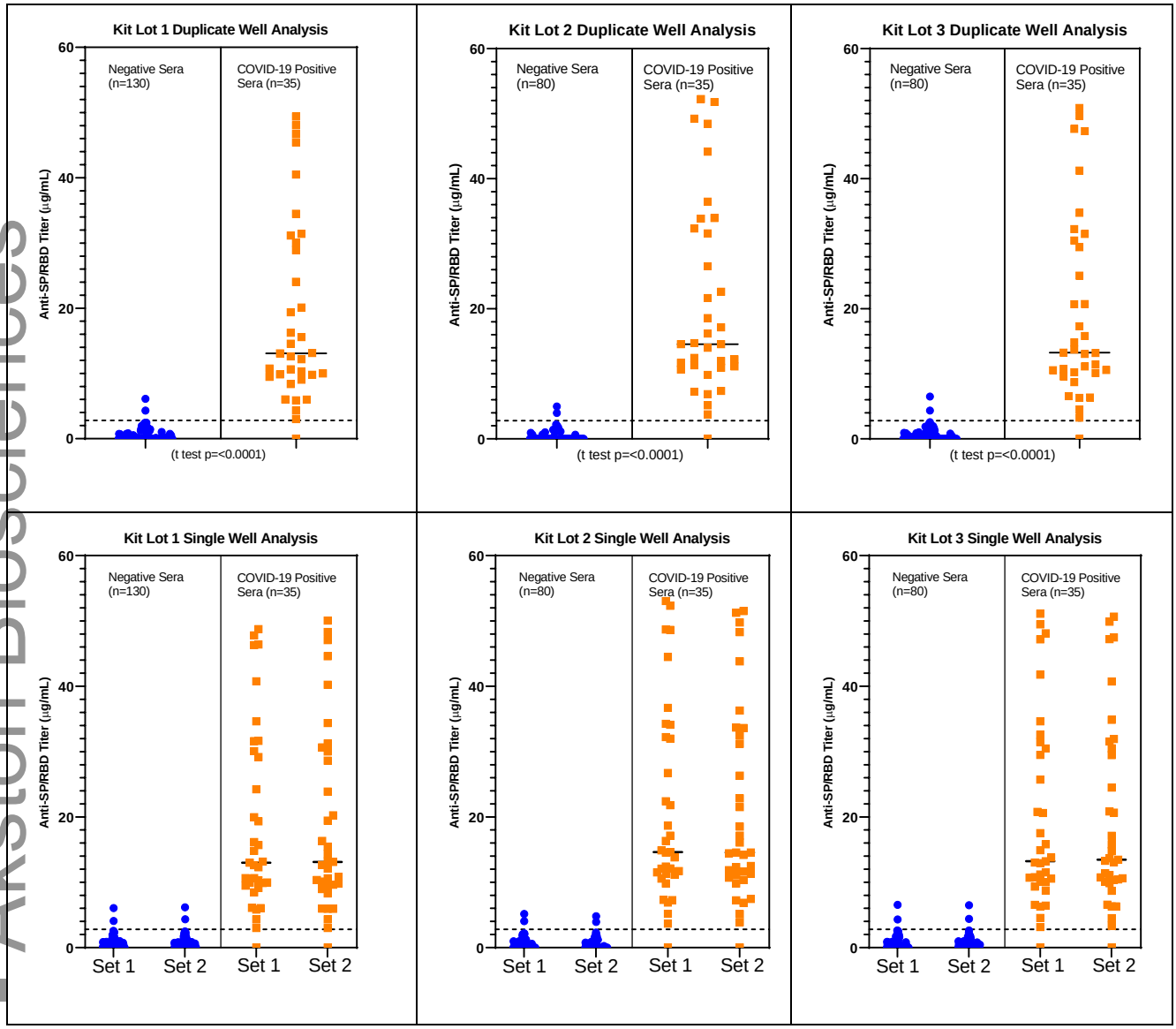

Figure 1. Testing of negative donor serum samples and COVID-19 positive patient serum samples in three (3) different lots of AntiCoV-ID™ IgG ELISA kits - **duplicate well and single well analysis**.

Sensitivity, Specificity, Positive Predictive Value, and Negative Predictive Value were analyzed for duplicate well assay format and single well assay format. The duplicate well assay and both sets of single well assays gave same results and clinical values (Table 2).

Table 2. AntiCoV-ID™ IgG ELISA performance 2 x 2 table.

Test Kit Lot 1 (N=130 negative serum samples and N=35 COVID-19 positive samples)

	Clinical Covid-19 Status						
AntiCoV-ID™ ELISA		Positive			Negative		
		Duplicate Well	Single well Set 1	Single well Set 2	Duplicate Well	Single well Set 1	Single well Set 2
	Positive	34	34	34	2	2	2
	Negative	1	1	1	128	128	128

		Test Kit Lot 1		
		Duplicate Well Analysis	Single Well Set 1 Analysis	Single Well Set 2 Analysis
Sensitivity (Positive Percent Agreement)	True Positives/ (True Positives + False Negatives)	97.1%	97.1%	97.1%
Specificity (Negative Percent Agreement)	True Negatives/ (True Negatives + False Positives)	98.5%	98.5%	98.5%
Positive Predictive Value (PPV)	True Positives/ (True Positives + False Positives)	94.4%	94.4%	94.4%
Negative Predictive Value (NPV)	True Negatives/ (True Negatives + False Negatives)	99.2%	99.2%	99.2%

Test Kit Lot 2 and 3 (N=80 negative serum samples and N=35 COVID-19 positive samples)

	Clinical Covid-19 Status						
AntiCoV-ID™ ELISA		**Positive**			**Negative**		
		Duplicate Well	Single well Set 1	Single well Set 2	Duplicate Well	Single well Set 1	Single well Set 2
	Positive	34	34	34	2	2	2
	Negative	1	1	1	78	78	78

		Test Kit Lot 2 and Lot 3		
		Duplicate Well Analysis	Single Well Set 1 Analysis	Single Well Set 2 Analysis
Sensitivity (Positive Percent Agreement)	True Positives/ (True Positives + False Negatives)	97.1%	97.1%	97.1%
Specificity (Negative Percent Agreement)	True Negatives/ (True Negatives + False Positives)	97.5%	97.5%	97.5%
Positive Predictive Value (PPV)	True Positives/ (True Positives + False Positives)	94.4%	94.4%	94.4%
Negative Predictive Value (NPV)	True Negatives/ (True Negatives + False Negatives)	98.7%	98.7%	98.7%

3) Antibody Class Specificity

Specificity of AntiCoV-ID™ IgG ELISA for highly selective detection of SARS-CoV-2 Spike Protein IgG antibodies was demonstrated by two different approaches.

3.1. Five normal donor negative serum samples were spiked with a spike protein RBD-specific IgG antibody or a spike protein RBD-specific IgM antibody or a mixture of the spike protein RBD-specific IgG and IgM antibodies at high and low concentration levels, and analyzed in AntiCoV-ID™ IgG ELISA, using the anti-human IgG-HRP detection antibody included in the kit or a separate anti-human IgM-HRP detection antibody. For quantitating the spike protein RBD IgM antibodies in the assay, a separate set of IgM coated calibrator standard strips were included in the assays.

All spike protein RBD IgM antibody spiked serum samples were negative in the AntiCoV-ID™ IgG ELISA demonstrating that the anti-human IgG-HRP detection antibody (enzyme conjugate) used in the assay kit does not have any cross reactivity to human IgM antibodies. All double (IgG and IgM) spiked serum samples were detected as positives and the IgG titer recovery was >90% for all spiked samples, demonstrating that the ability of human spike protein RBD IgM antibodies to compete with IgG and produce false negative results is minimal.

In order to determine the positive/negative cutoff values for IgM antibodies, N=36 serum samples were run in the AntiCoV-ID™ IgG ELISA using the IgM calibrator standards and anti-human IgM-HRP detection antibody. The cutoff for spike protein RBD IgM antibodies was determined as 0.8 µg/mL (average + 3 SD).

The assay results were also analyzed as duplicate wells as well as two separate sets of single wells for all samples. Both duplicate well analysis and single well analysis produced similar assay titer values and essentially the same clinical agreement regardless of IgM spike level (Table 3A and 3B).

Table 3A. Spike protein RBD specific IgG/IgM antibody spiked Serum Sample Analysis using anti-human IgG-HRP and anti-human IgM-HRP detection systems - **duplicate well analysis**. **Clinical (+/-)**: "+" denotes samples that were positive and above the AntiCoV-ID™ IgG ELISA cutoff value. "-" denotes samples that were negative and below the AntiCoV-ID™ IgG ELISA cutoff value.

	Serum ID	Anti-human IgG-HRP detection							Anti-human IgM-HRP detection					
		IgG only Spiked µg/mL	Clinical (+/–)	IgM only Spiked µg/mL	Clinical (+/–)	IgG + IgM Spiked µg/mL	Clinical (+/–)	Recovery of IgG titers	IgG only Spiked µg/mL	Clinical (+/–)	IgM only Spiked µg/mL	Clinical (+/–)	IgG + IgM Spiked µg/mL	Clinical (+/–)
High Spike	1	31.2	+	0	–	28.4	+	91%	0	–	20.5	+	20.0	+
	2	31.4	+	0	–	28.8	+	92%	0	–	21.3	+	19.5	+
	3	30.4	+	0.6	–	29.0	+	95%	0	–	21.8	+	19.0	+
	4	29.6	+	0	–	28.6	+	97%	0	–	21.6	+	18.9	+
	5	30.7	+	0	–	28.6	+	94%	0	–	20.9	+	19.5	+
Low Spike	1	9.2	+	0	–	8.9	+	97%	0	–	4.4	+	4.4	+
	2	9.1	+	0	–	8.9	+	98%	0	–	4.5	+	4.4	+
	3	9.0	+	0	–	8.8	+	98%	0.1	–	4.7	+	4.4	+
	4	9.0	+	0	–	9.1	+	101%	0	–	4.4	+	4.2	+
	5	9.1	+	0	–	9.3	+	102%	0.1	–	4.4	+	4.4	+

Table 3B. Spike Protein specific IgG/IgM antibody spiked Serum Sample Analysis using anti-human IgG-HRP and anti-human IgM-HRP detection systems – **single well analysis**. **Clinical (+/-)**: "+" denotes samples that were positive and above the AntiCoV-ID™ IgG ELISA cutoff value. "-" denotes samples that were negative and below the AntiCoV-ID™ IgG ELISA cutoff value.

	Serum ID	Anti-human IgG-HRP detection							Anti-human IgM-HRP detection						
		IgG only Spiked µg/mL	Clinical (+/–)	IgM only Spiked µg/mL	Clinical (+/–)	IgG + IgM Spiked µg/mL	Clinical (+/–)	Recovery of IgG titers	IgG only Spiked µg/mL	Clinical (+/–)	IgM only Spiked µg/mL	Clinical (+/–)	IgG + IgM Spiked µg/mL	Clinical (+/–)
High Spike	1	31.3	+	0	–	28.5	+	91%	0	–	20.6	+	20.1	+
		31.2	+	0	–	28.4	+	91%	0	–	20.3	+	19.9	+
	2	31.5	+	0	–	28.8	+	91%	0	–	21.5	+	19.6	+
		31.2	+	0	–	28.8	+	92%	0	–	21.1	+	19.4	+
	3	30.6	+	0.3	–	29.1	+	95%	0	–	21.7	+	19.0	+
		30.2	+	0.9	–	28.9	+	96%	0	–	21.8	+	18.9	+
	4	29.0	+	0	–	28.7	+	99%	0	–	21.8	+	19.0	+
		30.3	+	0	–	28.6	+	94%	0	–	21.4	+	18.8	+
	5	31.0	+	0	–	28.8	+	93%	0	–	21.0	+	19.5	+
		30.5	+	0	–	28.9	+	95%	0	–	20.7	+	19.5	+
Low Spike	1	9.1	+	0	–	9.0	+	99%	0	–	4.4	+	4.4	+
		9.2	+	0	–	8.8	+	96%	0	–	4.4	+	4.4	+
	2	9.2	+	0	–	8.9	+	97%	0	–	4.5	+	4.4	+
		9.1	+	0	–	8.9	+	98%	0	–	4.4	+	4.4	+
	3	9.1	+	0	–	8.7	+	96%	0.1	–	4.7	+	4.4	+
		8.9	+	0	–	8.9	+	99%	0.1	–	4.6	+	4.4	+
	4	9.0	+	0	–	9.0	+	100%	0	–	4.4	+	4.2	+
		8.9	+	0	–	9.1	+	102%	0	–	4.4	+	4.2	+
	5	9.1	+	0	–	9.3	+	102%	0.1	–	4.4	+	4.4	+
		9.0	+	0	–	9.4	+	104%	0.1	–	4.4	+	4.4	+

3.2. Seven COVID-19 positive patient serum samples were assayed in AntiCoV-ID™ IgG ELISA with and without treating with DTT to demonstrate that presence of spike protein RBD IgM antibodies does not have a potential to interfere with IgG antibodies and cause false negative results.

Table 4. Inactivation of IgM antibodies by DTT treatment to demonstrate IgG antibody specificity for the AntiCoV-ID™ IgG ELISA.

Covid-19 Positive Sample ID	Replicates	Results (µg/ml) No DTT Treatment IgG	Results (µg/ml) No DTT Treatment IgM	Results (µg/mL) DTT Treatment IgG	Results (µg/mL) DTT Treatment IgM	Expected Results with DTT Treatment IgG	Expected Results with DTT Treatment IgM	Results Agreement
		+/– Cutoff 2.8 µg/mL	+/– Cutoff 0.8 µg/mL	+/– Cutoff 2.8 µg/mL	+/– Cutoff 0.8 µg/mL			
ACS01	1	15.6 +	3.0 +	11.2 +	0.0 –	+	–	Yes
	2	15.2 +	3.3 +	11.2 +	0.0 –	+	–	Yes
ACS02	1	31.2 +	10.5 +	23.3 +	0.6 –	+	–	Yes
	2	31.1 +	10.8 +	23.2 +	0.6 –	+	–	Yes
ACS05	1	45.8 +	8.7 +	32.4 +	0.6 –	+	–	Yes
	2	45.7 +	8.9 +	32.1 +	0.5 –	+	–	Yes
ACS06	1	32.9 +	2.3 +	23.5 +	0.4 –	+	–	Yes
	2	32.7 +	2.4 +	23.6 +	0.4 –	+	–	Yes
*ACS07	1	12.8 +	0.2 –	9.8 +	0.0 –	+	–	Yes
	2	12.7 +	0.2 –	9.6 +	0.0 –	+	–	Yes
373379	1	15.6 +	2.7 +	13.5 +	1.2 +	+	–	Yes
	2	15.4 +	2.7 +	13.4 +	1.2 +	+	–	Yes
*369389	1	4.3 +	0.6 –	5.4 +	0.0 –	+	–	Yes
	2	3.9 +	0.6 –	5.2 +	0.0 –	+	–	Yes

Two serum samples (*ACS07 and *369389) had very low levels of IgM antibodies (below the cutoff for IgM) and reported negative for IgM even in the non-DTT treated samples, and the IgM levels became undetectable (0) after DTT treatment while IgG levels remained as positive. For one serum sample (373379), the IgM antibody levels were only partially decreased (>50% decrease) after DTT treatment. The remaining four serum samples were positive for both IgG and IgM in the non-DTT treated samples and became IgG+/IgM− after DTT treatment.

Both experiments described under 3.1 and 3.2 above demonstrated that potential for human IgM antibodies to compete with IgG or interfere in the assay and produce false negative results is minimal. The clinical results of the assay to detect spike protein RBD IgG antibodies remained unchanged regardless of the presence of IgM class antibodies in the samples.

4) Matrix Equivalency

N=11 serum samples and N=11 matching (paired) plasma samples (N=7 Na-Citrate Plasma and N=4 EDTA-Plasma) were analyzed both untreated and after heat inactivation at 56°C for 1 hour to demonstrate matrix equivalency. Both serum and plasma samples were run at 1:100 dilution in the AntiCoV-ID™ IgG ELISA per standard assay protocol and the results are summarized in Table 5A and 5B. Assay values between Serum and Plasma (either Citrate-Plasma or EDTA-Plasma) were comparable (90%–120% value agreement) and all samples detected as positives (100% clinical agreement). Only one plasma sample (#173949) showed a greater assay titer value that its paired serum sample, potentially due to some error during sample collection and processing. The assay titer value agreement between untreated serum and heat-inactivated serum, or between untreated plasma and heat-inactivated plasma was 93%–113% and 90%–100%, respectively. The untreated and heat-inactivated matrices showed 100% clinical agreement.

Table 5A. COVID-19 positive serum, citrate plasma and EDTA-plasma (untreated and heat-inactivated) Analysis – **Duplicate Well Analysis**. **Clinical (+/-)**: "+" denotes samples that were positive and above the AntiCoV-ID™ IgG ELISA cutoff value. "-" denotes samples that were negative and below the AntiCoV-ID™ IgG ELISA cutoff value.

Duplicate Well Analysis												
Patient ID COVID 19+	Serum				Citrate-Plasma				EDTA-Plasma			
	Untreated µg/mL	Clinical +/−	Heat Inactivated µg/mL	Clinical +/−	Untreated µg/mL	Clinical +/−	Heat Inactivated µg/mL	Clinical +/−	Untreated µg/mL	Clinical +/−	Heat Inactivated µg/mL	Clinical +/−
ACS01	14.2	+	14.5	+	13.1	+	12.9	+				
ACS02	29.8	+	33.7	+	28.2	+	30.5	+				
ACS03	18.5	+	19.0	+	16.9	+	16.3	+				
ACS04	25.3	+	23.6	+	23.3	+	21.0	+				
ACS05	45.8	+	46.9	+	44.0	+	43.3	+				
ACS06	32.8	+	31.1	+	30.0	+	28.6	+				
ACS07	11.9	+	12.5	+	10.7	+	11.2	+				
173949	4.9	+	4.9	+					8.3	+	8.4	+
173950	15.5	+	14.8	+					14.7	+	13.8	+
173951	14.0	+	14.2	+					16.3	+	16.7	+
173956	34.9	+	35.3	+					35.4	+	34.7	+

Table 5B. COVID-19 Positive Serum, Citrate-Plasma and EDTA-Plasma (Untreated and Heat-Inactivated) Analysis – **Single Well Analysis**. **Clinical (+/-)**: "+" denotes samples that were positive and above the AntiCoV-ID™ IgG ELISA cutoff value. "-" denotes samples that were negative and below the AntiCoV-ID™ IgG ELISA cutoff value.

Single Well Analysis												
Patient ID COVID 19+	Serum				Citrate-Plasma				EDTA-Plasma			
	Untreated µg/mL	Clinical +/−	Heat Inactivated µg/mL	Clinical +/−	Untreated µg/mL	Clinical +/−	Heat Inactivated µg/mL	Clinical +/−	Untreated µg/mL	Clinical +/−	Heat Inactivated µg/mL	Clinical +/−
ACS01	14.2	+	14.4	+	13.1	+	12.9	+				
ACS02	29.9	+	33.7	+	28.2	+	30.6	+				
ACS03	18.4	+	19.0	+	17.0	+	16.4	+				
ACS04	25.4	+	23.6	+	23.2	+	21.2	+				
ACS05	46.0	+	46.8	+	43.8	+	42.9	+				
ACS06	32.9	+	31.1	+	30.2	+	28.7	+				
ACS07	11.9	+	12.4	+	10.8	+	11.2	+				
173949	4.9	+	4.9	+					8.2	+	8.5	+
173950	15.5	+	14.9	+					14.8	+	13.7	+
173951	13.9	+	14.2	+					16.2	+	16.8	+
173956	35.4	+	36.3	+					35.2	+	34.8	+

Table 6A. Ratio and Clinical (+/−) Agreement between Serum and Plasma, and between untreated and heat-inactivated samples in **Duplicate Well Analysis**. **Clinical (+/-)**: "+" denotes samples that were positive and above the AntiCoV-ID™ IgG ELISA cutoff value. "-" denotes samples that were negative and below the AntiCoV-ID™ IgG ELISA cutoff value.

Duplicate Well Analysis						
Patient ID COVID 19+	Agreement between Serum and Plasma		Agreement between Untreated and Heat inactivated Serum		Agreement between Untreated and Heat inactivated Paasma	
	Ratio	Clinical +/−	Ratio	Clinical +/−	Ratio	Clinical +/−
ACS01	92%	100%	102%	100%	99%	100%
ACS02	95%	100%	113%	100%	108%	100%
ACS03	91%	100%	103%	100%	96%	100%
ACS04	92%	100%	93%	100%	90%	100%
ACS05	96%	100%	102%	100%	98%	100%
ACS06	92%	100%	95%	100%	95%	100%
ACS07	90%	100%	105%	100%	105%	100%
173949	169%	100%	100%	100%	101%	100%
173950	95%	100%	95%	100%	94%	100%
173951	117%	100%	101%	100%	102%	100%
173956	102%	100%	101%	100%	98%	100%

Table 6B. Ratio and Clinical (+/−) Agreement between Serum and Plasma, and between untreated and heat-inactivated samples in **Single Well Analysis**. **Clinical (+/-)**: "+" denotes samples that were positive and above the AntiCoV-ID™ IgG ELISA cutoff value. "-" denotes samples that were negative and below the AntiCoV-ID™ IgG ELISA cutoff value.

Single Well Analysis						
Patient ID COVID 19+	Agreement between Serum and Plasma		Agreement between Untreated and Heat inactivated Serum		Agreement between Untreated and Heat inactivated Paasma	
	Ratio	Clinical +/−	Ratio	Clinical +/−	Ratio	Clinical +/−
ACS01	92%	100%	102%	100%	99%	100%
ACS02	95%	100%	113%	100%	109%	100%
ACS03	92%	100%	103%	100%	97%	100%
ACS04	91%	100%	93%	100%	92%	100%
ACS05	95%	100%	102%	100%	98%	100%
ACS06	92%	100%	95%	100%	95%	100%
ACS07	91%	100%	104%	100%	104%	100%
173949	166%	100%	100%	100%	103%	100%
173950	95%	100%	96%	100%	93%	100%
173951	116%	100%	102%	100%	104%	100%
173956	100%	100%	103%	100%	99%	100%

5) Cross reactivity – other viruses

Serum collected from patients with known IgG titers against the following non-coronaviruses was tested on the assay: Mumps (N=8), Measles (N=8), Epstein-Barr virus (EBV; N=8), Cytomegalovirus (CMV; N=8), Varicella zoster virus (VZV; N=8), influenza virus (N=10). None of the samples from any of these donors demonstrated any cross-reactivity on the AntiCoV-ID IgG ELISA assay (Figure 2A, 2B; Table 7A).

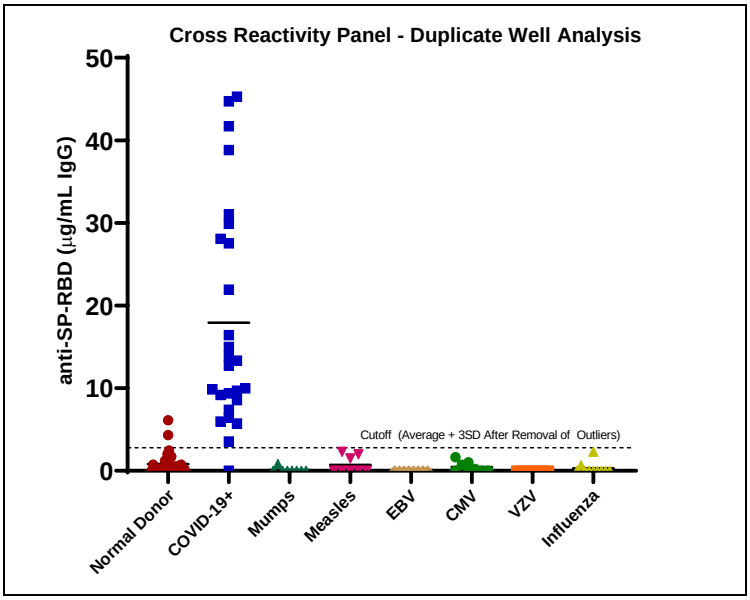

Figure 2A. Performance of AntiCoV-ID™ IgG ELISA with samples from donors with known IgG titers against various viruses – **Duplicate Well Analysis**.

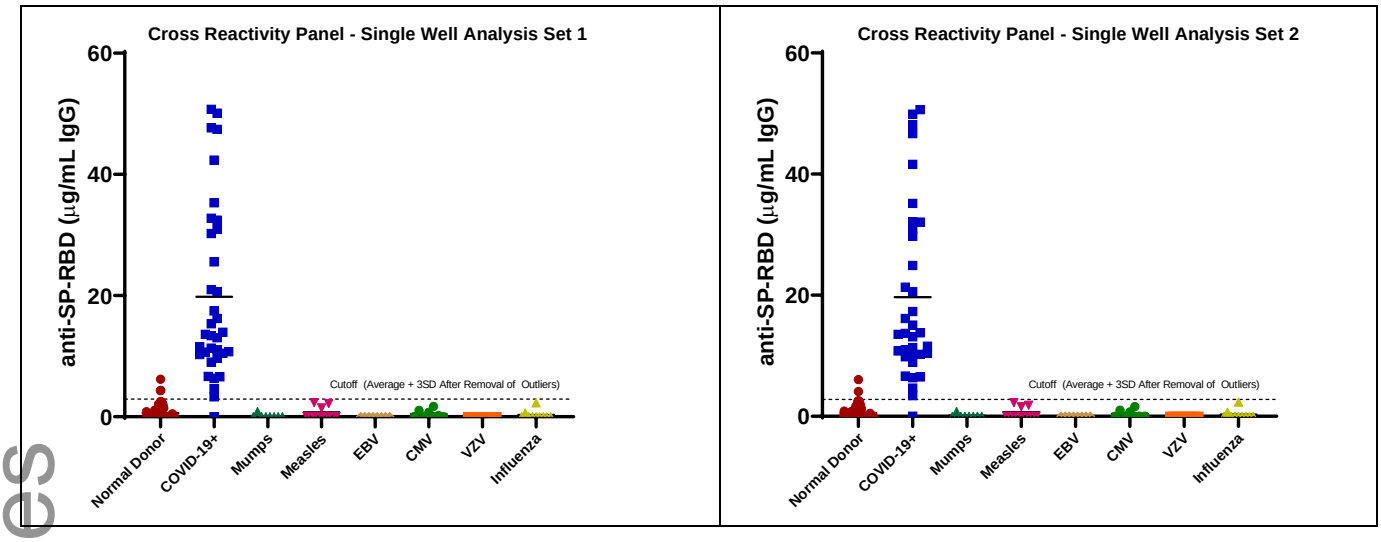

Figure 2B. Performance of AntiCoV-ID™ IgG ELISA with samples from various types of donors - **Single Well Analysis**.

Table 7A. AntiCOV-ID™ IgG ELISA shows no cross reactivity with serum from patients with non-coronavirus IgG titers.

ID	Panel	N	AntiCov-ID ELISA	
			Negatives	% Negative
1	Anti-Mumps IgG Antibodies	8	8	100%
2	Anti-Measles IgG Antibodies	8	8	100%
3	Anti-EBV/Anti-Epstein-Barr Nuclear Antigen IgG Antibodies	8	8	100%
4	Anti-CMV IgG Antibodies	8	8	100%
5	Anti-VZV IgG Antibodies	8	8	100%
6	Anti-Influenza IgG Antibodies	10	10	100%

Cross reactivity – other non-SARS coronaviruses and additional virus panel

Serum collected from N=105 patients with known IgG titers against additional non-SARS coronaviruses (229E, HKU1, NL63 and OC43) and still other viruses are provided in Table 7B below. This study was conducted at a Tier 1 university hospital system, and the AntiCoV-ID IgG ELISA was demonstrated to have a significantly better false positive rate than a competitor qualitative IgG ELISA kit (competitor kit has EUA approval; 5% false positive rate in panel; data not shown) run with matched samples.

Table 7B. AntiCoV-ID™ IgG ELISA performance in non-SARS coronavirus and other virus cross-reactivity panel run at leading Tier 1 university hospital system.

Serum Panel Containing IgG's Against	N	Negative	%Negative
Adenovirus E	4	4	100%
Coronavirus 229E	8	8	100%
Coronavirus HKU1	6	6	100%
Coronavirus NL63	9	8	89%
Coronavirus OC43	15	15	100%
Coxsackie/Echovirus	12	12	100%
Influenza A	2	2	100%
Influenza B	6	6	100%
Influenza A, novel H1N1	8	8	100%
Parainfluenzavirus 1	2	2	100%
Parainfluenzavirus 3	2	2	100%
Parainfluenzavirus 4	2	2	100%
Metapneumovirus (A,B)	2	2	100%
Rhinovirus	11	11	100%
Rhinovirus & Coxsackie/Echovirus	14	14	100%
RSVA	2	2	100%
Total	**105**	**104**	
False Positives	**1**		
False Positive Rate in Panel	**< 1.0%**		

7) Accuracy and Precision

In order to validate analytical accuracy and precision of the AntiCoV-ID™ IgG ELISA, three levels of Validation Samples (High QC, Mid QC, Low QC) were prepared by spiking an anti-SARS-CoV-2 spike protein specific human IgG1 chimeric antibody in sample dilution buffer, and these samples were then stored frozen at -20°C as small-volume aliquots for single-thaw use and used for validation runs. Sample dilution buffer was used as unspiked Negative QC. The QCs were tested in multiple runs to establish their nominal values and the assigned nominal values were used to evaluate Accuracy, Precision, and other validation parameters. At least six core assay runs were performed by ≥ 3 analysts over at least two days, with no more than two runs per day per analyst.

Validation Samples (High QC, Mid QC, Low QC) were assayed and analyzed both as duplicate well and single well assay runs, and the data are summarized in Tables 8A and 8B, and the full data set is shown in Appendix 1.

Table 8A. AntiCoV-ID™ IgG ELISA Accuracy & Precision Summary – **Duplicate Well Analysis**.

	Criteria	Results	
Accuracy			
Intra-assay Accuracy	± 20% of for HQC and MQC ± 25% for LQC of assigned nominal values	HQC 95%-116% MQC 94%-115% LQC 94%-113%	PASS PASS PASS
Inter-assay Accuracy	± 20% of for HQC and MQC ± 25% for LQC	HQC 102% MQC 102% LQC 101%	PASS PASS PASS
Precision			
Intra-assay Precision	≤ 20% of for HQC and MQC ≤ 25% for LQC of assigned nominal values	HQC 1%-4% MQC 1%-2% LQC 0%-3%	PASS PASS PASS
Inter-assay Precision	≤ 25% CV in across all runs	HQC 6.7% MQC 6% LQC 6.7%	PASS PASS PASS

Table 8B. AntiCoV-ID™ IgG ELISA accuracy and precision summary – **Single Well Analysis**.

	Criteria	Results	
Accuracy			
Intra-assay Accuracy	±20% of for HQC and MQC ±25% for LQC of assigned nominal values	HQC 95%-117% MQC 94%-115% LQC 94%-114%	PASS PASS PASS
Inter-assay Accuracy	±20% of for HQC and MQC ±25% for LQC	HQC 102% MQC 102% LQC 101%	PASS PASS PASS
Precision			
Intra-assay Precision	≤ 20% of for HQC and MQC ≤ 25% for LQC of assigned nominal values	HQC 1%-3% MQC 1%-2% LQC 1%-4%	PASS PASS PASS
Inter-assay Precision	≤ 25% CV in across all runs	HQC 7.0% MQC 6.1% LQC 6.7%	PASS PASS PASS

8) Spike Recovery

Recovery of the analyte in sample matrix (normal human serum and normal human plasma samples) was evaluated by spiking a COVID-19 high positive sample at High, Medium, and Low levels and assessing the recovery of spiked values. The positive sample spiked in sample dilution buffer was used as the control for calculating the recovery. Un-spiked serum and plasma were also diluted in the sample dilution buffer and assayed for subtraction of background signals.

Three levels of spiked samples were analyzed. The observed concentrations of at least two-thirds of the spiked samples at each level should demonstrate a recovery of ± 25% of their nominal values (± 30% for lower concentration sample) to meet the acceptance criteria for the spike recovery (see Table 9 below).

Table 9. Spike Recovery in serum and plasma.

	Duplicate Well Analysis			Single Well Analysis		
	High Spike	**Mid Spike**	**Low Spike**	**High Spike**	**Mid Spike**	**Low Spike**
Serum	98%–102%	97%–109%	100%–118%	99%–104%	97%–107%	100%–118%
Plasma	87%–102%	79%–107%	83%–114%	86%–101%	78%–110%	83%–115%

All N=12 spiked serum samples showed recovery between 79% and 118% of spiked values. Only one out of N=12 plasma sample showed 69% recovery, and all remaining plasma samples gave

acceptable recovery between 78% and 118%. A complete spike recovery data set is shown in Appendix 2.

9) Limits of Quantitation

The limit of Detection (LOD) in the AntiCoV-ID™ IgG ELISA is the lowest point of the calibration curve (0.0125 µg/mL). The lower limit of quantitation (LLOQ) was determined by spiking spike protein RBD human IgG1 chimeric antibody in sample dilution buffer at multiple concentration levels near the lower end of the curve (e.g. serial dilutions in small increments) and then testing them in 3 assays to determine the LLOQ. The lowest concentration of the analyte that gave ≤ 25% CV and values within ± 30% spiked concentration in repeated assays was selected as the LLOQ. From both duplicate well analysis and single well analysis, the LLOQ was determined as **0.013 µg/mL**, i.e. sample assay values ≥ 0.013 µg/mL should be considered as reliable. Details of the LLOQ data are summarized in Appendix 3A.

Similarly, the upper limit of quantitation (ULOQ) was evaluated by assaying several closely spaced concentrations at the upper end of the curve in 3 assay runs. The highest concentration of the analyte that gave ≤ 25% CV and values within ± 25% spiked concentration in repeated assays was selected as the ULOQ. The ULOQ was determined as 0.530 µg/mL in duplicate well analysis and 0.540 µg/mL in single well analysis, hence sample assay titer values **≤ 0.540 µg/mL** should be considered as reliable and the end user should consider reanalyzing samples that yield values above the this level with ≥ 4X additional dilution (e.g. 1:400 dilution or higher) to get more reliable titer values. Details of the ULOQ data are summarized in Table 10 below and Appendix 3B.

The effective dynamic assay range therefore is established as 0.013 µg/mL to 0.540 µg/mL.

Table 10. LLOQ and ULOQ for the AntiCoV-ID™ IgG ELISA.

LLOQ and ULOQ	Criteria	Results
LLOQ	% Recovery ± 30% and ≤ 25% CV for lowest spiked	LLOQ = 0.013 µg/mL (Recovery 122%; CV 9%)
ULOQ	% Recovery ± 30% and ≤ 25% CV for highest spiked	ULOQ = 0.540 µg/mL (Recovery 114%; CV 5%)

10) Sample Stability

Sample stability was evaluated by spiking a negative serum sample with spike protein RBD-specific human IgG1 chimeric antibody at High, Mid, and Low levels. The spiked QC samples were stored frozen in small aliquots and thawed and used for the stability testing. The three spiked QC Samples were exposed to room temperature (20–25°C) for ≥ 4 hours or kept in the refrigerator (2–8°C) for 24-48 hours and assayed along with a set of freshly-thawed QC samples. Samples were also exposed to one, two, and three freeze-thaw (F/T) cycles with a minimum of 12 hours of freezing between thaws. The measured anti-spike protein RBD IgG concentrations in 2–8°C and RT exposed QC samples, and 1X, 2X, and 3X F/T QC samples were compared to the results of freshly-thawed samples.

Stability Samples (High QC, Mid QC, Low QC) were analyzed both as duplicate wells and single wells, and the data are summarized in Table 11A and 11B. The complete data set is shown in Appendix 4.

Table 11A. Sample Stability Summary – **Duplicate Well Analysis**.

	Criteria	Results	
Sample Stability		% Recovery	
Samples exposed to room temp for 4 hrs	75 – 125% Recovery	101 – 103%	PASS
Samples exposed to2-8°C for 24 hrs	75 – 125% Recovery	99 – 102%	PASS
Samples exposed to 1 X Freeze-thaw	75 – 125% Recovery	103 – 105%	PASS
Samples exposed to 2 X Freeze-thaw	75 – 125% Recovery	96 – 100%	PASS
Samples exposed to 3 X Freeze-thaw	75 – 125% Recovery	98 – 99%	PASS

Table 11B. Sample Stability Summary – **Single Well Analysis**.

	Criteria	Results	
Sample Stability		% Recovery	
Samples exposed to room temp for 4 hrs	75 – 125% Recovery	102 – 105%	PASS
Samples exposed to2-8°C for 24 hrs	75 – 125% Recovery	100 – 102%	PASS
Samples exposed to 1 X Freeze-thaw	75 – 125% Recovery	100 – 105%	PASS
Samples exposed to 2 X Freeze-thaw	75 – 125% Recovery	96 – 101%	PASS
Samples exposed to 3 X Freeze-thaw	75 – 125% Recovery	99 – 100%	PASS

11) Summary

The AntiCoV-ID™ IgG ELISA is currently the only quantitative, validated ELISA assay for the measurement of human IgG antibodies against the SARS-CoV-2 virus receptor binding domain. The assay was validated over multiple production lots of kits, and the validation passed all parameters both for the technical portion and clinical portion of the validation. Moreover, the low %CVs demonstrate by the kit allowed for the validation of the assay with samples in duplicate wells, but also allowed for validation in single well mode.

The assay was proven sensitive and selective in the clinical analysis of Covid-19+ and normal patient serum, with a PPA of 97.1%, an NPA of 98.5%, a PPV of 99.4%, and a NPV of 99.2%, which makes the assay one of the best performing ELISA assays currently available. A cutoff value was established to delineate negative samples from Covid-19+ samples on the assay. Additionally, good clinical agreement was obtained for heat inactivated and non-heat inactivated serum and plasma samples. Furthermore, the assay was also shown to not cross-react with other types of serum containing IgM nor other serum samples containing IgG titers against other viruses.

The highly sensitive and selective AntiCoV-ID™ IgG ELISA assay is also competitive against other high-throughput techniques. When run in duplicate well mode and run over a three hour protocol, the assay is capable of quantitating 38 patient samples (~5.0 minutes per patient-test), or in singlet well mode the assay is capable of quantitating 76 patient samples (~2.5 minutes per patient-test). Often a well-trained operator or automated system can further boost these patient-test productivities by running more than one assay kit simultaneously.

12) Production update and regulatory status

The AntiCoV-ID™ IgG ELISA EUA application is currently under review at FDA. The kits are now IVD, and are being produced in large production lots. The kits are fully able to be used for diagnostic purposes by laboratories certified to perform high complexity testing. They are not to be used for at-home testing. The kits are sold in the United States under Section IV.D of the Policy for Diagnostic Tests for Coronavirus Disease-2019 during the Public Health Emergency. The AntiCoV-ID™ IgG ELISA kit is registered with the FDA with the Device Listing number D409900 under Akston Biosciences' Owner/Operator number 10075756.