For additional clarity, we provide three example specimens with sample data for evaluation:

	Zika Ag OD_{450}	CCA OD_{450}	NCA OD_{450}
Positive Control – Replicate #1	1.640	0.165	0.075
Positive Control – Replicate #2	1.584	0.184	0.081
Negative Control – Replicate #1	0.081	0.064	0.062
Negative Control – Replicate #2	0.071	0.066	0.069
Sample #1	1.379	0.085	0.062
Sample #2	0.120	0.946	0.049
Sample #3	0.114	0.099	0.108

Step 1: ***Evaluate the QC Criteria***

Evaluating the QC Criteria, we find the following:

Factor (For Assay Verification)	Calculated Value	Acceptance Criteria	Criteria met? (Yes/No)
Average Positive Control OD_{450} in Zika Antigen	1.612	> 0.500	Yes
Positive Control Zika Immune Status Ratio (Zika ISR)	9.21 (1.612 ÷ 0.175)	≥ 4.00	Yes
Average Negative Control OD_{450} with Zika Antigen, CCA and NCA	0.076 / 0.065 / 0.066	< 0.120	Yes
Average Negative Control OD_{450} with Zika Antigen	0.076	> 0.030	Yes
Negative Control Zika Immune Status Ratio (Zika ISR)	1.17 (0.076 ÷ 0.065)	< 2.00	Yes
Negative Control CCA/NCA Ratio	0.98 (0.065 ÷ 0.066)	< 2.00	Yes

The criteria were met, so we may continue the analysis.

Step 2: ***Determine the Threshold Zika Ag OD_{450}***

We next calculate the Threshold Zika Ag OD_{450} = 0.130 + Average Negative Control OD_{450} with the Zika Antigen. That is **Threshold Zika Ag OD_{450}** = 0.130 + 0.076 = **0.206.**

Step 3: ***Calculate the Zika ISR and CCA / NCA ratio***

The Zika ISR and CCA ÷ NCA ratios are calculated and shown below.

	Zika Ag OD_{450}	CCA OD_{450}	NCA OD_{450}	Zika ISR	CCA / NCA
Average Positive Control	1.612	0.175	0.078	9.21	2.24
Average Negative Control	0.076	0.065	0.066	1.17	0.98
Sample #1	1.379	0.085	0.062	16.22	1.37
Sample #2	0.120	0.946	0.049	0.13	19.31
Sample #3	0.114	0.099	0.108	1.15	0.92

Step 4: ***Categorize the Presumptive Zika Positive specimens***

Any specimen with a raw Zika Ag OD_{450} ≥ Threshold Zika Ag OD_{450} AND a Zika ISR > 1.90 is considered Presumptive Zika Positive. For this example, the Threshold Zika Ag OD_{450} per step 2 is 0.206.

	Zika Ag OD_{450}	CCA OD_{450}	NCA OD_{450}	Zika ISR	CCA / NCA	Presumptive Zika Positive?
Average Positive Control	1.612	0.175	0.078	9.21	2.24	YES
Average Negative Control	0.076	0.065	0.066	1.17	0.98	NO
Sample #1	1.379	0.085	0.062	16.22	1.37	YES
Sample #2	0.120	0.946	0.049	0.13	19.31	NO
Sample #3	0.114	0.099	0.108	1.15	0.92	NO

Step 5: ***Determine if the specimen requires duplicate repeat testing***

None of the samples meet both criteria that Zika antigen raw OD_{450} value ≥ Threshold OD_{450} AND 1.50 ≤ Zika ISR ≤ 1.90. Hence, no samples require duplicate repeat testing.

Step 6: ***Categorize the Presumptive Other Flavivirus (non-Zika) specimens***

We then evaluate all of the remaining specimens that are not categorized as Zika positive. If a (non-Zika) specimen has a CCA ÷ NCA ≥ 5.00, then the sample is considered Presumptive Other Flavivirus Positive (non-Zika).

	Zika Ag OD_{450}	CCA OD_{450}	NCA OD_{450}	Zika ISR	CCA / NCA	Presumptive Zika Positive?	Presumptive Other Flavivirus (non-Zika)?
Average Positive Control	1.612	0.175	0.078	9.21	2.24	YES	N/A
Average Negative Control	0.076	0.065	0.066	1.17	0.98	NO	NO
Sample #1	1.379	0.085	0.062	16.22	1.37	YES	N/A
Sample #2	0.120	0.946	0.049	0.13	19.31	NO	YES
Sample #3	0.114	0.099	0.108	1.15	0.92	NO	NO

Step 7: ***Categorize the Negative specimens***

If a specimen is not categorized as Presumptive Zika Positive or as Presumptive Other Flavivirus (non-Zika), then the sample should be considered Negative. All specimens are now categorized and can be interpreted appropriately.

	Zika Ag OD_{450}	CCA OD_{450}	NCA OD_{450}	Zika ISR	CCA / NCA	Interpretation
Average Positive Control	1.612	0.175	0.078	9.21	2.24	Presumptive Zika
Average Negative Control	0.076	0.065	0.066	1.17	0.98	Negative
Sample #1	1.379	0.085	0.062	16.22	1.37	Presumptive Zika
Sample #2	0.120	0.946	0.049	0.13	19.31	Presumptive Other Flavivirus
Sample #3	0.114	0.099	0.108	1.15	0.92	Negative

EXPECTED VALUES/REFERENCE RANGE

Of 609 subjects enrolled for the clinical studies, 466 subjects from the non-endemic and endemic sites reported both age and gender, and did not provide serial draws. The serum samples were prospectively collected from these subjects. The reactivities of the ZIKV *Detect*™ 2.0 IgM Capture ELISA with the endemic and non-endemic populations are shown in the tables below.

Expected results from an endemic site

				ZIKV *Detect*™ 2.0 IgM Capture ELISA results		
Age Group (years)	**Total No. of Subjects**	**number of males**	**number of females**	**number reactive**	**number non-reactive**	**% reactive**
5-18	72	35	37	0	72	0.0%
19-30	83	42	41	2	81	2.5%
31-49	70	37	33	3	67	4.5%
50-64	18	9	9	0	18	0.0%
65+	7	3	4	0	7	0.0%

Expected results from a non-endemic site

				ZIKV *Detect*™ 2.0 IgM Capture ELISA results		
Age Group (years)	**Total No. of Subjects**	**number of males**	**number of females**	**number reactive**	**number non-reactive**	**% reactive**
5-18	7	3	4	0	7	0.0%
19-30	54	22	32	2	52	3.8%
31-49	68	32	36	0	68	0.0%
50-64	51	22	29	0	51	0.0%
65+	36	13	23	1	35	2.9%

PERFORMANCE CHARACTERISTICS

Clinical Studies:

Test samples were collected from endemic sites (both presumed positive and presumed negative samples) and from non-endemic sites (presumed negative samples). Of the 609 subjects, 31 provided serial draws after confirmation of zika infection. These subjects returned for serum collections up to five times, ranging from 0-84 days post symptoms onset. Another 50 subjects from zika endemic areas provided paired acute/convalescent draws. A total of 807 unique samples were provided by the 609 subjects.

All samples were shipped to InBios for aliquoting and randomization and were then distributed among three sites in the United States for testing using the ZIKV *Detect*™ 2.0 IgM Capture ELISA. Test results with the ZIKV *Detect*™ 2.0 IgM Capture ELISA were compared to a composite reference method that included a validated Zika RT-PCR and CDC Zika MAC-ELISA. Positive percent agreement (PPA) and negative percent agreement (NPA) for the endemic and non-endemic subjects are presented in tables 1 and 2 respectively.

Specific days Post Symptom Onset (PSO) of collection was known for 744 of 807 samples. Positive percent agreement (PPA) and negative percent agreement (NPA) for combined endemic and non-endemic specimens are presented by days Post Symptom Onset (PSO) in table 3. As expected for an IgM assay, the PPA is lower for PSO < 7 days. For samples collected ≥7 days PSO, PPA is > 90%.

Table 1. ZIKV *Detect*™ 2.0 IgM Capture ELISA - Agreement results for the endemic subjects

		Composite Reference Method Results			
		Positive	**Equivocal**	**Negative**	**Total**
ZIKV *Detect*™ 2.0 IgM Capture ELISA Result	**Positive**	84	0	2	86
	Other Flavivirus	5	1	19	25
	Negative	4[a]	0	238	242
	Total	93	1	259	353
	PPA; 95% CI	89.4% (84/94): 95% CI: 81.5%-94.8%)			
	NPA; 95% CI	99.2% (257/259): 95% CI: 97.2%-99.9%)			

[a]Two of four samples were collected <7 days PSO. PPA is 91.34% without counting these two samples.

Table 2. ZIKV *Detect*™ 2.0 IgM Capture ELISA - Agreement results for the non-endemic subjects

		Composite Reference Method Results			
		Positive	**Equivocal**	**Negative**	**Total**
ZIKV *Detect*™ 2.0 IgM Capture ELISA Result	**Positive**	13	0	10	23
	Other Flavivirus	0	0	1	1
	Negative	3[b]	0	229	232
	Total	16	0	240	256
	PPA; 95% CI	81.3% (13/16): 95% CI: 54.4%-96.0%)			
	NPA; 95% CI	95.8% (230/240): 95% CI: 92.5%-98.0%)			

[b]The samples were collected <7 days PSO. PPA is 100% without counting these three samples.

***Note*:** Samples with high OD_{450} values for both Zika Ag and CCA may be misclassified by ZIKV *Detect*™ 2.0 IgM Capture ELISA as "Presumptive Other Flavivirus Positive" rather than "Presumptive Zika Positive". Further confirmatory testing is recommended.

Table 3. ZIKV *Detect*™ 2.0 IgM Capture ELISA - Agreement results for combined endemic and non-endemic specimens by days Post Symptom Onset (PSO)

Days PSO	Number of Specimens	Number of True Positives	Number of Reference Positives	PPA	Number of True Negatives	Number of Reference Negatives	NPA
0-2	283	2	53	3.8% (2/53)	228	230	99.1% (228/230)
3-6	223	14	34	41.2% (14/34)	187	189	98.9% (187/189)
7-14	70	32	35	91.4% (32/35)	34	35	97.1% (34/35)
15-21	47	36	38	94.7% (36/38)	9	9	100.0% (9/9)
22-28	39	34	37	91.9% (34/37)	2	2	100.0% (2/2)
29-42	51	45	48	93.8% (45/48)	3	3	100.0% (3/3)
43-84	31	30	31	96.8% (30/31)	0	0	N/A

Analytical Sensitivity:

The purpose of this study was to estimate the limit of detection (LOD) for the ZIKV *Detect*™ 2.0 IgM Capture ELISA using the World Health Organization (WHO) 1st International Standard for anti-Asian lineage Zika virus antibody (human). Multiple dilutions of the antibody were tested in replicates of twenty. The lowest concentration at which ≥95% of replicates tested Presumptive Zika positive was considered the LOD. LOD was determined to be 225 IU/mL.

Table 4: Analytical Sensitivity of the ZIKV *Detect*™ 2.0 IgM Capture ELISA

	275 IU/mL	250 IU/mL	225 IU/mL	200 IU/mL
Replicates positive	20	20	20	14
Replicates negative	0	0	0	6
Detection rate	100%	100%	100%	70%

Reproducibility Study:

The reproducibility study of the ZIKV *Detect*™ 2.0 IgM Capture ELISA was performed at three sites by two different individuals at each site for 5 separate days. Each operator ran one blinded panel of specimens in triplicate on each day. In addition, three lots of ZIKV *Detect*™ 2.0 IgM Capture ELISA were provided for each site. For *each lot* of ZIKV *Detect*™ 2.0 IgM Capture ELISA, a total of 3 replicates x 3 sites x 2 operators x 5 days = 90 total replicates were performed for each panel member. As there were three lots provided, a final total of 270 replicates for each panel member was evaluated. A panel consisting of five samples, including a 'negative', 'high negative', 'low positive,' 'moderate positive,' and a 'retest' specimen, were tested in this study. The ZIKV *Detect*™ 2.0 IgM Capture ELISA's total precision %CV (from the "total" standard deviation) for the ISR values ranged from 13.0% - 29.6%, depending upon the sample.

Table 5: Reproducibility of the ZIKV *Detect*™ 2.0 IgM Capture ELISA

			Repeatability		Between-Operator		Between-Days		Between-Lot		Between-Sites		Total Precision	
Sample ID	**Mean Value**	**N**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**	**SD**	**%CV**
Low Positive	4.08	270	0.43	10.5	0.30	7.33	0.49	12.0	0.45	10.9	0.86	21.1	1.21	29.6
Moderate Positive	7.85	270	0.79	10.0	0.91	11.6	0.87	11.1	0.53	6.76	1.27	16.2	2.03	25.8
Re-test	1.83	270	0.24	13.0	0.14	7.45	0.14	7.83	0.21	11.6	0.18	9.84	0.42	22.7
Negative	1.03	270	0.09	9.06	0.00	0.00	0.06	5.76	0.05	4.64	0.06	5.34	0.13	13.0
High Negative	1.07	270	0.14	13.0	0.00	0.00	0.06	5.52	0.04	3.79	0.07	6.33	0.17	16.1
%CV coefficient of variation expressed as a percentage; SD standard deviation. Zero cell means the variance estimate was below zero.														

Cross-reactivity Study:

Cross-reactivity of the ZIKV *Detect*™ 2.0 IgM Capture ELISA was evaluated by testing specimens from patients with confirmed IgM antibodies to other microorganisms which could potentially cause false positive results. The study utilized a panel of IgM positive serum specimens sourced from patients who have been infected with potentially cross-reactive microorganisms. Results for cross-reactivity are presented in table 6.

Table 6: Cross-reactivity of the ZIKV *Detect*™ 2.0 IgM Capture ELISA

Specimen Type	# of samples	# Zika Positive	# Other Flavivirus Positive	# Non-Reactive
Dengue††	39	1[a]	38	0
West Nile Virus	28	2[a]	19	7
Japanese Encephalitis	11	0	4	7
Eastern Equine Encephalitis Virus (EEEV)	3	0	0	3
Varicella-Zoster Virus	10	0	0	10
St. Louis Encephalitis Virus	10	0	1	9
Yellow Fever Vaccine Recipients	24	5[a,b]	1	18
Chikungunya	57	5[a,c]	2	50
Malaria	9	1[a]	0	8
Syphilis	8	0	1	7
Rubella	10	0	0	10
Herpes Simplex Virus[d]	20	0	0	20
Lyme	10	1[a]	0	9
Hepatitis B	10	0	0	10
Hepatitis C	10	0	0	10
Leptospirosis	9	0	0	9
Babesiosis	15	3	0	12
Parvovirus	12	0	0	12
Epstein-Barr Virus	15	0	0	15
Cytomegalovirus	10	0	0	10

RF	16	0	0	16
HAMA	15	2[a, c]	0	13
ANA	10	0	0	10

Total:	346	17	66	263

[tt]Dengue specimens included Dengue-1 (n = 10), Dengue-2 (n = 9), Dengue-3 (n = 10) and Dengue-4 (n = 10). Serotypes were confirmed with acute phase samples but IgM seropositivity was confirmed with convalescent phase sample draws. The ZIKV *Detect*™ 2.0 IgM Capture ELISA was performed with the convalescent phase sample draw.

[a]The following number of ZIKV *Detect*™ 2.0 IgM Capture ELISA Zika Positive specimens also tested as Zika Positive with the CDC Zika MAC-ELISA: 1 Dengue specimen, 1 Yellow Fever Vaccine recipient [an additional 2 specimens were Equivocal], 1 Malaria specimen, 1 Chikungunya specimen [an additional 1 specimen was Equivocal], and 2 HAMA specimens. The West Nile Virus, Lyme, and 3 Chikungunya specimens were negative with CDC Zika MAC-ELISA testing.

[b]The yellow fever vaccine recipients that were positive with the ZIKV *Detect*™ 2.0 IgM Capture ELISA were sourced from Colombia during a Zika virus outbreak in 2016.

[c]Chikungunya and HAMA specimens that tested Zika positive with ZIKV *Detect*™ 2.0 IgM Capture ELISA were tested with PRNT. Four Chikungunya and 2 HAMA specimens demonstrated neutralization activity with ZIKV PRNT90.

[d]Ten (10) specimens are HSV-1 IgM positive; ten (10) specimens are HSV-2 IgM positive.

In addition, viral vector that was used to prepare Zika recombinant antigen was tested for cross-reactivity. Supernatants from cells transformed with a plasmid containing the same vector backbone as that used to generate Zika VLPs show no reactivity against samples positive or negative for zika in ZIKV *Detect*™ 2.0 IgM Capture ELISA. The reactivity from these supernatants is comparable to the reactivity from cell supernatants without plasmid transformation (NCA).

Interference Study:

Potentially interfering substances commonly occurring in serum were evaluated with the ZIKV *Detect*™ 2.0 IgM Capture ELISA. Interfering substances included conjugated and unconjugated bilirubin (0.4 mg/mL), hemoglobin (20 mg/mL), albumin (60 mg/mL), cholesterol (5 mg/mL), triglycerides (30 mg/mL), HAMA (~800 and ~80 ng/mL), and rheumatoid factor (2060 IU/mL). These interfering substances were spiked into low reactive (n=3) and normal human serum samples (n=3) to evaluate their impact on assay performance. Of the interfering substances tested, only very high levels of HAMA seemed to have a deleterious effect by decreasing Zika Ag reactivity, resulting in false negative results with the panel tested. At the lower HAMA concentration tested, no interference was observed.

Table 7: Interference of the ZIKV *Detect*™ 2.0 IgM Capture ELISA

Interfering Substance	Concentration Tested	Effect on Low Reactive Specimens	Effect on Negative Specimens
Bilirubin unconjugated	0.4 mg/mL	None observed (0/3)	None observed (0/3)
Bilirubin conjugated	0.4 mg/mL	None observed (0/3)	None observed (0/3)
Hemoglobin	20 mg/mL	None observed (0/3)	None observed (0/3)
Human Serum Albumin	60 mg/mL	None observed (0/3)	None observed (0/3)
Cholesterol	5 mg/mL	None observed (0/3)	None observed (0/3)
Intralipids (triglycerides)	30 mg/mL	None observed (0/3)	None observed (0/3)
HAMA	798.7 ng/mL	*Interference observed (3/3)*	None observed (0/3)
	79.9 ng/mL	None observed (0/3)	None observed (0/3)
RF	2060 IU/mL	None observed (0/3)	None observed (0/3)

Example Plate Layout

An example plate layout is shown below which indicates a method for screening 28 specimens against Zika Ag, CCA and NCA.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Positive Control**	**Positive Control**	Sample #13	Sample #21	**Positive Control**	**Positive Control**	Sample #13	Sample #21	**Positive Control**	**Positive Control**	Sample #13	Sample #21
B	**Negative Control**	**Negative Control**	Sample #14	Sample #22	**Negative Control**	**Negative Control**	Sample #14	Sample #22	**Negative Control**	**Negative Control**	Sample #14	Sample #22
C	Sample #1	Sample #7	Sample #15	Sample #23	Sample #1	Sample #7	Sample #15	Sample #23	Sample #1	Sample #7	Sample #15	Sample #23
D	Sample #2	Sample #8	Sample #16	Sample #24	Sample #2	Sample #8	Sample #16	Sample #24	Sample #2	Sample #8	Sample #16	Sample #24
E	Sample #3	Sample #9	Sample #17	Sample #25	Sample #3	Sample #9	Sample #17	Sample #25	Sample #3	Sample #9	Sample #17	Sample #25
F	Sample #4	Sample #10	Sample #18	Sample #26	Sample #4	Sample #10	Sample #18	Sample #26	Sample #4	Sample #10	Sample #18	Sample #26
G	Sample #5	Sample #11	Sample #19	Sample #27	Sample #5	Sample #11	Sample #19	Sample #27	Sample #5	Sample #11	Sample #19	Sample #27
H	Sample #6	Sample #12	Sample #20	Sample #28	Sample #6	Sample #12	Sample #20	Sample #28	Sample #6	Sample #12	Sample #20	Sample #28
	Ready to Use ZIKV Antigen (Zika Ag)				Cross-reactive Control Antigen (CCA)				Normal Cell Antigen (NCA)			

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