Supplementary Materials for

Rapid and specific detection of Asian- and African-lineage Zika viruses

Authors: Nunya Chotiwan[1,2†], Connie D. Brewster[1†], Tereza Magalhaes[2,8], James Weger-Lucarelli[1,2], Nisha K. Duggal[3], Claudia Rückert[1,2], Chilinh Nguyen[1,2], Selene M. Garcia Luna[1,2], Joseph R. Fauver[1,2], Barb Andre[1], Meg Gray[1,2], William C. Black IV[1,2], Rebekah C. Kading[1,2], Gregory D. Ebel[1,2], Guillermina Kuan[4], Angel Balmaseda[5], Thomas Jaenisch[6,7], Ernesto T. A. Marques[8,9], Aaron C. Brault[3], Eva Harris[10], Brian D. Foy[1,2], Sandra L. Quackenbush[1], Rushika Perera[1,2], Joel Rovnak[1]*

Affiliations:

[1]Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA.

[2]Arthropod-borne Infectious Disease Laboratories, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA.

[3]Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, CO 80521, USA.

[4]Centro de Salud Sócrates Flores Vivas, Ministry of Health, Managua, Nicaragua.

[5]Laboratorio Nacional de Virología, Centro Nacional de Diagnóstico y Referencia, Ministry of Health, Managua, Nicaragua.

[6]Section Clinical Tropical Medicine, Department for Infectious Diseases, Heidelberg University Hospital, Heidelberg, Germany.

[7]German Centre for Infection Research (DZIF), partner site Heidelberg, Germany

[8]Laboratory of Virology and Experimental Therapeutics, Centro de Pesquisas Aggeu Magalhaes, Fundacao Oswaldo Cruz, Recife-PE, Brazil.

[9]Center for Vaccine Research, School of Public Health, University of Pittsburgh, Pittsburgh, PA 15261, USA.

[10]Division of Infectious Diseases and Vaccinology, School of Public Health, University of California, Berkeley, Berkeley, CA 9472s, USA.

†These authors contributed equally to this work.

*Correspondence to: joel.rovnak@colostate.edu.

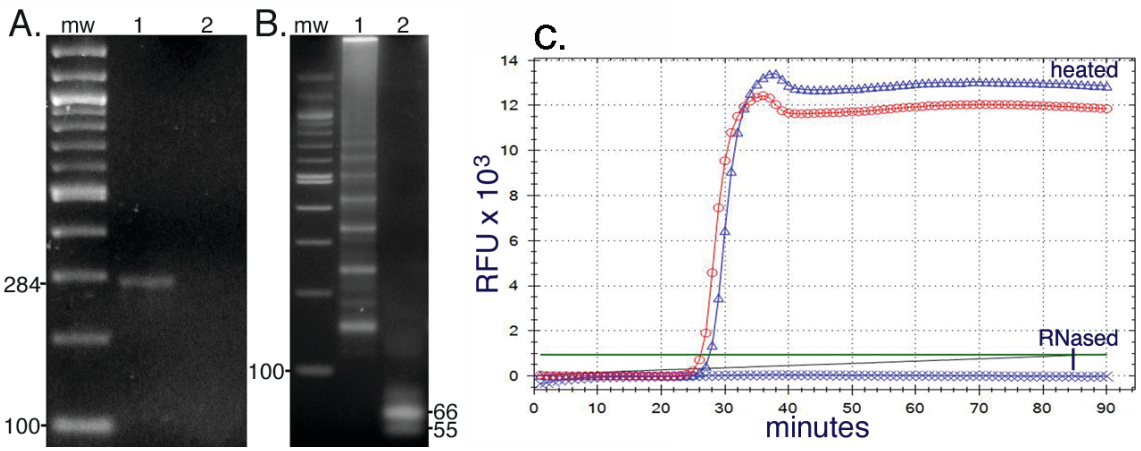


Fig. S1. Zika-specific, RNA-dependent LAMP amplification was confirmed. (**A**) PCR amplification of 1 μl of a 1:500 dilution of the PRVABC59 LAMP product with B3 and F3 LAMP primers to produce a single, predicted 284-bp amplicon. (**B**) PRVABC59 LAMP product (lane 1) was digested with *AluI* restriction endonuclease to yield predicted 55 and 66-bp fragments (lane 2). Molecular weight standards, 100-bp ladders. (**C**) RNase A digestion abrogates LAMP signal. Total RNA from PRVABC59-infected cells were treated with RNase A and the RNase heat inactivated prior to PRVABC59 LAMP. RNased and heat-treated positive-control RNA (blue crosses, below threshold), heat-treated positive-control RNA (blue triangles), positive-control RNA (red circles), buffer (black line, below threshold).

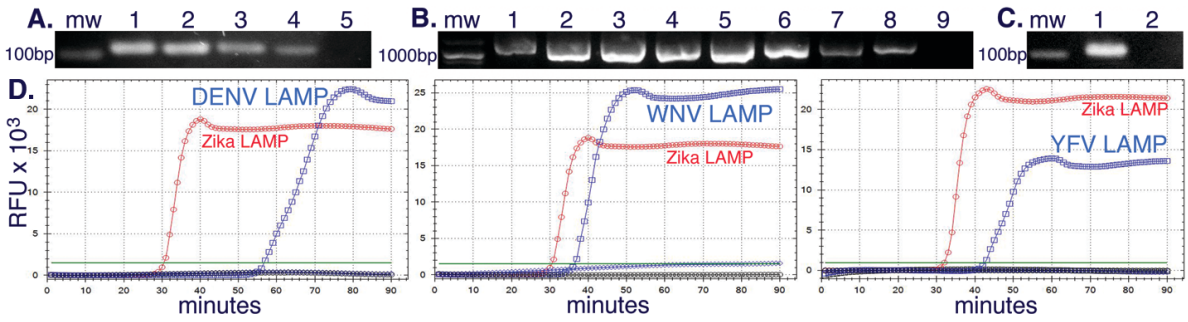
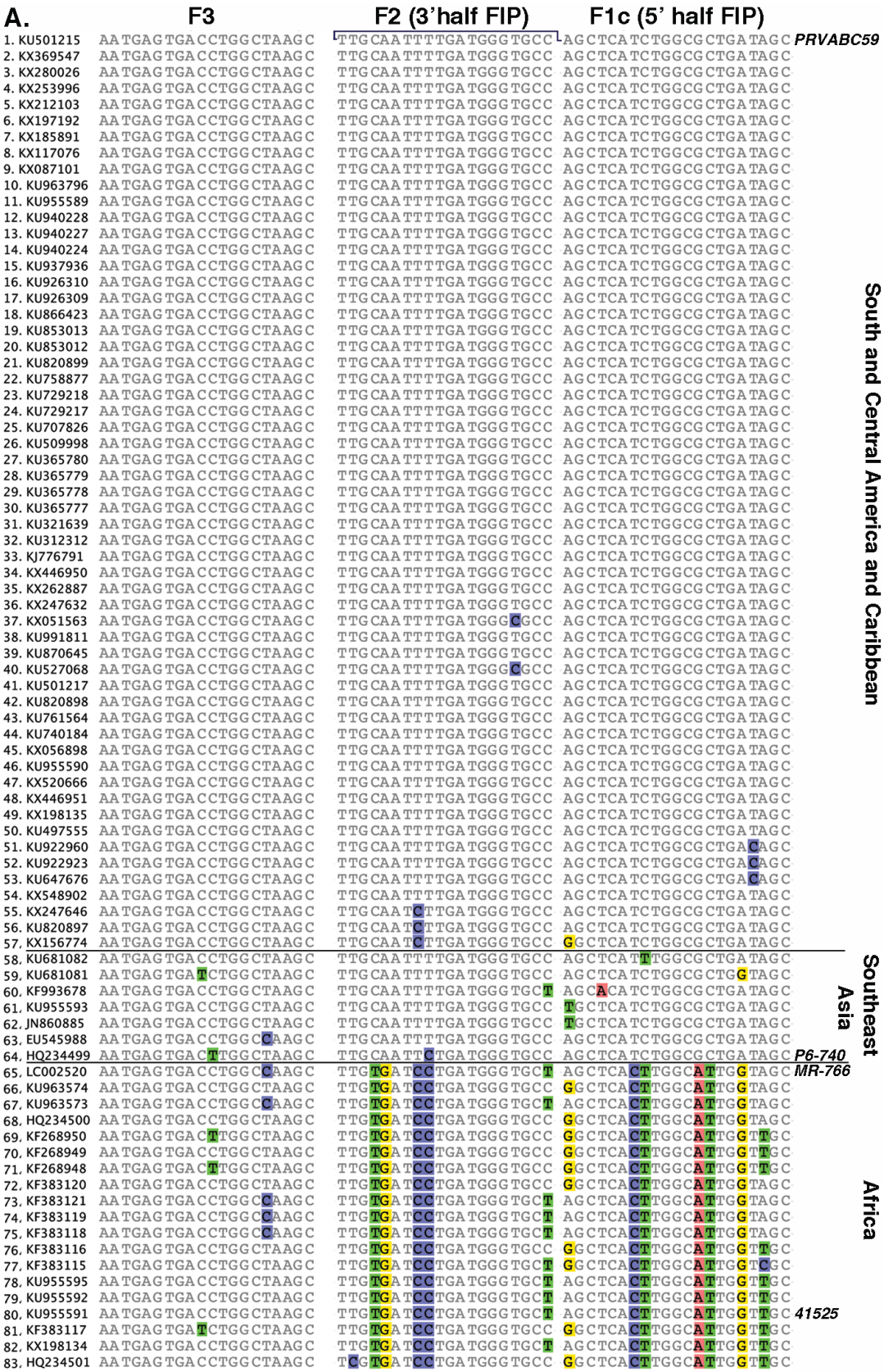


Fig. S2. Control samples contain virus RNA. (**A**) RT-PCR amplifications of Zika virus RNA preparations with Zika virus-specific primers yield the predicted 109-bp amplicon from all four Zika virus RNA preparations; lanes 1-5, PRVABC59, MR-766NIID, 41525, P6-740 and buffer control. Position of 100-bp molecular weight marker is indicated. (**B**) RT-PCR amplifications of flavivirus RNA preparations with pan-flavivirus primers yield predicted ~1000-bp amplicons from 8 different viruses; lanes 1-9, Bussuquara, St. Louis encephalitis, Langat, Powassan, Ilheus, West Nile, DENV-2, YFV and buffer control. Position of 1000-bp molecular weight marker is indicated. (**C**) RT-PCR amplification of CHIKV RNA preparation with CHIKV virus-specific primers yield the predicted 112-bp amplicon (lane 1), buffer control (lane 2). Position of 100-bp molecular weight marker is indicated. (**D**) LAMP amplification of DENV-2, WNV, and YFV RNAs with their respective, specific LAMP primers (blue boxes) and amplification PRVABC59 RNA with PRVABC59-specific primers (red circles). DENV-2, WNV, and YFV RNAs were not amplified with PRVABC59-specific primers (blue diamonds, below threshold), and PRVABC59 RNA was not amplified with DENV-, WNV-, or YFV-specific LAMP primers (black circles, below threshold).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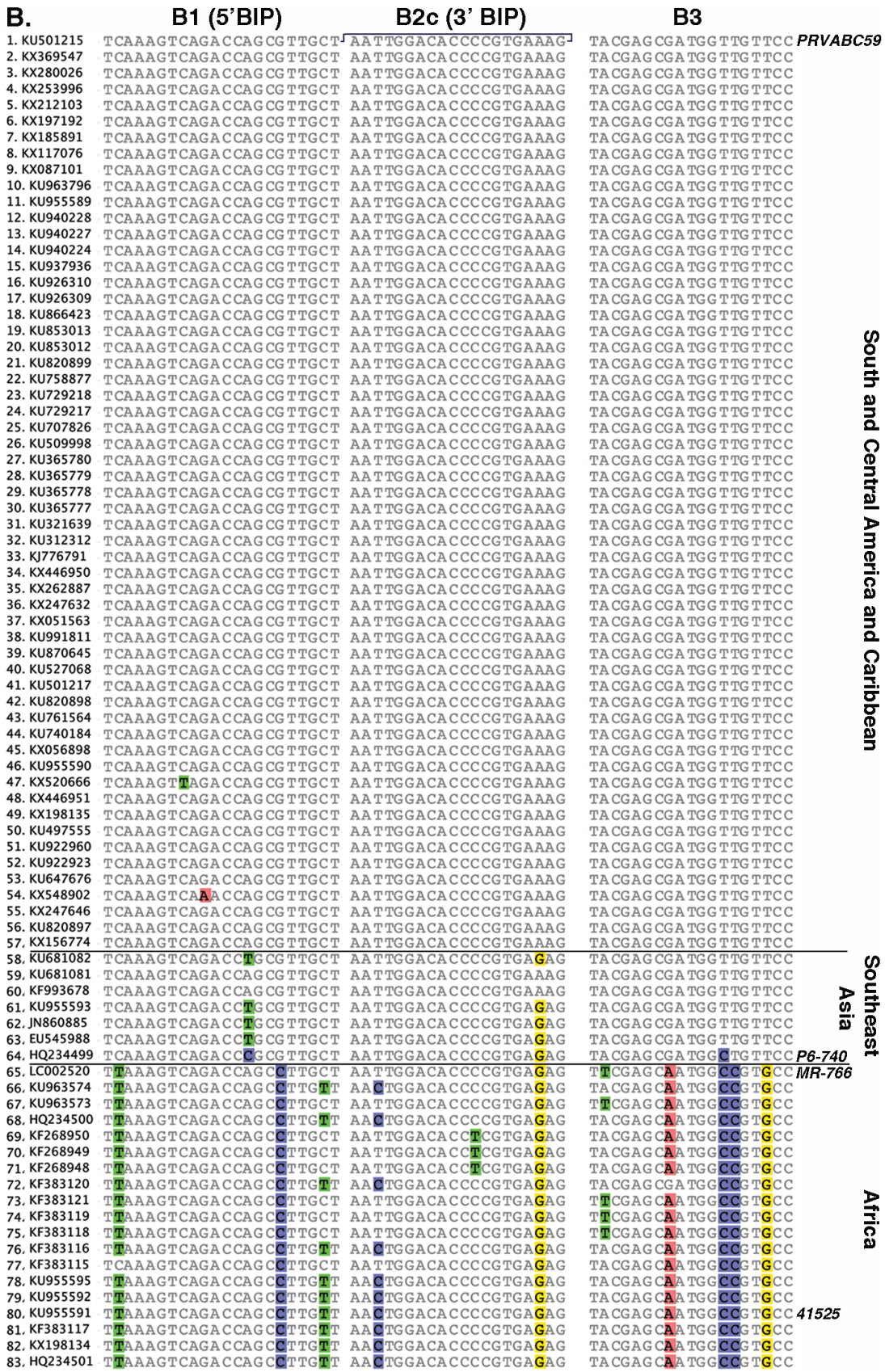
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Southeast Asia
Africa

Fig. S3. Alignment of Zika LAMP primer sequences with target sequences in all known Zika virus sequences were carried out. Primer sequences F3, F2 and F1c (**A**) and B1, B2c and B3 (**B**) are presented in 5' to 3' orientation according to position in the virus genome. FIP and BIP primers (Table S1) are fusions of F2 and the inverse complement of F1c and B1 and the inverse complement of B2c, respectively (Fig.1A). BIP and FIP junctions are indicated by connecting lines above the PRVABC59 sequence (KU501215). Blast alignments were based on the entire 284-bp, LAMP amplicon bound by primers F3 and B3 (Fig. 1A). The sequences of the four strains tested, PRVABC59, P7-740, MR-766NIID, and 41525, are indicated as are the geographic regions where strains were isolated: South and Central America and Caribbean, Southeast Asia (including Micronesia), and Africa. Multiples of identical whole virus sequences with different accession numbers were excluded from the alignment.

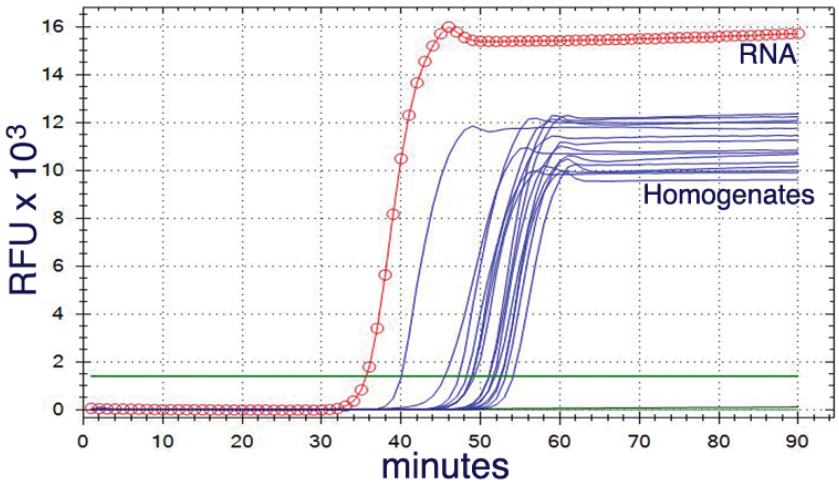

Fig. S4. Direct detection of Zika virus in mosquitoes was accomplished. LAMP amplification of 15 individual homogenates of infected mosquitoes at 7 days post infection (blue lines), positive control RNA (red circles), buffer control (black line, below threshold).

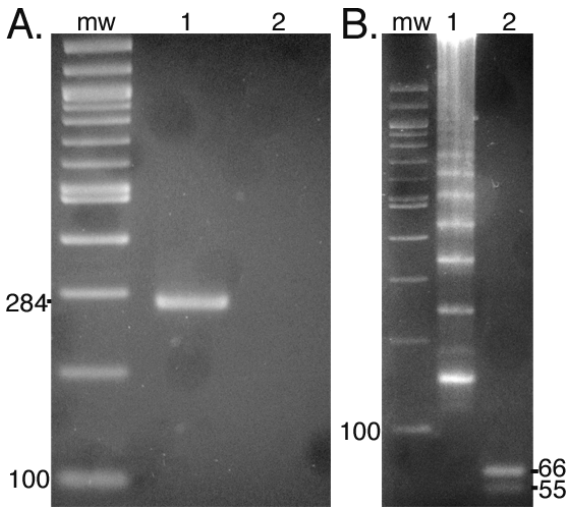

Fig. S5. Zika-specific LAMP amplification from mosquito homogenates was confirmed. (**A**) PCR amplification of 1 μl of a 1:100 dilution of PRVABC59 LAMP product with B3 and F3 LAMP primers produced the predicted 284-bp amplicon. (**B**) PRVABC59 LAMP product (lane 1) was digested with *AluI* restriction endonuclease to yield predicted 55 and 66-bp fragments (lane 2). Molecular weight standards, 100-bp ladders.

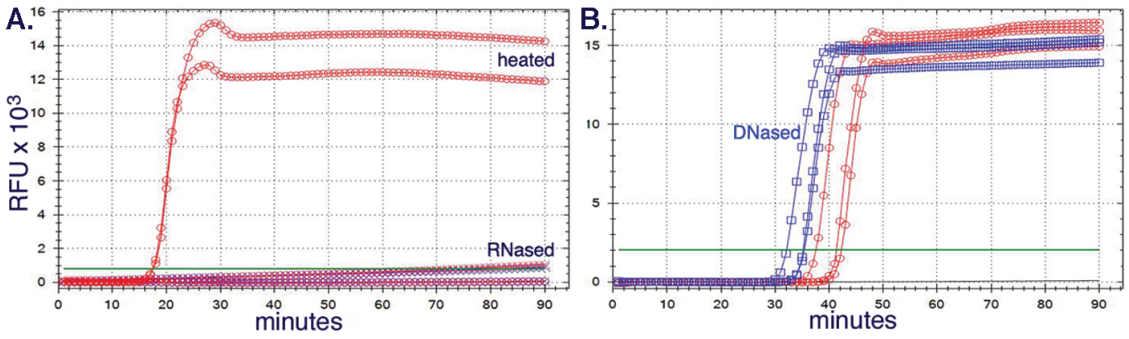

Fig. S6. Zika LAMP assay is dependent upon RNA. (**A**) RNase A digestion abrogates LAMP signal from mosquitoes. Mosquito homogenates from 2 mock-infected and 2 infected animals were incubated with or without RNase A and heat inactivated prior to PRVABC59 LAMP: infected and RNased (red crosses, below threshold), infected and mock-RNased (red circles), mock-infected and RNased (blue crosses, below threshold), mock-infected and non-RNased homogenate (blue circles, below threshold), buffer control (black line, below threshold). (**B**) DNase I digestion enhances LAMP amplification. An infected mosquito homogenate was incubated with or without DNase I and heat inactivated prior to PRVABC59 LAMP amplification in triplicate: DNase-digested (blue squares), mock-digested (red circles) and buffer control (black line, below threshold).

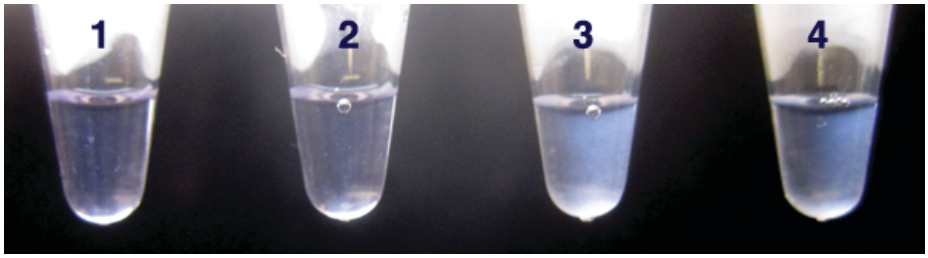

Fig. S7. A visual LAMP assay was tested using mosquito homogenates. Mock and infected mosquito homogenates were amplified and reactions inspected for turbidity. Tube 1, buffer control; tube 2, mock-infected mosquito homogenate; tube 3, infected mosquito homogenate; tube 4, infected Vero cell RNA.

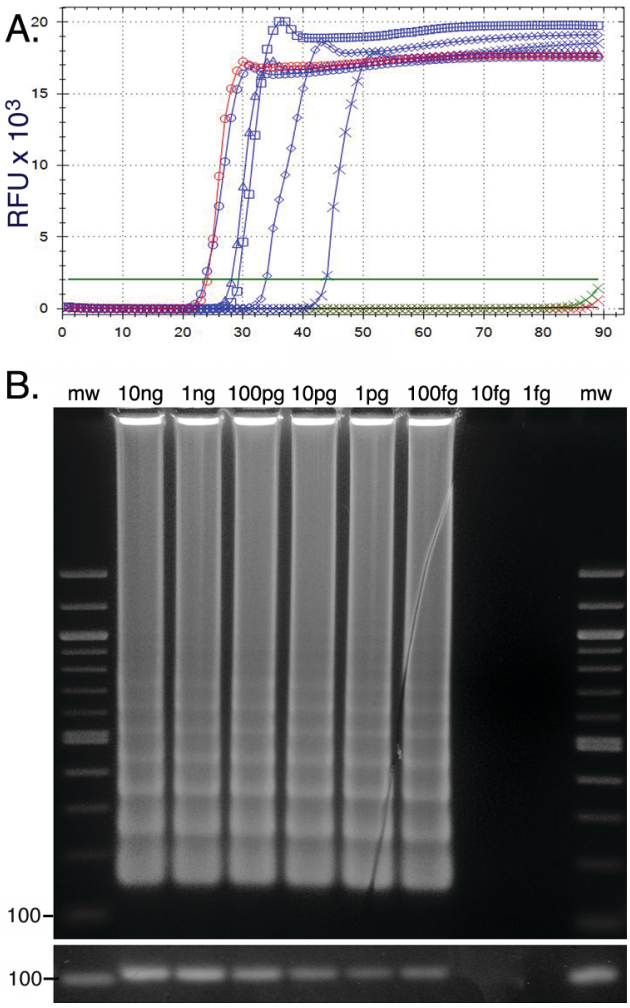

Fig. S8. Limits of PRVABC59-LAMP detection match RT-PCR. (**A**) PRVABC59-specific LAMP of serial, tenfold dilutions of 10 ng of total RNA. 10 ng (red circles), 1 ng (blue circles), 100 pg (blue triangles), 10 pg (blue squares), 1 pg (blue diamonds), 100 fg (blue crosses), 10 fg (green crosses, below threshold), 1 fg (red crosses, below threshold), buffer control (black line, below threshold). (**B**) Agarose gel electrophoresis of LAMP products in (**A**) and RT-PCR products from the same dilution series produced with Zika-specific PCR primers after reverse transcription (**lower panel**). Predicted amplicon from RT-PCR is 109 bp. Positions of 100-bp molecular weight markers are indicated. Molecular weight standards are 100-bp ladders.

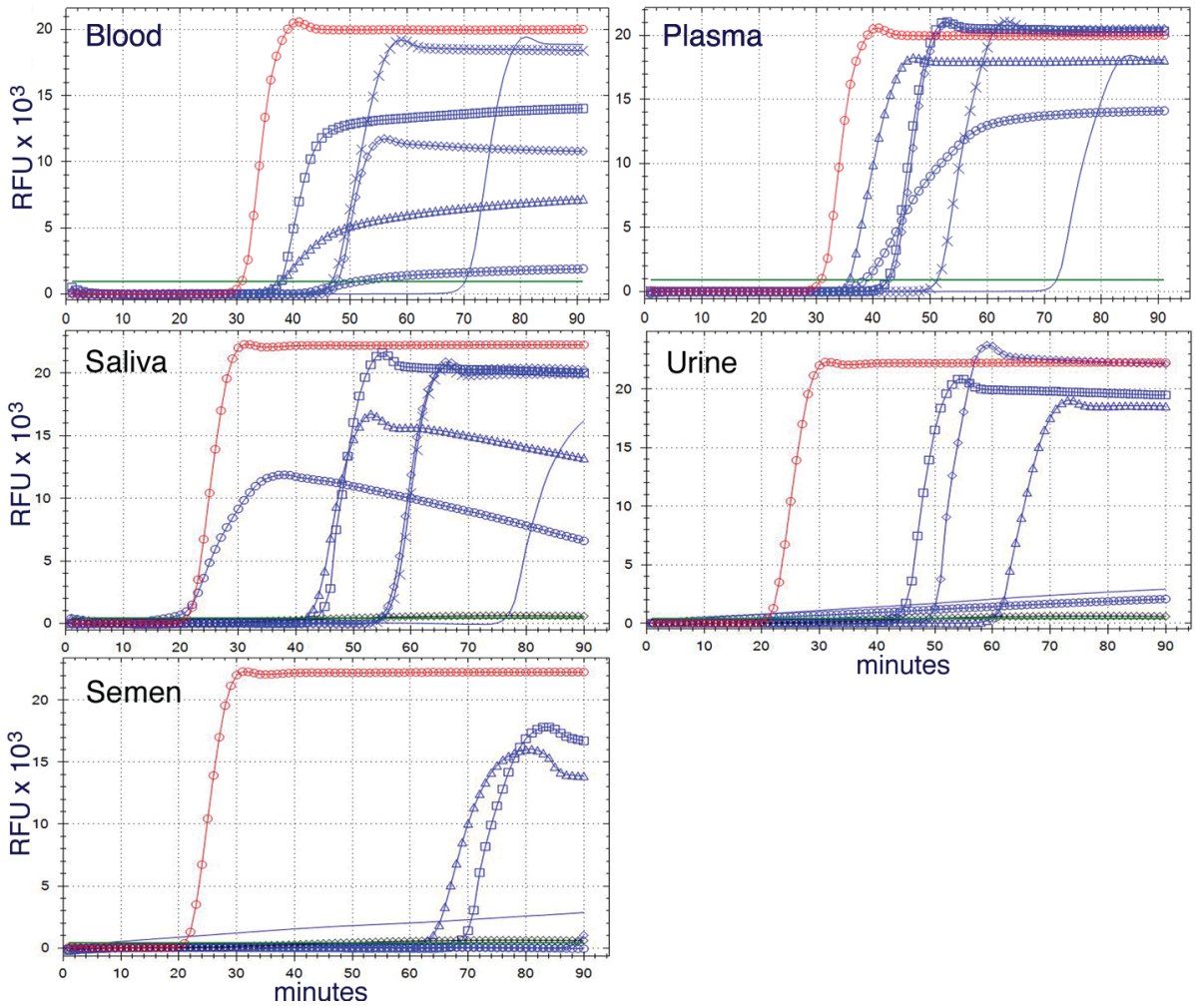

Fig. S9. The LAMP assay detects PRVABC59 virus in human biofluids. (**A**) LAMP of 2 μl of serial tenfold dilutions in water of blood, plasma, urine, saliva and semen containing PRVABC59 virus at $1x10^6$ PFU/ml. Undiluted (blue circles), 10^{-1} (blue triangles), 10^{-2} (blue squares), 10^{-3} (blue diamonds), 10^{-4} (blue crosses) and 10^{-5} (blue line, no symbol, below threshold). Positive control RNA (red circles) and un-inoculated biofluids (black line, below threshold).

Table S1. Zika LAMP primers

Primer	Sequence (5'-3')	Position (nt)
F3 Asian	AATGAGTGACCTGGCTAAGC	3694-3713
B3 Asian	GGAACAACCATCGCTCGTA	3978-3960
FIP Asian	GCTATCAGCGCCAGATGAGCTTTGCAATTTTGATGGGTGCC	3786-3766 3715-3733
BIP Asian	TCAAAGTCAGACCAGCGTTGCTCTTTCACGGGGTGTCCAATT	3792-3813 3855-3836
F3 African	AATGAGTGACCTGGCCAAGC	3694-3713
B3 African	GGCACGGCCATTGCTCGAA	3978-3960
FIP African	GCTACCAATGCCAAGTGAGCTTTGTGATCCTGATGGGTGCT	3786-3766 3715-3733
BIP African	TTAAAGTCAGACCAGCCTTGCTCTCTCACGGGGTGTCCAATT	3792-3813 3855-3836

Table S2. Plaque titration of split, infected mosquitoes.

mosq #	dilutions (1^{Ex})				PFU/ml
	-1	-2	-3	-4	
1	TM	TM	TM	53	$2.1x10^6$
2	TM	TM	TM	33	$1.3\ x10^6$
3	TM	21	0	0	$8.4\ x10^3$
4	TM	TM	TM	46	$1.8\ x10^6$
5	TM	TM	TM	61	$2.4\ x10^6$
6	TM	TM	TM	85	$3.4\ x10^6$
7	TM	TM	TM	45	$1.8\ x10^6$
8	TM	TM	TM	47	$1.9\ x10^6$
9	TM	TM	TM	33	$1.3\ x10^6$
10	TM	TM	TM	58	$2.3\ x10^6$

TM = too many to count

Table S3. Plaque titration of midguts (MG), salivary glands (SG), and carcass

Mosquito number	tissue	# of plaques				titer (PFU/ml)	LAMP
		-1	-2	-3	-4		
11	Carcass		TM	TM	11	8.8×10^5	-
	SG	TM	TM	100		8.0×10^5	+
	MG	2	0	0		160	-
12	Carcass		0	0	0	0	-
	SG	0	0	0		0	-
	MG	0	0	0		0	+
13	Carcass		0	0	0	0	-
	SG	0	0	0		0	+
	MG	0	0	0		0	+
14	Carcass		0	0	0	0	+
	SG	TM	TM	>150		$>1.2 \times 10^6$	+
	MG	9	4	0		1.96×10^3	+
15	Carcass		0	0	0	0	-
	SG	0	0	0		0	+
	MG	0	0	0		0	+

Grey = not tested, TM = too many to count

Table S4A. LAMP assay LOD for genome copies

genome copies	# Pos./Total
100000	6/6
10000	6/6
1000	6/6
100	6/6
50	4/6
25	2/6
13	2/6
10	0/6
6	0/6
1	0/6
0	0/6

LOD 111 genome copies (95% CI: 58- 1148)

Table S4B. TaqMan LOD for genome copies

genome copies	# Pos./Total	Mean Cq
100000	4/4	23.61
10000	4/4	26.96
1000	4/4	30.53
100	4/4	34.93
50	4/5	
25	4/5	
10	4/16	37.82
6	1/6	
5	4/18	
3	1/12	
1.3	1/12	
1	1/4	
0.7	0/12	
0.1	0/4	

LOD 53 genome copies (95% CI: 38 – 98)

Table S5A. Plasma 1:1000 LOD PFU/ml	PFU/ RXN	# pos./Total
1000000	2	6/6
100000	.2	6/6
50000	.1	6/6
25000	.05	6/6
12500	.025	3/6
6000	.012	1/6
1000	.002	0/6
0	0	0/6

LOD 0.05 PFU (95% CI: 0.03 - 0.42)

Table S5B. Plasma 1:1000 turbidity LOD PFU/ml	PFU/ RXN	# pos./Total
1000000	2	6/6
100000	.2	6/6
50000	.1	5/6
25000	.05	4/6
12500	.025	3/6
6000	.012	2/6
1000	.002	0/6
0	0	0/6

LOD 0.17 PFU (95% CI: 0.08 - 2.52)

Table S6. Blood 1:1000 LOD

PFU/ml	PFU/RXN	# pos./Total
1000000	2	6/6
100000	0.2	6/6
50000	.1	6/6
25000	.05	6/6
12500	.025	3/6
6000	.012	2/6
500	.001	0/6
0	0	0/6

LOD 0.05 PFU (95% CI: 0.03 - 0.25)

Table S7. Urine 1:1000 LOD PFU/ml	PFU/RXN	# pos./Total
1000000	2	6/6
500000	1	6/6
250000	0.5	6/6
125000	0.25	5/6
60000	0.12	1/6
10000	0.02	0/6
0	0	0/6

LOD 0.32 PFU (95% CI: 0.22 - 3.26)

Table S8. Saliva 1:1000 LOD

PFU/ml	PFU/RXN	# pos./Total
1000000	2	6/6
100000	0.2	6/6
50000	0.1	5/6
25000	0.05	2/6
12500	0.025	0/6
6000	0.012	0/6
1000	0.002	0/6
0	0	0/6

LOD 0.13 PFU (95% CI: 0.09 - 0.86)

Table S9. Semen 1:1000 LOD

PFU/ml	PFU/RXN	# pos./Total
1000000	2	5/6
500000	1	2/6
250000	0.5	1/6
125000	0.25	1/6
60000	0.12	0/6
10000	0.02	0/6
0	0	0/6

LOD 5.57 PFU (CI:2.18608.14)

Table S10. Nicaraguan serum and serum RNA samples

Sample #	Cq (rep1)	Cq (rep2)	Cq Mean	Cq Error	RNA qRT-PCR	RNA LAMP	Direct LAMP	qRT-PCR Code
1	33.21	32.6	32.91	0.43	+	+	+	+
2	31.74	31.18	31.46	0.4	+	+	+	+
3	31.67	31.39	31.53	0.2	+	+	+	+
4	27.05	27.08	27.07	0.02	+	+	+	+
5	35	35.52	35.26	0.37	+	+	+	+
6	-	-	-	-	-	-	+	-
7	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	+	-
9	-	-	-	-	-	-	-	-
10	37.67	-	37.67	0	+	+	+	+
11	31.71	31.63	31.67	0.06	+	+	+	+
12	34.23	34.65	34.44	0.3	+	+	+	+
13	30.05	30.12	30.09	0.05	+	+	+	+
14	34.62	35.49	35.06	0.62	+	+	+	+
15	33.42	33.63	33.53	0.15	+	+	+	+
16	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-
19	-	-	-	-	-	+	+	-
20	31.53	31.53	31.53	0	+	+	+	+
21	35.26	35.03	35.15	0.16	+	+	+	+
22	37.93	-	37.93	0	+	+	+	+
23	34.78	34.15	34.47	0.45	+	+	-	+
24	31.28	31.56	31.42	0.2	+	+	-	+
25	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	+	-
28	-	-	-	-	-	-	-	-
29	25.79	25.79	25.79	0	+	+	+	+
30	37.31	37.76	37.54	0.32	+	-	+	+
31	-	-	-	-	-	+	+	+
32	25.92	25.92	25.92	0	+	+	+	+
Total Positive					19	20	22	20

Table S11. Brazilian plasma and RNA samples

Sample #	qRT-PCR		Serology		Combined qRT-PCR Serology	LAMP plasma	LAMP RNA	LAMP Plasma and RNA
	Ct	Result	IgM	Result				
1	31.70	+	5.2	+	+	+	+	+
2	30.43	+	n.d.	n.d.	+	-	n.d.	-
3	32.41	+	n.d.	n.d.	+	+	+	+
4	35.72	+	n.d.	n.d.	+	+	+	+
5	35.17	+	7.1	+	+	+	-	+
6	34.74	+	15.5	+	+	+	+	+
7	34.17	+	14.6	+	+	+	+	+
8	34.21	+	n.d.	n.d.	+	-	+	+
9	32.96	+	8.0	+	+	+	n.d.	+
10	-	-	1.5	-	-	+	+	+
11	-	-	11.3	+	+	-	n.d.	-
12	-	-	19.2	+	+	+	n.d.	+
13	-	-	9.9	+	+	+	n.d.	+
14	35.66	+	14.4	+	+	-	-	-
15	35.13	+	n.d.	n.d.	+	+	+	+
16	35.94	+	17.6	+	+	+	-	+
17	36.97	+	n.d.	n.d.	+	-	-	-
18	-	-	5.9	+	+	+	n.d.	+
19	-	-	14.7	+	+	+	n.d.	+
20	-	-	1.9	-	-	-	n.d.	-
21	-	-	1.0	-	-	+	n.d.	+
22	31.14	+	7.6	+	+	+	+	+
23	33.39	+	13.6	+	+	+	+	+
24	28.36	+	n.d.	n.d.	+	+	+	+
25	34.87	+	14.3	+	+	-	+	+
26	36.13	+	n.d.	n.d.	+	+	-	+
27	36.09	+	16.7	+	+	+	+	+
28	-	-	0.9	-	-	+	n.d.	+
29	-	-	n.d.	n.d.	-	-	n.d.	-
30	-	-	1.0	-	-	+	n.d.	+
31	24.48	+	n.d.	n.d.	+	+	+	+
32	-	-	1.8	-	-	+	n.d.	+
33	-	-	1.3	-	-	-	n.d.	-
34	-	-	0.9	-	-	+	n.d.	+
35	33.71	+	5.2	+	+	-	+	+
36	30.73	+	10.7	+	+	-	-	-
37	36.73	+	n.d.	n.d.	+	-	+	+
38	34.42	+	n.d.	n.d.	+	-	+	+
39	-	-	1.8	-	-	+	+	+
40	-	-	2.9	+	+	+	n.d.	+
41	-	-	0.8	-	-	+	n.d.	+
42	-	-	1.1	-	-	-	n.d.	-
43	-	-	1.9	-	-	+	n.d.	+
44	-	-	1.0	-	-	+	-	+
45	-	-	0.9	-	-	-	n.d.	-
46	-	-	0.9	-	-	-	n.d.	-
47	-	-	1	-	-	+	n.d.	+
48	34.18	+	n.d.	n.d.	+	+	-	+
49	-	-	1.1	-	-	+	n.d.	+
Total +	25		19/36		31	33	18/26	38