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Supplementary Materials for

Rapid antigen tests for dengue virus serotypes and Zika virus in patient serum

Irene Bosch, Helena de Puig, Megan Hiley, Marc Carré-Camps, Federico Perdomo-Celis, Carlos F. Narváez, Doris M. Salgado, Dewahar Senthoor, Madeline O'Grady, Elizabeth Phillips, Ann Durbin, Diana Fandos, Hikaru Miyazaki, Chun-Wan Yen, Margarita Gélvez-Ramírez, Rajas V. Warke, Lucas S. Ribeiro, Mauro M. Teixeira, Roque P. Almeida, José E. Muñóz-Medina, Juan E. Ludert, Mauricio L. Nogueira, Tatiana E. Colombo, Ana C. B. Terzian, Patricia T. Bozza, Andrea S. Calheiros, Yasmine R. Vieira, Giselle Barbosa-Lima, Alexandre Vizzoni, José Cerbino-Neto, Fernando A. Bozza, Thiago M. L. Souza, Monique R. O. Trugilho, Ana M. B. de Filippis, Patricia C. de Sequeira, Ernesto T. A. Marques, Tereza Magalhaes, Francisco J. Díaz, Berta N. Restrepo, Katerine Marín, Salim Mattar, Daniel Olson, Edwin J. Asturias, Mark Lucera, Mohit Singla, Guruprasad R. Medigeshi, Norma de Bosch, Justina Tam, Jose Gómez-Márquez, Charles Clavet, Luis Villar, Kimberly Hamad-Schifferli,* Lee Gehrke*

*Corresponding author. Email: kim.hamad@umb.edu (K.H.-S.); lgehrke@mit.edu (L.G.)

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Other Supplementary Material for this manuscript includes the following:
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Table S5 (Microsoft Excel format). Individual subject-level data.

Supplementary Materials

Materials and Methods

Flow cytometry analysis of the hybridoma supernatants

To ensure that the antibodies expressed by hybridomas recognized native NS1 proteins, hybridoma supernatants were used as a source of anti-NS1 antibodies for immunostaining Vero cells that were infected by DENV or ZIKV. Vero cells (90% confluency) were infected with DENV or ZIKV at multiplicity of infection (MOI) of 1 and incubated for 24 hours. The cells (50,000 cells per well of a 96-well plate) were prepared for immunostaining by using the BD cytofix/cytoperm reagent, following the protocol provided by the manufacturer (BD Biosciences). Washed and fixed cells were incubated with 100 ul of primary mouse hybridoma media for 1 hour, washed and incubated for 1 hour with phycoerythrin (PE)-labeled anti-mouse secondary antibody. Immuno-reacted cells were analyzed using a Guava flow cytometer (Millipore). Live cells were gated and fluorescence was quantified using a positive control antibody anti-E flavivirus detecting dengue 1-4 or Zika infected cells to assess infection levels.

Epitope mapping with peptide arrays

Linear epitope mapping was used for further antibody characterization and also to inform the interpretation of the paired antibody immunochromatography assays. Linear epitopes were analyzed by two methods. Libraries of tiled DENV NS1 peptides (BEI Resources) were resuspended according to manufacturer's instructions. Peptides were diluted to a working concentration of 100 ug/ml in water. Each library (200 ng of each peptide) was spotted onto Protran Premium 0.2 um nitrocellulose (GE Life Sciences) and allowed to air dry. Membranes were blocked with 5% Blotto in TBST for 1 hour. Membranes were rinsed with Tris-buffered saline-Tween (TBST) and incubated with each anti-NS1 antibody (at 5 ug/ml) for 1 hour. Membranes were washed 3 times with TBST. Anti-mouse HRP antibody was used as a secondary (diluted 1:5000). Membranes were incubated in secondary for 30 minutes. After another 3 washes, membranes were developed with Western Lightning plus-ECL. After washing, positive signals were detected using an anti-mouse IgG antibody coupled to horseradish peroxidase for signal development. Alternatively, tiled peptides were synthesized on glass slides (JPT Technologies, Germany) and incubated with each of the antibodies. Positive signals were detected by immunofluorescence microscopy and scored.

Supplementary Figures

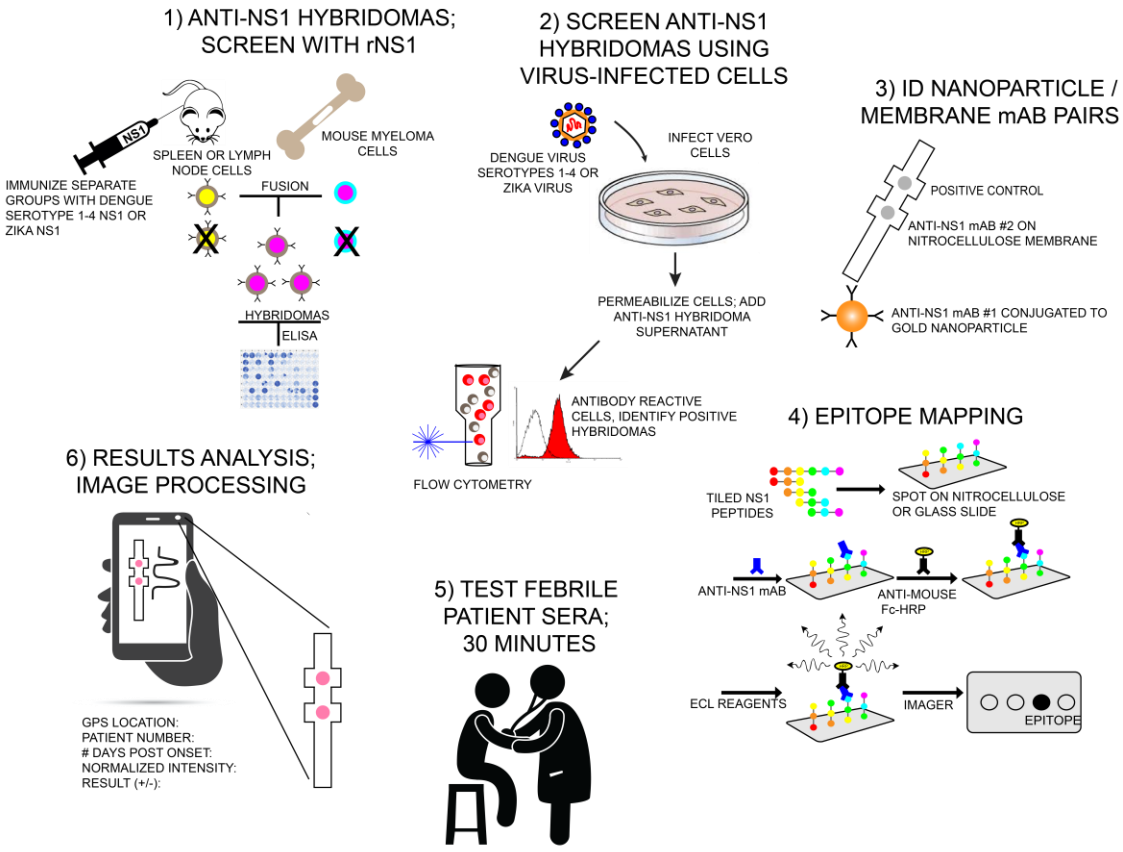

fig. S1. Stepwise strategy for identifying mAbs that differentiate the closely related NS1 proteins of DENV1–4 and ZIKV. Step 1: mouse hybridomas are generated by immunizing mice with viral NS1 protein antigen, and the resulting hybridomas are screened using ELISA against multiple recombinant NS1 proteins. Step 2: hybridoma cell supernatants containing monoclonal antibodies are screened using flow cytometry for recognition of native NS1 proteins released by Vero cells infected with DENV serotypes or ZIKV. Step 3: purified monoclonal antibodies are tested in pairs for specific recognition of DENV or ZIKV NS1 proteins in immunochromatography rapid tests that run in about 30 minutes. Step 4: linear epitope mapping is performed to identify NS1 sites recognized by monoclonal antibodies used in the rapid tests. Step 5: patient serum from DENV- or ZIKV-infected patients is tested using the immunochromatography dipsticks. Step 6: images of test results are captured using image recognition on a mobile phone camera, and analyzed objectively to quantify signals.

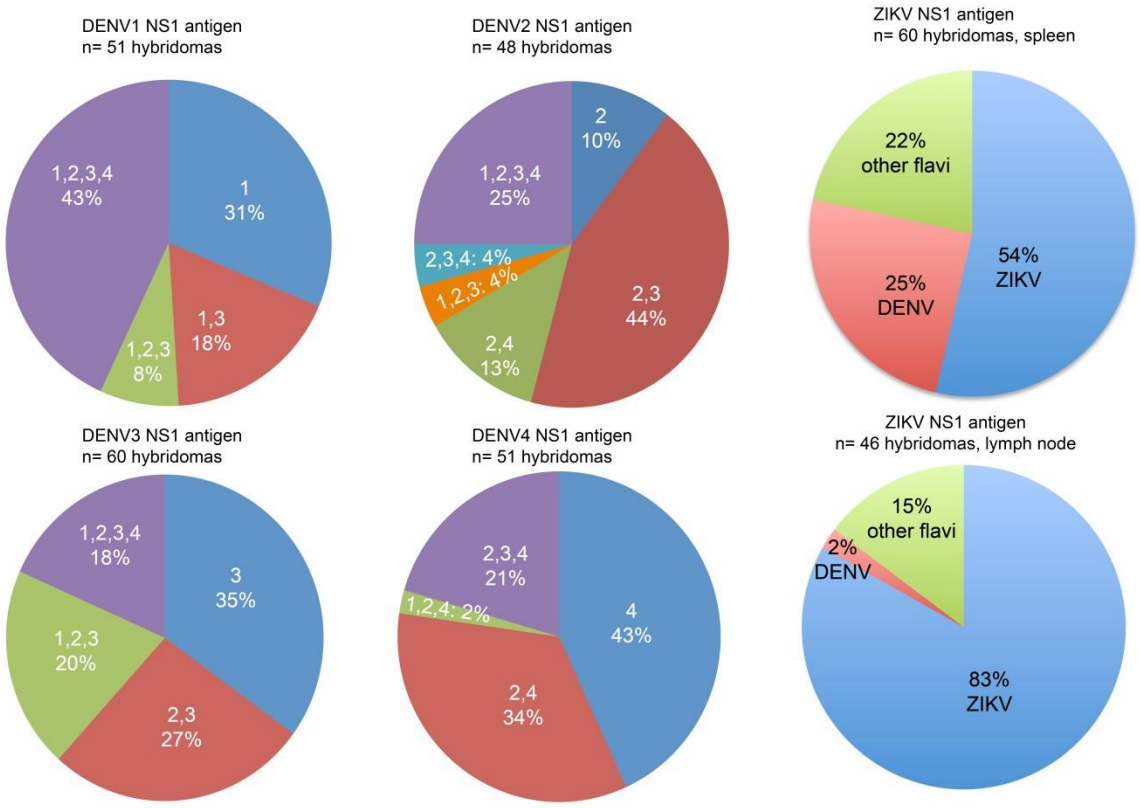

fig. S2. Repertoire of antigen-specific antibodies among DENV and ZIKV mAbs. The pie charts show the percentages of dengue serotype-specific or Zika virus-specific monoclonal antibodies as defined during screening. The values were determined by screening the hybridomas against the antigens labeled on the figures. The ZIKA virus monoclonal antibody preparation was done by two methods (splenic B cells and lymph node tissue).

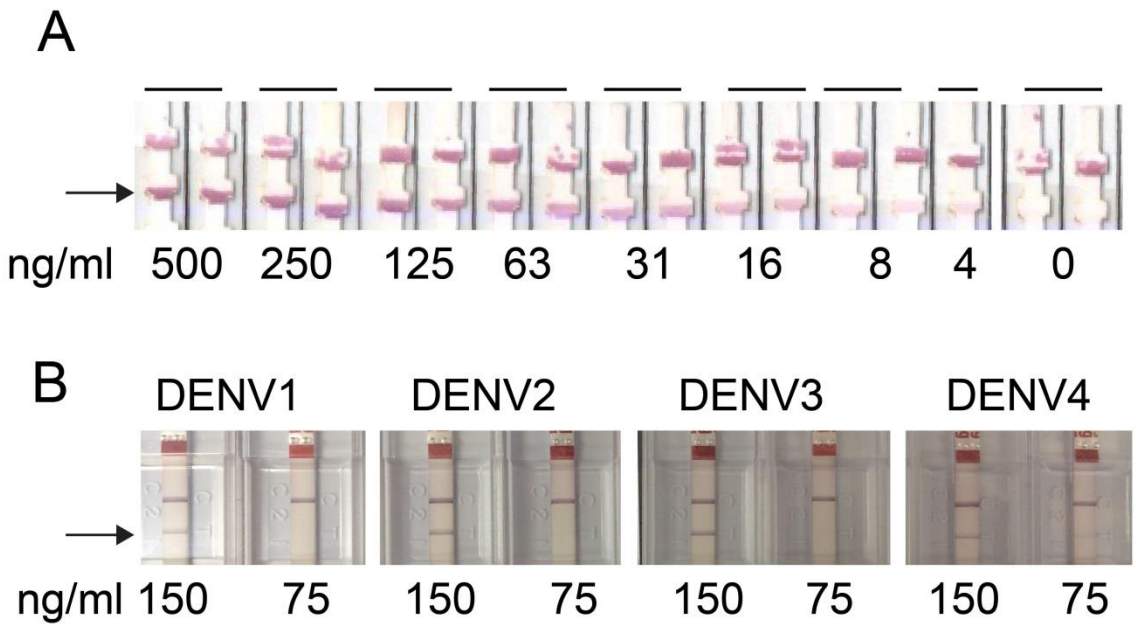

fig. S3. Limit of detection comparison. (**A**) Bosch *et al.* dengue NS1 rapid test and (**B**) Standard Diagnostic dengue NS1 test. In panel A, the dipsticks were chromatographed with solutions containing the indicated recombinant dengue 1 NS1 concentrations. Panel B shows Standard Diagnostic tests chromatographed with solutions of 150 ng/ml or 75 ng/ml of recombinant DENV NS1 proteins. The results of this analysis suggest that the limit of detection of the Bosch *et al.* rapid test is below 10 ng/ml, whereas the limits of detection for the Standard Diagnostic test for dengue NS1 protein are between 75 and 150 ng/ml. The arrows mark the NS1 signal band while the upper band is the positive control.

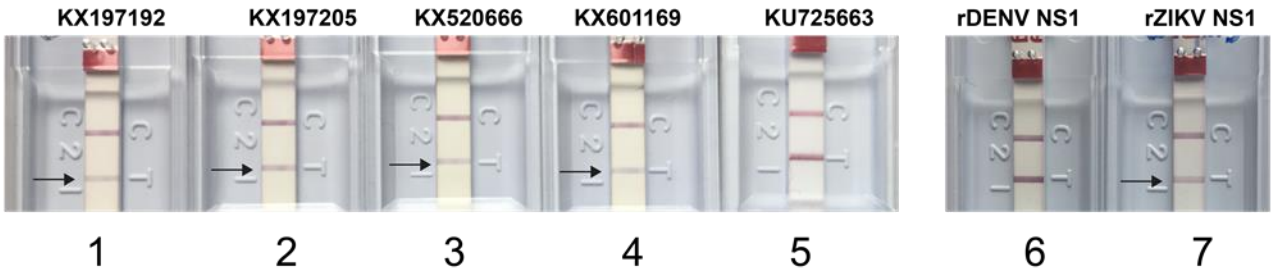

fig. S4. The Standard Diagnostics DENV NS1 test cross-reacts with ZIKV NS1 protein. Images show Standard Diagnostic dengue NS1 tests run with cell culture media from Vero cells infected with the indicated ZIKV strains (lanes 1-4), positive control [lane 5, culture media supernatant from Vero cells infected with DENV2 16681 and recombinant DENV NS1 (500 ng/ml, lane 6)], or recombinant ZIKV NS1 protein (MR766 Uganda; 500 ng/ml, lane 7). Arrows mark the cross-reactive NS1 signals in strips 1-4 and 7.

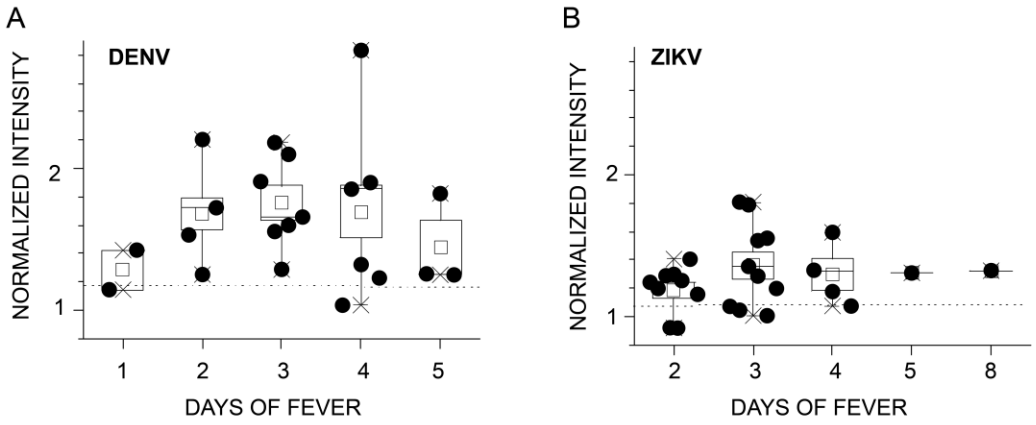

fig. S5. DENV NS1 and ZIKV NS1 detection expressed as days after onset of fever symptoms. (A) DENV samples from Colombia, India, and Guatemala were chromatographed on pan-DENV tests. Image processing methods were used to quantify the signals, which were normalized and plotted to show NS1 detection on days 1-5 post onset of fever. (B) ZIKV samples from the Dominican Republic were chromatographed on ZIKV test strips. Image processing methods were used to quantify the signals, which were normalized and plotted to show NS1 detection on days 2-8 post onset of fever. Box and whiskers plots: the X symbols represent the maximum and minimum measured normalized intensity values, the small square box represents the mean value, and the larger rectangles represent the 25-75% range of the data. Individual filled circles represent individual patient samples measured. The horizontal dashed line represents the cutoff value, above which the samples were considered positive tests, and below which were considered negative tests.

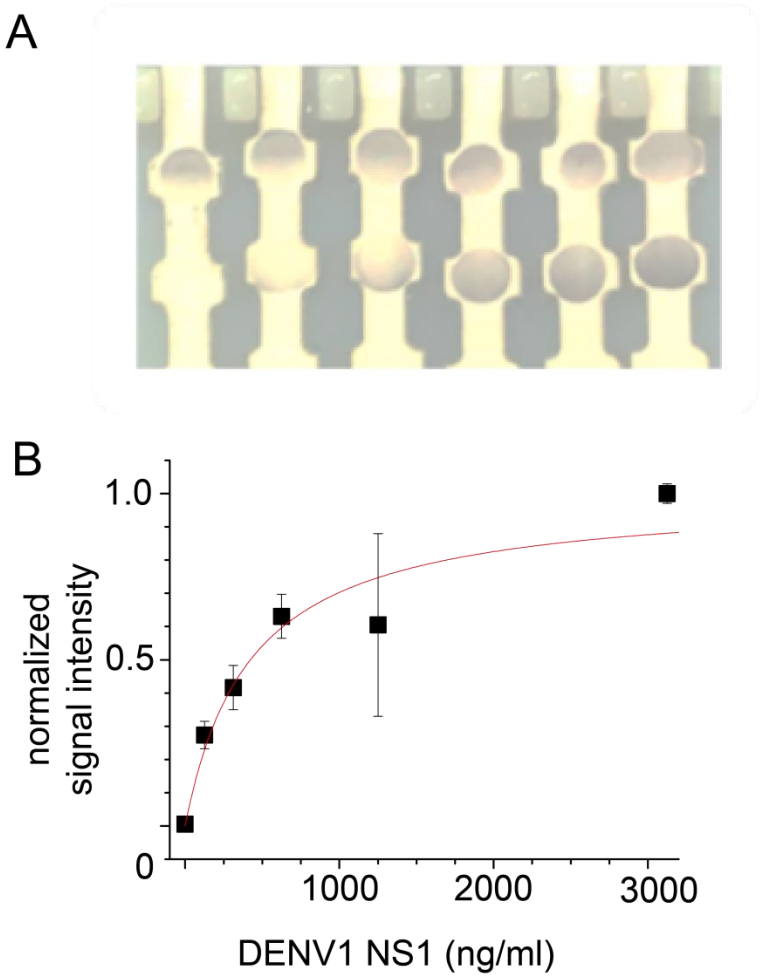

fig. S6. Laboratory-made gold nanoparticles for detecting DENV NS1 in rapid test format. (**A**) An NS1 protein dilution series was performed to define limit of detection for DENV1 NS1 using the lab-made nanoparticles. Antibodies were adsorbed to the nitrocellulose membrane by manually pipetting 0.3 µl of a 2 mg/ml solution of antibodies onto the control or test areas of the nitrocellulose membrane. For the test area, monoclonal antibodies against ZIKV-NS1 were applied. The positive control area on the nitrocellulose membrane was spotted with goat antibodies recognizing the crystallizable fragment (Fc) of mouse immunoglobulins. The dipstick was placed in a tube containing a test solution comprised of 4 µl of 50% w/v sucrose in water, 8 µl of 1% v/v Tween 80 in PBS, 1 µl of the antibody-nanoparticle conjugates, and 17 µl of the analyte (total volume of 30 µl, prepared by spiking DENV1 NS1 protein into filtered human serum). (**B**) The signal intensities at each of the test areas were quantified, normalized, and plotted to define the limit of detection. N=3 for each point; the error bars represent standard deviation. The limit of detection for DENV1 NS1 in these experiments is 19 ng/ml.

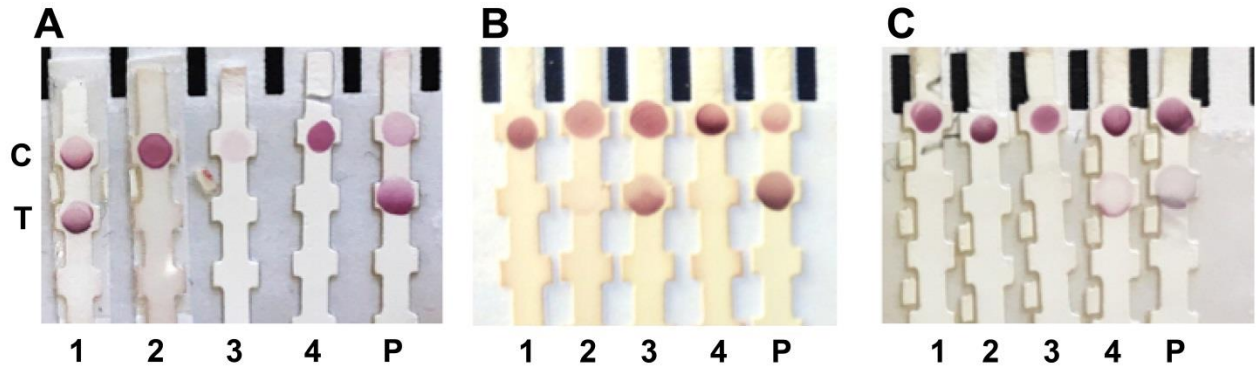

fig. S7. NS1 detection by rapid tests in secondary infections. Three representative examples are presented to illustrate NS1 detection at day two of fever in secondary dengue infection cases. Hemagglutination inhibition was used to define the dengue secondary infection status. In a hemagglutination experiment, virus binds to red blood cells and causes agglutination. In the presence of anti-envelope antibodies, expressed during a virus infection, hemagglutination is inhibited. Here, a standard hemagglutination inhibition (HI) assay was used to quantify anti-dengue E protein antibody levels as a function of dilution required to reflect inhibition of hemagglutination *(46, 47)*. Secondary virus infections are defined by the presence of high concentrations of anti-E such that dilutions of greater than 1:1280 are needed in order to observe decreased hemagglutination inhibition. Alternatively, serum samples with low antibody levels show inhibition at dilutions less than 1:1280. Each panel (**A-C**) has five strips (1-5), detecting DENV1-DENV4 and pan-DENV (P). Positive signals in lanes 1-4 define the DENV serotype, whereas the pan-DENV signal (P) confirms dengue virus infection. At the time of NS1 rapid test analysis (A-C), all sera tested had HI titers ≤1280, indicating low anti-NS1 antibody levels. At 7 days later the same patients were tested again to obtain a confirmatory hemagglutination inhibition assay (HI) titer. At the 7-day point, there was at least a four-fold increase in HI levels in the convalescent sample (+7 days illness) compared to the first sample, confirming secondary infection status. The values of HI titers are shown for each of the serotypes tested were A) 10,240 (DENV1); B) 5,120 (DENV3); C) 10,240 (DENV4). The data demonstrate that NS1 was detected in serum samples from secondary infection patients. C: control signal; T: NS1 test signal.

STEP #	ASSAY	GOAL/FUNCTION	number of mAb analyzed
1 immunize mice with viral NS1 protein	ELISA	screen hybridoma supernatants against NS1 proteins	DENV: 209 ZIKV: 104
2 first mAb selection	Flow Cytometry	test reactivity of monoclonal antibodies to native NS1 antigen expressed by virus infected cells	DENV: 32 ZIKV: 16
3 second selection	Immunochromatography	identify antibody pairs that detect NS1 antigens with high discrimination	DENV: 726 combinations ZIKV: 300 combinations
4 characterize selected mAb	linear epitope mapping	identify protein domains recognized by mAbs	DENV: 8 ZIKV: 2
5 patient samples	immunochromatography	validate the dipstick tests using serum from virus-infected patients	DENV: 8 ZIKV: 2

table S1. Stepwise description of the approaches used to define antibodies that detect and distinguish DENV1–4 NS1 and the ZIKV NS1 proteins. In Step 3, an 11 X 11 matrix was used to test all combinations of 11 anti-DENV NS1 pairs using each of the four DENV NS1 proteins. In addition, a 10 X 10 matrix was used to test all combinations of 10 anti-ZIKV NS1 pairs using ZIKV NS1 protein as well as each of the four DENV serotype NS1 proteins. The matrix-based screening identified optimal monoclonal antibody pairs that detected NS1 without crossover interference. DENV: dengue virus; ZIKV: Zika virus.

AMINO ACID IDENTITY

	DENV1	DENV2	DENV3	DENV4	ZIKV
DENV1		74	80	69	54
DENV2	86		75	73	54
DENV3	90	86		73	55
DENV4	82	85	85		54
ZIKV	73	72	73	72	

AMINO ACID HOMOLOGY

table S2. Amino acid homology and identity among DENV NS1 and ZIKV NS1 proteins. The matrix shows comparisons of percentage amino acid identity (exact positional amino acid matches in the amino acid sequences) and percentage amino acid homology (positional amino acid substitutions by amino acids with similar physicochemical properties) for the DENV and ZIKV NS1 proteins. Dengue virus and Zika virus NS1 sequences were extracted from deposited data (Zika: KX702400.1; DENV1: GU131834.1; DENV2: GQ868641.1;DENV3: KF955487.1; DENV4: GQ868645.1. These sequences represent viruses that were isolated from the same geographic area in years 2001-2016 for a representative sampling. The BLAST algorithm from the National Center for Biotechnology Information, U.S. National Library of Medicine was used to generate the identity and similarity scores among the NS1 proteins *(48).*

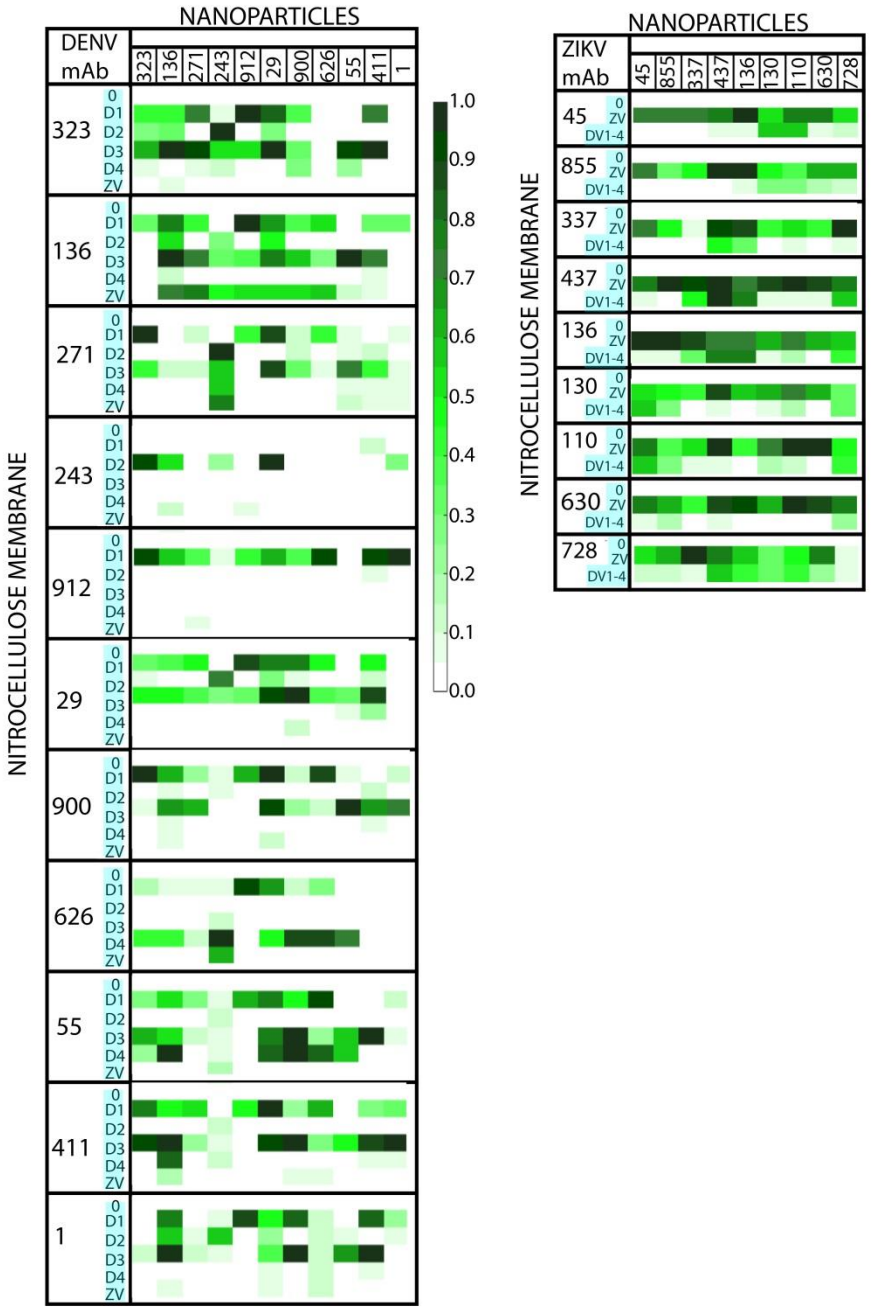

table S3. Matrix of mAb pair trials. To define functional antibody pairs, one antibody was conjugated to gold nanoparticles, and one antibody was adsorbed to nitrocellulose membrane. The resulting nanoparticle conjugates-membrane pairs were tested using the specific serotype recombinant NS1 protein (on the left axis: mock, DENV1, DENV2, DENV3, DENV 4, ZIKV, or a DV1-4 mix of all four NS1 proteins; highlighted in blue color). These proteins were present at a concentration of 1.6 ug/ml in the testing. Because of the large number of tests performed, the strips were analyzed and normalized in groups corresponding to nanoparticle lots and antibody dilutions. The signal for each test strip was quantified using ImageJ. Background signal intensity

was subtracted and the signal of greatest intensity was set at a value of "1". For remaining strips, background was subtracted from signal intensity to yield a corrected intensity value. The corrected intensity value was then divided by the maximal signal value to yield a value between 0 and 1 that was plotted above to generate a relative binding affinity map. Dark green color: strong visual signal in the rapid test; light green color: weak visual signal in the rapid test; gray color: no signal in the rapid test. These tests were performed using lab-made gold nanoparticles, which perform equivalently in comparison to commercial Innova particles (see also Figs. 2 and 3). The antibody pairs were tested at a sample concentration of 3μg/ml of recombinant NS1 protein. Left panel: dengue virus antibodies; Right panel: Zika virus antibodies, with the exception of 136, a dengue NS1 antibody that binds Zika virus NS1 with high affinity.

	ELISA RELATIVE BINDING (FOB)	ELISA RELATIVE BINDING (FOB)	ELISA RELATIVE BINDING (FOB)	ELISA RELATIVE BINDING (FOB)	IMMUNOCHROMATOGRAPHY APPLICATION
DENV mAb #	ag: DENV1 NS1	ag: DENV2 NS1	ag: DENV3 NS1	ag: DENV4 NS1	dipstick strip identification
mAb 271 ag: DENV3 NS1	87	72	64	1.3	membrane, dipstick 1 (DENV serotype 1) nanoparticles, pan-DENV test
mAb 912 ag: DENV1 NS1	65	3	2	0	nanoparticles DENV serotype 1 test
mAb 1 ag: DENV1 NS1	57	5.8	16.3	1	membrane, dipstick 2 (DENV serotype 2)
mAb 243 ag: DENV2 NS1	0.7	46	0.6	0.6	nanoparticles, DENV serotype 2 nanoparticles, pan-DENV test
mAb 55 ag: DENV3 NS1	3	70	63	72	membrane, dipstick 3 (DENV serotype 3)
mAb 411 ag: DENV3 NS1	36	74	68	72	nanoparticles, DENV serotype 3 nanoparticles, pan-DENV test
mAb 55 ag: DENV3 NS1	3	70	63	72	membrane, dipstick 4 (DENV serotype 4)
mAb 626 ag: DENV4 NS1	8	40	0.2	52	nanoparticles, dipstick 4 (DENV serotype 4) nanoparticles, pan-DENV test
mAb 323 ag: DENV3 NS1	82	73	65	79	membrane, dipstick pan-DENV
ZIKV mAb #	ag: ZIKV NS1	ag: pooled DENV 1-4 NS1	pooled flavivirus NS1 panel		
mAb 110 ag: ZIKV UG NS1	65	0	0		membrane, ZIKV dipstick
mAb 130 ag: ZIKV UG NS1	69	0	0		nanoparticle, ZIKV test

table S4. List of mAbs used in the rapid tests, relative binding values, and summary of final use in the DENV and ZIKV immunochromatography tests. The antibody name is shown at the left column with the antigen (ag) that was used to inject the mice to generate the antibody. The immunochromatography application of each antibody (conjugated to nanoparticle or adsorbed to nitrocellulose membrane) is in the right column. The center columns show "fold over background" (FOB) ELISA values for each of the antigens used as bait on the ELISA plates (DENV 1–4 NS1; upper, or ZIKV NS1 or pooled flavivirus NS1 panel; lower). These numbers define differential recognition recognition of each of the four dengue NS1 antigens during initial screening (fig. S1, step 1). This differential recognition formed the initial selection of the antibodies for use in the final test.