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A nanoparticle pseudo pathogen for rapid detection and diagnosis of virus infection

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ABSTRACT

We herein report a new rapid blood test for virus infection detection and diagnosis. A citrate gold nanoparticle is first coated with a virus lysate to form a gold nanoparticle pseudo pathogen. The gold nanoparticle pseudo virus is then mixed with a blood plasma or serum samples. If the blood sample is from a positive patient, the activated immune molecules in the blood such as antibodies, complement proteins and others will react with the nanoparticle pseudo virus, leading to nanoparticle aggregate formation. The nanoparticle aggregate formation is detected and measured using a particle sizing technique called dynamic light scattering. In this study, we applied this test for Zika virus infection detection. We tested blood plasma samples from 85 Zika positive patients, 40 Dengue positive patients, 10 Chikungunya positive patients, and 78 non-patient control samples collected from both endemic and non-endemic locations. The study shows that the new test has a higher sensitivity compared to some existing commercial tests in the market, while maintaining a similar specificity. Within 7 days from the symptom onset, the new test can detect 43% of the infected patients while a commercial anti-Zika IgM test detects only 26% of the infected patients. Within 14 days from the symptom onset, our new test detects 73% of the infected patients while the same commercial anti-Zika IgM test detects 53% of the infected patients. The test is extremely simple, easy to develop, with test results obtained within minutes. This new test platform may be potentially adapted for the detection and diagnosis of a wide range of viral infectious diseases, for example, the currently ongoing COVID-19.

1. Introduction

The current outbreak of Novel Coronavirus (COVID-19) around the globe is a clear reminder of how severe threat emerging infectious diseases may pose to our life and society [1,2]. Almost every time when such an outbreak occurs, there is a lack of rapid diagnostic tests for timely screening and diagnosis of infected patients [3,4], and this problem has been causing great challenges in early control of emerging infectious diseases. This situation has just occurred in China, the first epidemic center of COVID-19 within few months ago, and now the US and many other countries are facing the same problem. Between 2015 and 2017, the outbreak of Zika virus (ZIKV) in Brazil and its subsequent spread to the whole continental America and the Caribbean Islands is another example manifesting the needs for rapid diagnostic tests for high-risk emerging infectious diseases. At the early stage of Zika outbreak, the lack of rapid tests caused similar delays in diagnosing positive patients. ZIKV infection is linked to pregnancy complications including microcephaly, central nervous system malformation, spontaneous abortion, stillbirth, hydranencephaly, and placental insufficiency [5–7]. The long waiting time for the testing results not only caused much anxiety among pregnant women or women at pregnancy age, but also delayed decision process that can be very critical for the health of both infected mothers and unborn babies. For viral infectious diseases, molecular diagnostic testing, the detection of viral DNAs or RNAs from infected patients, remains the gold standard [8,9]. While these tests are highly sensitive and specific, they

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tests, such as serology tests based on the detection of virus-specific antibodies, are not readily available in small clinics and hospitals. Other diagnostic tests and not suitable for point-of-care applications. Molecular tests are also expensive and require laboratory equipment that are not rapid and simple enough for point-of-care applications. Although many innovative new tests and test platforms for point-of-care applications are in the development pipeline [11,12], most of these new tests have not reached the market yet.

Here we report the development of an extremely simple and rapid blood test, D2Dx™ test, for virus infection detection, screening and diagnosis. Although the study we present here was focused on ZIKV infection, our new approach may be potentially adapted for other virus infections, including the currently occurring COVID-19. The design and the process to perform the test are illustrated in Fig. 1. The test uses a gold nanoparticle (AuNP) pseudo virus pathogen to detect active humoral immune responses from the infected patients’ blood plasma or serum samples. The AuNP pseudo virus pathogen is made by simply coating a citrate-AuNPs with a virus lysate solution. In the case of ZIKV detection, the AuNP surface is coated with a solution of ZIKV lysate. Proteins, especially the envelope proteins, along with lipids and membranes, and other envelope components will adsorb and assemble spontaneously to the surface of the AuNPs to form a corona that resembles the envelope structure of a real virus. Then, upon mixing the AuNP pseudo virus solution (60 μL) with a blood plasma sample (3 μL), activated immune molecules such as IgM, IgG, complement proteins from the humoral immune system in the blood will react with the nanoparticle pseudo virus, introducing a nanoparticle aggregate formation. The nanoparticle aggregate formation is detected by measuring the average particle size of the assay solution using dynamic light scattering (DLS). A test score is obtained by calculating the ratio of the average particle size of the assay solution, $D_2$, versus the average particle size of the original AuNP pseudo virus solution, $D_1$. Because this test is based on nanoparticle size measurement, the test was given the name of D2Dx™ (from diameter to diagnostics).

There are several important aspects of this new test that we would like to highlight here. First, the D2Dx™ test, different from any other immunoassay techniques, is not detecting any single, particular immune molecules, such as IgM, or IgG, or any specific complement proteins alone. Rather, it is detecting the humoral immune response that would occur in vivo. In a real biological body, immune responses are extremely complicated processes involving collective and highly orchestrated reactions and interactions between and among various molecules from the immune system and the invading pathogens [13]. The D2Dx™ test is designed to capture and detect this humoral immune response. Our recently reported studies have shown that when a gold nanoparticle material is mixed with blood serum samples, the blood serum interacts with the nanoparticle as if it is a pathogen, and three most important immune-related proteins, IgG and IgM antibody, and complement proteins, are directly involved in such interactions [14].

Second, with its principle explained, one should not make an assumption that D2Dx™ test is non-specific or non-quantitative. On the contrary, data presented in this study will show that the new blood test is highly specific to its intended virus infection detection, and the test...
provides quantitative information. The specificity is achieved through the coating of the AuNP with envelop proteins and lipids derived from the specific virus that the test is intended for.

Finally, we want to emphasize that the development process of the D2Dx™ test is extremely simple and easy: all that is needed is the virus lysate solutions, which can be typically obtained by simply adding mild detergent such as Triton X-100 to the purified virus stock solution [15, 16]. The AuNP pseudo virus can be made in situ just prior to conducting the test by simply mixing a citrate-AuNP solution with a small amount of virus lysate solution and such made AuNP pseudo virus solutions can be used directly for testing without additional purification steps. Potentially, our new test platform can be adapted rapidly to develop new diagnostic tests for a broad range of virus infectious diseases, especially envelope viruses such as the current ongoing COVID-19.

### Table 1

The test results of 45 single draw and two-time draw Zika-positive clinical samples.

| Sample ID | Days | Aptima Zika RT-PCR | InBios anti-Zika IgM test results | Interpretation of InBios test | D2Dx test score | b) Interpretation of D2Dx-test |
|-----------|------|--------------------|----------------------------------|-------------------------------|-----------------|-------------------------------|
| **Single draw** |
| 1 | 1043-TDS-0148 | 2 | 35.49 | 1.49 | 11.9 | Positive |
| 2 | 1043-TDS-0348 | 2 | 29.88 | 1.07 | 2.4 | |
| 3 | 1043-TDS-0371 | 2 | 31.04 | 1.13 | 11.3 | Positive |
| 4 | 1043-TDS-0449 | 2 | 32.52 | 1.14 | 2.3 | |
| 5 | 1043-TDS-0474 | 2 | 33.74 | 1.12 | 4.0 | |
| 6 | 1043-TDS-0134 | 2 | 33.07 | 4.62 | Positive | 4.7 | |
| 7 | 1043-TDS-0214 | 2 | 33.1 | 4.13 | Positive | 8.0 | Positive |
| 8 | 1043-TDS-0363 | 2 | 30.65 | 3.35 | Positive | 4.4 | |
| 9 | 1043-TDS-0388 | 2 | 32.11 | 1.76 | 2.6 | |
| 10 | 1043-TDS-0012 | 5 | 30.19 | 1.09 | 3.8 | |
| 11 | 1043-TDS-0021 | 6 | 32.58 | 1.16 | 4.6 | |
| 12 | 1043-TDS-0024 | 3 | 32.9 | 1.32 | 5.6 | Positive |
| 13 | 1043-TDS-0026 | 3 | 31.47 | 1.03 | 3.2 | |
| 14 | 1043-TDS-0028 | 2 | 18.7 | 6.71 | Positive | 5.2 | Positive |
| 15 | 1043-TDS-0032 | 4 | 13.72 | 8.05 | Positive | 7.8 | Positive |
| 16 | 1043-TDS-0033 | 4 | 30.41 | 1.06 | 6.4 | Positive |
| 17 | 1043-TDS-0045 | 4 | 31.72 | 1 | |
| 18 | 1043-TDS-0050 | 4 | 15.83 | 20.89 | Positive | 5.4 | |
| 19 | 1043-TDS-0056 | 7 | 33.06 | 0.84 | 3.6 | |
| 20 | 1043-TDS-0005 | 4 | 31.58 | 0.82 | 14.4 | Positive |
| 21 | 1043-TDS-0018 | 4 | 30.98 | 1.49 | 7.3 | |
| 22 | 1043-TDS-0029 | 4 | 33.03 | 1.03 | 2.7 | |
| 23 | 1043-TDS-0030 | 3 | 30.41 | 1.05 | 2.2 | |
| **Sensitivity** | | | | | 6/23 – 26% | 10/23 – 43% |
| **Two-time blood draw** |
| 24 | 1043-TDS-0163 | 2 | 33.47 | 1.08 | 3.6 | |
| 25 | 1043-TDS-0163v2 | 9 | 0 | 12.53 | Positive | 7.7 | |
| 26 | 1043-TDS-0175 | 4 | 33.67 | 1.88 | Positive | 5.4 | |
| 27 | 1043-TDS-0175v2 | 14 | 20.2 | 15.9 | Positive | 6.8 | |
| 28 | 1043-TDS-0122 | 5 | 32.42 | 1.13 | 7.7 | |
| 29 | 1043-TDS-0122v2 | 9 | 0 | 5.6 | Positive | 10.4 | |
| 30 | 1043-TDS-0127 | 3 | 32.95 | 1.12 | 7.6 | |
| 31 | 1043-TDS-0127v2 | 7 | 0 | 1.24 | 6.6 | |
| 32 | 1043-TDS-0223 | 2 | 33.64 | 1.26 | 5.3 | |
| 33 | 1043-TDS-0223v2 | 8 | 30.48 | 3.78 | Positive | 11.6 | |
| 34 | 1043-TDS-0225 | 2 | 33.18 | 1.74 | 4.6 | |
| 35 | 1043-TDS-0225v2 | 7 | 0 | 4.88 | Positive | 10.1 | |
| 36 | 1043-TDS-0141 | 4 | 32.72 | 1.02 | 4.8 | |
| 37 | 1043-TDS-0141v2 | 7 | | 3.0 | |
| 38 | 1043-TDS-0144 | 2 | 32.6 | 1.15 | 8.0 | |
| 39 | 1043-TDS-0144v2 | 5 | | 12.1 | |
| 40 | 1043-TDS-0219 | 5 | 34.28 | 8.9 | Positive | 7.0 | |
| 41 | 1043-TDS-0219v2 | 11 | | 7.3 | |
| 42 | 1043-TDS-0221 | 6 | 36.37 | 15.41 | Positive | 5.3 | |
| 43 | 1043-TDS-0221v2 | 12 | | 3.8 | |
| 44 | 1043-TDS-0228 | 5 | 19.59 | 4.06 | Positive | 4.8 | |
| 45 | 1043-TDS-0228v2 | 10 | | 8.4 | |
| **Sensitivity** | | | | | 9/17 – 53% | 16/22 – 73% |

a) Days: days between the blood collection and symptom onset.

b) For D2Dx™ test, a test score of 5.0 is used as the clinical cutoff value, equal to or above 5.0: positive; lower than 5.0: negative.

c) For the Interpretation column, only positive diagnostic interpretation is marked. Negative and equivocal diagnostic interpretations are left blank.
2. Materials and methods

2.1. Chemicals and materials

Citrate AuNP with an average hydrodynamic diameter around 90 nm was received as a gift from Nano Discovery Inc. (Orlando, Florida). Zika virus lysate (catalog number 0810521) was manufactured by Zeptometrix, using virus strain MR766, propagated using cell line LLC-mk2, and the lysate has a total protein concentration of 1.18 mg/mL. According to the manufacturer, the lysate was made by treating purified Zika virus stock solution with Triton X-100, with a concentration of 0.5%. A human anti-Zika E protein IgM antibody (manufacturer: Absolute Antibody, catalog number Ab00779 – 15.0) at a concentration of 1.0 mg/mL was used to test the binding activity of the Zika virus lysate-coated AuNP.

2.2. Preparation of Zika virus lysate-coated AuNPs (AuNP-ZIKV)

15 μL Zika virus lysate solution was added to 1.5 mL citrate-AuNP in an Eppendorf centrifuge tube. After thorough mixing, the mixture was allowed to sit at room temperature for 20 min. The AuNP-ZIKV probe was then be ready for testing without additional purification. The prepared the AuNP pseudo virus particle has an average hydrodynamic diameter of 105 nm, measured using a dynamic light scattering assay reader, D2Dx-R, manufactured by Nano Discovery Inc. (Orlando, Florida).

2.3. Blood test procedure

To perform the test on blood plasma samples, 3 μL of undiluted human blood plasma sample was mixed with 60 μL AuNP-ZIKV pseudo virus solution in a mini-glass tube. After vortex mixing for 10 s, the assay solution was left to stand still at room temperature for 20 min. The average particle size of the assay solution was then measured using D2Dx-R. The ratio of the average particle size of the assay solution ($D_2$) versus the average size of the AuNP pseudo virus particle ($D_1$) is calculated as the test score.

### Table 2

| Sample ID     | Days | Aptima Zika RT-PCR | InBios anti-Zika IgM test | Interpretation of InBios test | D2Dx test score | Interpretation of D2Dx test |
|--------------|------|--------------------|---------------------------|-------------------------------|----------------|----------------------------|
| 1043-TDS-0067  | 3    | 34.64              | 7.9                       | Positive                      | 14.0           | Positive                   |
| 1043-TDS-0067V2 | 27   | 32.59              | 16.54                     | Positive                      | 15.6           | Positive                   |
| 1043-TDS-0067V3 | 39   | 33.24              | 13.19                     | Positive                      | 3.6            | Positive                   |
| 1043-TDS-0067V4 | 46   | 32.43              | 9.83                      | Positive                      | 9.8            | Positive                   |
| 1043-TDS-0067V5 | 53   | 33.85              | 9.84                      | Positive                      | 2.4            | Positive                   |
| 1043-TDS-0067V6 | 60   | 33.32              | 6.1                       | Positive                      | 10.9           | Positive                   |
| 1043-TDS-0067V7 | 67   | 35.3               | 4.74                      | Positive                      | 3.4            | Positive                   |
| 1043-TDS-0067V8 | 74   | 36.1               | 4.88                      | Positive                      | 5.5            | Positive                   |
| 1043-TDS-0067V10 | 135  | 36.14              | 3.76                      | Positive                      | 2.5            | Positive                   |

**Pregnant woman**

2. 1043-TDS-0143 5 33.09 1.09 Positive 5.0 Positive

2. 1043-TDS-0143V2 27 32.59 16.54 Positive 15.6 Positive

2. 1043-TDS-0143V3 39 33.24 9.84 Positive 2.4 Positive

2. 1043-TDS-0150V2 7 8.19 0.175 9.8 Positive

2. 1043-TDS-0150V3 11 0 11.58 17.0 Positive

2. 1043-TDS-0150V4 14 0 10.96 7.9 Positive

2. 1043-TDS-0150V5 21 0 1.53 12.8 Positive

2. 1043-TDS-0150V6 28 0 1.12 8.8 Positive

2. 1043-TDS-0150V7 34 0 0.93 14.0 Positive

2. 1043-TDS-0150V8 42 0 0.86 4.1 Positive

**Non-pregnant woman**

3. 1043-TDS-0156 4 34.33 1.05 13.2 Positive

3. 1043-TDS-0156V2 15 0 5.15 9.1 Positive

3. 1043-TDS-0156V3 24 0 5.84 13.5 Positive

3. 1043-TDS-0156V4 31 0 3.5 4.6 Positive

3. 1043-TDS-0156V5 38 0 3.18 13.2 Positive

3. 1043-TDS-0156V6 45 0 2.12 12.0 Positive

3. 1043-TDS-0156V7 52 0 1.81 5.8 Positive

3. 1043-TDS-0156V8 59 0 2.1 4.8 Positive

**Male**

4. 1043-TDS-0263 4 33.4 1.01 8.3 Positive

4. 1043-TDS-0263V2 18 0 5.52 3.1 Positive

4. 1043-TDS-0263V3 26 0 3.51 15.8 Positive

4. 1043-TDS-0263V4 32 0 3.12 5.0 Positive

4. 1043-TDS-0263V5 40 0 2.29 2.2 Positive

4. 1043-TDS-0263V6 47 0 1.59 3.3 Positive

4. 1043-TDS-0263V7 54 0 1.55 12.3 Positive

4. 1043-TDS-0263V8 61 0 1.09 2.8 Positive

**Non-pregnant woman**

**Sensitivity**

29/40 – 73%

### Footnotes

a Days: days between the blood collection and symptom onset.

b For D2Dx™ test, a test score of 5.0 is used as the clinical cutoff value, equal or above 5.0: positive; lower than 5.0: negative.

c For the Interpretation column, only positive diagnosis is listed. Negative and equivocal diagnostic interpretations are left blank.
Table 3
The D2Dx™ test results of negative control blood plasma samples collected in a location within the United States, where there had been no Zika case reported.

| Sample ID          | Test score | Interpretation |
|--------------------|------------|----------------|
| D000030363         | 2.2        |                |
| D000030364         | 4.5        |                |
| D000030365         | 2.9        |                |
| D000030366         | 3.3        |                |
| D000030367         | 3.8        |                |
| D000030368         | 2.1        |                |
| D000030369         | 3.3        |                |
| D000030370         | 3.9        |                |
| D000030371         | 14.2       | False positive |
| D000030372         | 3.9        |                |
| D000030373         | 2.8        | False positive |
| D000030374         | 2.5        |                |
| D000030375         | 2.5        |                |
| D000030376         | 2.5        |                |
| D000030377         | 3.6        |                |
| D000030378         | 2.8        |                |
| D000030379         | 2.8        |                |
| D000030380         | 4.4        |                |
| D000030381         | 3.4        |                |
| D000030382         | 4.8        |                |
| D000030383         | 4.3        |                |
| D000030384         | 2.8        | False positive |
| D000030385         | 3.0        |                |
| D000030386         | 6.9        |                |
| D000030387         | 3.0        |                |
| D000030388         | 3.9        |                |
| D000030389         | 2.8        | False positive |
| D000030390         | 6.3        |                |
| D000030391         | 3.4        |                |
| D000030392         | 4.9        |                |
| D000030393         | 2.9        |                |
| D000030394         | 4.2        |                |
| D000030395         | 3.8        |                |
| D000030396         | 3.5        |                |
| D000030397         | 3.9        |                |
| D000030398         | 3.9        |                |
| D000030399         | 3.9        |                |
| D000030400         | 3.1        |                |
| D000030401         | 3.7        |                |
| D000030402         | 2.7        |                |

False positive rate 3/40 – 7.5%

*For D2Dx test interpretation, a test score of 5.0 is used as the clinical cutoff value, equal or above 5.0: positive; lower than 5.0: negative. The samples were collected in December 2017 from a state in the United States where no Zika case was reported. False positive rate: 3/40 = 7.5%.

2.4. Source of human blood plasma samples

All blood samples used in this study are EDTA-K3 human blood plasma samples. All samples were purchased from a commercial vendor, Boca Biolistics (Boca Raton, Florida). Because such samples were received as de-identified samples, per NIH guideline, the study reported here is not a human subject research, therefore, Institutional Review Board (IRB) approval is not required. During the period of ZIKV outbreak (2015–2017), Boca Biolistics collected a large number of blood samples from countries and regions where ZIKV endemic took place. The plasma samples were aliquoted, frozen and stored at –80°C as soon as they were processed from the blood and separated by centrifuge. Aliquots were shipped to our laboratory, and thawed at 4°C overnight before testing. Boca Biolistics was listed in FDA Emergency Use Authorization (EUA) guidelines as one source to obtain clinical samples for ZIKV diagnostic test validation study. Boca Biolistics conducted in house testing of collected blood samples using FDA-approved or authorized clinical tests to confirm the positive or negative disease status of the samples. Two tests were used to confirm the status of Zika positive samples: Holologic Aptima Zika RT-PCR nucleic acid test and/or InBios anti-Zika IgM antibody test. The samples used in our current study have complete clinical data regarding the clinical diagnosis and in house testing results by Boca Biolistics. In addition to Zika positive samples and negative control samples, we also tested certain numbers of other flavivirus positive samples, including Dengue and Chikungunya positive samples, to determine the cross reactivity of the current test for ZIKV detection. The positive status of Dengue or Chikungunya samples were confirmed using InBios anti-Dengue or anti-Chikungunya IgM antibody test.

We obtained and tested the following samples in this study: (1) 85 confirmed ZIKV symptomatic and positive samples collected in Dominican Republic between late 2015 to middle 2016; (2) 40 negative control samples collected in the United States from a state in December 2017 where no Zika case was ever reported; (3) 10 Chikungunya-positive samples collected in Dominican Republic in February 2015, prior to the report of ZIKV outbreak; (4) 25 Dengue-positive samples from Dominican Republic between August to December 2015 and 15 Dengue-positive samples from Peru in May 2017; (5) 38 ZIKV-asymptomatic samples collected in Dominican Republic towards the end of the Zika endemic in the region, in September 2016.

2.5. Statistical analyses

P values as presented in the figures were determined by two-tailed unpaired Student’s t-test. P values < 0.05 were considered as significant difference. The numbers of asterisks indicate significance levels of P values, for example, the symbols of *, **, ***, and **** represent P values of ≤0.05, <0.01, ≤0.001, and ≤0.0001, respectively. If there is no significance difference (P > 0.05) between the groups, the results are presented as “ns”, namely, not significant.

3. Results and discussions

3.1. Preparation of the AuNP pseudo virus (AuNP-ZIKV)

We first prepared the Zika virus lysate - coated AuNP pseudo virus particles (AuNP-ZIKV). The preparation is extremely simple, by mixing 15 μL Zika virus lysate solution to 1.5 mL citrate-AuNP solution. After incubating at room temperature for about 20 min, the nanoparticle pseudo virus reagent is ready for use. This 1.5 mL AuNP-ZIKV solution allows for testing of 25 samples (60 μL for each test). The successful coating of Zika lysate to the AuNP is supported by an average particle size increase of the coated AuNPs. The hydrodynamic diameter of the original uncoated AuNP is approximately 90 nm. Following the lysate coating, the average hydrodynamic diameter increased to 105 ± 5 nm. Additionally, the coated AuNP was tested for its reactivity with a human anti-ZIKV IgM antibody. As shown in Fig. 2, when mixed with the human anti-ZIKV IgM antibody, the P value is less than 0.0001, which indicates a significant difference.
antibody (60 μL AuNP-ZIKV solution mixed with 3 μL of anti-ZIKV IgM antibody solution), there is a steady and linear increase of the average particles size of the mixture solution with increased human anti-ZIKV IgM antibody concentration. This test can be used to examine and monitor the quality of the so-prepared AuNP-ZIKV pseudo virus reagent.

3.2. Sensitivity of the D2Dx test and comparison with anti-Zika IgM serology test

We then tested 85 Zika positive patient samples and 40 negative control samples. Tables 1 and 2 are the summary of D2Dx™ test results of 85 Zika positive patient samples along with their test results using Aptima Zika RT-PCR test and/or InBios anti-Zika IgM test. In both Tables 1 and 2, the days between blood draw and symptom onset were provided for each sample. All Zika positive samples were collected in Dominican Republic during the outbreak of Zika endemic (2015–2016). Among the 85 Zika positive samples, 23 samples were taken from 23 individual patients (single blood draw), and 22 samples from 11 patients (two-time blood draw). Table 1 summarizes the results of these 45 samples. For single blood draw samples, the ZIKV positive status was confirmed by either Aptima Zika RT-PCR test or InBios anti-ZIKV IgM test, or both. For the two-blood draw samples, if one of the two blood draws is positive, that is, either Aptima RT-PCR test or InBios anti-Zika IgM test is positive, then the patient is considered Zika positive. Table 2 is the test results of 40 serial blood draw samples collected from 5 Zika positive patients. Table 3 is the test results of 40 negative control samples. The 40 negative samples were collected from volunteers in a State at the United States, where no Zika case was reported during the outbreak. These samples were not tested for Zika, however, because they were collected from a place where no Zika case was ever reported, these samples can be presumed to be Zika-negative.

![Fig. 4. Sensitivity comparison of D2Dx™ test versus InBios anti-ZIKV IgM test.](image)

![Fig. 5. False positive rate of the D2Dx™ test on negative control samples (N = 40), Chikungunya positive samples (N = 10), and two groups of Dengue positive samples collected from Zika epidemic (Dominican Republic, N = 25) and non-epidemic locations (Peru, N = 15).](image)

![Table 4 Test results of Chikungunya positive samples. These samples were collected in February 2015 in Dominican Republic, a time period when Zika case had not been reported yet in this country. The samples were tested as Chikungunya positive using InBios anti-CHIKV IgM test kit, and Zika-negative using InBios anti-Zika IgM test kit.](image)

![Table 3 is the test results of 40 negative control samples. The 40 negative samples were collected from volunteers in a State at the United States, where no Zika case was reported during the outbreak. These samples were not tested for Zika, however, because they were collected from a place where no Zika case was ever reported, these samples can be presumed to be Zika-negative.](image)

![Fig. 3 is the comparison of the average D2Dx™ test scores of 85 Zika positive samples versus 40 negatively control samples. The average test score of Zika positive group is 7.8, and the average test score of the negative control group is 3.8. Student t-test reveals a p value far less than 0.0001. The difference between the Zika positive group and the negative control group is statistically significant.](image)
Table 5

Test results of Dengue positive samples. These samples were from two sources: (1) collected between August to December 2015 in Dominican Republic; and (2) collected in May 2017 in Peru. Peru did not have Zika endemic but had an ongoing Dengue epidemic. Samples from Dominican Republic were tested negative in the Aptima Zika RT-PCR test, however, not necessarily negative in the InBios anti-Zika IgM test. Most samples from Dominican Republic were tested as “Possible Zika positive” or “Presumptive other flavivirus” based on InBios anti-Zika IgM test. Peru samples were confirmed as Dengue positive by symptoms and positive InBios anti-Dengue IgG assay. Peru samples were also confirmed by Aptima Zika RT-PCR test as negative and InBios anti-Zika IgM test as negative.

| Sample ID   | InBios anti-Dengue IgM | Interpretation of InBios anti-Dengue IgM test | D2Dx test score | *Interpretation of D2Dx test | InBios anti-Dengue IgG test | InBios anti-Zika IgM test |
|-------------|-------------------------|-----------------------------------------------|-----------------|-------------------------------|-----------------------------|---------------------------|
| From Dominican Republic |                         |                                               |                 |                               |                             |                           |
| 1043-DNG-0224 | 2.78                    | Equivocal                                     | 3.6             | 7.93                          | Other flavivirus            |
| 1043-DNG-0229 | 2.46                    | Equivocal                                     | 9.1             | Positive                      | Other flavivirus            |
| 1043-DNG-0236 | 1.96                    | Equivocal                                     | 11.6            | Positive                      | Other flavivirus            |
| 1043-DNG-0237 | 2.1                     | Equivocal                                     | 6.0             | Positive                      | Possible Zika               |
| 1043-DNG-0246 | 2.19                    | Equivocal                                     | 2.2             | 0.24                          | Negative                    |
| 1043-DNG-0248 | 1.85                    | Equivocal                                     | 3.6             | 11.44                         | Negative                    |
| 1043-DNG-0259 | 1.86                    | Equivocal                                     | 11.0            | Positive                      | Negative                    |
| 1043-DNG-0262 | 1.71                    | Equivocal                                     | 3.4             | 30.8                          | Negative                    |
| 1043-DNG-0212 | 3.29                    | Positive                                       | 4.4             | 12.32                         | Other flavivirus            |
| 1043-DNG-0215 | 8.26                    | Positive                                       | 3.4             | 21.21                         | Possible Zika               |
| 1043-DNG-0222 | 6.36                    | Positive                                       | 2.5             | 1.08                          | Other flavivirus            |
| 1043-DNG-0286 | 6.81                    | Positive                                       | 3.5             | 11.57                         | Other flavivirus            |
| 1043-DNG-0293 | 14.94                   | Positive                                       | 2.1             | 11.18                         | Other flavivirus            |
| 1043-DNG-0299 | 4.38                    | Positive                                       | 2.9             | 12.66                         | Other flavivirus            |
| 1043-DNG-0235 | 5.6                     | Positive                                       | 16.4            | Positive                      | Other flavivirus            |
| 1043-DNG-0256 | 16.71                   | Positive                                       | 3.1             | 2.32                          | Other flavivirus            |
| 1043-DNG-0274 | 4.59                    | Positive                                       | 14.9            | Positive                      | Other flavivirus            |
| 1043-DNG-0294 | 7.25                    | Positive                                       | 2.1             | 19.98                         | Other flavivirus            |
| 1043-DNG-0260 | 10.06                   | Positive                                       | 9.2             | Positive                      | Possible Zika              |
| 1043-DNG-0245 | 11.51                   | Positive                                       | 14.6            | Positive                      | Possible Zika              |
| 1043-DNG-0251 | 5.14                    | Positive                                       | 13.2            | Possible Zika                |
| 1043-DNG-0270 | 6.44                    | Positive                                       | 4.6             | 16.4                          | Other flavivirus            |
| 1043-DNG-0279 | 10.82                   | Positive                                       | 12.2            | 9.04                          | Possible Zika              |
| 1043-DNG-0290 | 3.49                    | Positive                                       | 1.6             | 13.96                         | Possible Zika              |
| 1043-DNG-0291 | 7.88                    | Positive                                       | 3.0             | 13.06                         | Possible Zika              |
| Positive rate | 10/25 = 40%             |                                               |                 |                               |                             |                           |
| From Peru |                         |                                               |                 |                               |                             |                           |
| D000020551 | 1.19                    | Negative                                       | 3.2             | 4.28                          | Negative                    |
| D000020554 | 1.25                    | Negative                                       | 2.8             | 2.98                          | Negative                    |
| D000020555 | 1.13                    | Negative                                       | 1.8             | 15.62                         | Negative                    |
| D000021155 | 1.35                    | Negative                                       | 2.5             | 18.29                         | Negative                    |
| D000021160 | 1.51                    | Negative                                       | 2.2             | 17.14                         | Negative                    |
| D000021138 | 18.52                   | Positive                                       | 7.5             | Positive                      | Other flavivirus            |
| D000021142 | 2.0                     | Equivocal                                      | 2.2             | 19.48                         | Zika positive               |
| D000021149 | 1.81                    | Equivocal                                      | 5.7             | Positive                      | Other flavivirus            |
| D000021152 | 1.81                    | Equivocal                                      | 4.8             | 18.95                         | Negative                    |
| D000021154 | 7.01                    | Positive                                       | 2.4             | 11.44                         | Possible Zika              |
| D000021156 | 4.13                    | Positive                                       | 3.2             | 7.3                           | Possible Zika              |
| D000021159 | 5.7                     | Positive                                       | 2.1             | 12.53                         | Other flavivirus            |
| D000021163 | 18.04                   | Positive                                       | 2.9             | 8.45                          | Possible Zika              |

(continued on next page)
Table 5 (continued)

| Sample ID   | Date | Result | Antibody | Positive Rate |
|-------------|------|--------|----------|---------------|
| D000020556 | 2.1  | Equivocal | 1.8 | 15.54 |
| D000020558 | 3.7  | Positive | 1.6 | 14.42 |

*Positive rate 2/15 – 13.3%* All positive Dengue IgG

For a D2Dx™ test interpretation, a test score of 5.0 is used as the clinical cutoff value, equal or above 5.0: positive; lower than 5.0: negative.

From a closer look of the 40 negative samples, there are three samples appear to be out of the range from other 37 samples (Table 3). While most samples show a test score between 2.0 and 5.0, mostly between 3.0 and 4.0, these three samples (#371, 386 and 390) have test score of 14.2, 6.9 and 6.3, respectively. Using the outliers function in Excel, we can indeed treat these three samples as outliers of the data set. It is possible that these three donors have natural immunity to Zika virus or they have been infected or vaccinated with other types of viruses that would cross react with Zika virus. In either case, these three samples can be regarded as giving false positive test results. If we eliminate these three samples, calculate the mean test score of the rest 37 samples from the negative group, the mean test score of the negative group is 3.4, and the standard deviation is 0.7. One method to determine the cutoff value between normal and disease group is to calculate the two standard deviations of the difference between mean values of the two groups under the independence assumption [17]. According to this method, it was calculated that the cutoff D2Dx™ test score can be 4.8. For clinical applications, the sensitivity and specificity of a diagnostic test has to be balanced. Typically, a reciprocal operation curve (ROC) should be developed by selecting different levels of sensitivity and specificity from a large set of testing data. In our current study, because of the limited number of data set, ROC cannot be properly constructed. With all factors considered, we chose a D2Dx™ test score of 5.0 as the cutoff value for test result interpretation: a test score of equal to or above 5.0 was considered as positive; and a test score below 5.0 is considered as negative. This cutoff value was used throughout the current study to determine the sensitivity and specificity of the D2Dx™ test.

Using the test score cutoff value of 5.0, we first examined the sensitivity of the D2Dx™ test and compared it with InBios anti-Zika IgM test. According to this cutoff value, samples with a D2Dx™ score exceeding 5.0 is labelled as positive. During the time of our study, InBios anti-Zika IgM test was the serology test that received emergency use authorization (EUA) from FDA. InBios anti-Zika IgM test is an immunoassay serology test that detects the anti-Zika IgM antibody produced in the body following infection. For infectious disease detection and diagnosis, it is important to know the date of the symptom onset: in the first few days post infection, the virus load in the body is high, and virus DNA or RNA tests are used to detect the presence of virus in the body. After 7–10 days, the body will start to produce anti-virus IgM antibody against the virus, and anti-virus IgM serology test should be applied for the detection and diagnosis. After 7–10 days, virus particles may or may not be present in the body, therefore, the RT-PCR virus nucleic acid test may become negative. For this reason, the days between symptom onset and blood draw of each sample were recorded and listed in Tables 1 and 2. If one of the test of the blood sample, i.e. Aptima RT-PCR nucleic acid test or InBios anti-Zika IgM test is positive, the sample is considered as a “true” positive sample. All 85 samples we selected for the study, as listed in Tables 1 and 2, are “true” Zika positive samples.

By comparing the positive rate detected by the D2Dx™ test versus the number of “true” positive samples, we obtained the sensitivity of the D2Dx™ test on samples collected on different days from symptom onset, as summarized in Tables 1 and 2. We further highlight the sensitivity study results in Fig. 4. Within 7 days of symptom onset, from single blood draw, the InBios anti-Zika IgM test has a sensitivity of 26% while the D2Dx™ test has a sensitivity of 43%. Within 14 days of symptom onset, from two-time blood draw, the InBios anti-Zika IgM test has a sensitivity of 53% while the D2Dx™ test has a sensitivity of 73%. In both scenarios, D2Dx™ test has higher sensitivity than the InBios anti-Zika IgM test.

Although our current study is based on a relatively small number of samples, we believe D2Dx™ test has shown some advantages compared to the traditional antibody serology test. While the traditional antibody serology test detects a single immune molecule such as anti-virus IgM antibody produced in the body, D2Dx™ test detects the whole humoral immune response in the infected blood. As we reported in previous studies, not only different antibodies such as IgG and IgM, but also other humoral immune related molecules such as complement may be involved in the interaction with the nanoparticle probes. D2Dx™ test detects an immune response as an integral process, not individual molecules. We believe this is the reason why D2Dx™ test has higher sensitivity than the traditional serology test. More extensive studies need to be conducted to confirm our preliminary findings and hypothesis.

### 3.3. Specificity and cross reactivity study of the D2Dx test

The specificity of the D2Dx™ test was determined by analyzing the false positive rate of various control samples. These study results are summarized in Fig. 5. When selecting negative control samples to validate new diagnostic tests for infectious diseases, one needs to be cautious about where the samples are collected. For epidemic and pandemic viral infectious diseases, many people may contract the virus, develop an immune response in the body, however, never develop any symptom. The best negative control samples should be collected from a non-epidemic region, where there is a great probability that the population has never been exposed to the virus. For this reason, we obtained blood samples collected in a location at the United States where no case of Zika infection was ever reported. As shown in Table 3, among 40 samples, only 3 samples showed test scores above the cutoff value. Therefore, the false positive rate of the D2Dx™ test on true negative samples is 7.5%. As explained earlier, it is possible that the three false positive samples were from donors who have natural immunity to Zika virus, or they have been vaccinated or infected with a virus that may cross react with Zika virus.

Zika is a flavivirus. It is known that serology test designed to detect Zika infection may potentially cross-react with other flavivirus infections. In this study, we included two flavivirus infections, Dengue and Chikungunya, for cross reactivity study. 10 Chikungunya positive samples collected in Dominican Republic in February 2015 when Zika case had not been reported in the region yet, were analyzed. Among the 10 samples, only one has a D2Dx™ test score exceeding 5.0. Therefore, the false positive rate from the Chikungunya positive samples is 10% (see Table 4).

For cross reactivity study with Dengue infection, we obtained Dengue positive samples from two locations: 25 samples from Dominican Republic, collected from August 2015 to December 2015; and 15 samples from Peru in May 2017. The first case of Zika in Dominican Republic was reported in January 2016. Around May 2017, Peru did not have a Zika outbreak. From the analysis of the 25 Dengue positive samples from Dominican Republic, we found a 40% positive rate using D2Dx™ test, while the Peru sample group only showed a 13.3% positive rate (see Table 5). We believe some of the Dengue positive samples from Domin-ican Republic may be actually Zika positive. Our suggestion is partially supported by the InBios testing results. Most of these samples from Dominican Republic were tested as “Possible Zika positive” or “Presumptive other flavivirus” based on InBios anti-Zika IgM test. Peru samples, on the other hand, were confirmed by both Aptima Zika RT-PCR test and InBios anti-Zika IgM test as negative, and we found a much lower false positive rate of 13.3% from the Peru sample cohort.
Table 6
Summary of test results of 38 Zika-asymptomatic samples collected in Dominican Republic in September 2016, a period close to the end of Zika epidemic.

| Sample ID   | InBios anti-Zika IgM | Interpretation of InBios anti-Zika IgM test | Aptima Zika RT-PCR | *Interpretation of D2Dx test score | *Interpretation of D2Dx test | DiaPro anti-Zika IgG | Interpretation of DiaPro Zika IgG |
|-------------|----------------------|---------------------------------------------|--------------------|------------------------------------|-----------------------------|---------------------|-------------------------------|
| 1043-TDA-0127 | 0.96                 | Negative                                    | Negative           | 5.3                                | Positive                    | 5.17                | Positive                     |
| 1043-TDA-0128 | 1.38                 | Negative                                    | Negative           | 2.3                                | Positive                    | 1.44                | Positive                     |
| 1043-TDA-0129 | 0.95                 | Negative                                    | Negative           | 3.2                                | Positive                    | 0.3                 |                              |
| 1043-TDA-0130 | 0.98                 | Negative                                    | Negative           | 11.9                               | Positive                    | 0.88                |                              |
| 1043-TDA-0131 | 1.56                 | Negative                                    | Negative           | 12.8                               | Positive                    | 3.75                | Positive                     |
| 1043-TDA-0132 | 0.75                 | Negative                                    | Negative           | 10.0                               | Positive                    | 0.87                |                              |
| 1043-TDA-0133 | 0.89                 | Negative                                    | Negative           | 9.5                                | Positive                    | 0.29                |                              |
| 1043-TDA-0134 | 1.16                 | Negative                                    | Negative           | 6.4                                | Positive                    | 2.39                | Positive                     |
| 1043-TDA-0135 | 1.12                 | Negative                                    | Negative           | 7.8                                | Positive                    | 5.37                | Positive                     |
| 1043-TDA-0136 | 0.96                 | Negative                                    | Negative           | 7.1                                | Positive                    | 3.21                | Positive                     |
| 1043-TDA-0137 | 1.48                 | Negative                                    | Negative           | 3.1                                | Positive                    | 3.89                | Positive                     |
| 1043-TDA-0138 | 1.38                 | Negative                                    | Negative           | 6.1                                | Positive                    | 7.61                | Positive                     |
| 1043-TDA-0139 | 1.26                 | Negative                                    | Negative           | 8.6                                | Positive                    | 0.46                |                              |
| 1043-TDA-0140 | 1.15                 | Negative                                    | Negative           | 4.0                                | Positive                    | 7.61                | Positive                     |
| 1043-TDA-0141 | 1.12                 | Negative                                    | Negative           | 4.3                                | Positive                    | 2.36                | Positive                     |
| 1043-TDA-0142 | 1.15                 | Negative                                    | Negative           | 2.3                                | Positive                    | 0.85                |                              |
| 1043-TDA-0147 | 1.11                 | Negative                                    | Negative           | 12.8                               | Positive                    | 2.59                | Positive                     |
| 1043-TDA-0148 | 1.2                  | Negative                                    | Negative           | 3.6                                | Positive                    | 3.33                | Positive                     |
| 1043-TDA-0150 | 1.54                 | Negative                                    | Negative           | 7.8                                | Positive                    | 1.6                 | Positive                     |
| 1043-TDA-0152 | 0.86                 | Negative                                    | Negative           | 7.8                                | Positive                    | 0.14                |                              |
| 1043-TDA-0153 | 1.17                 | Negative                                    | Negative           | 5.2                                | Positive                    | 4.25                | Positive                     |
| 1043-TDA-0154 | 1.1                  | Negative                                    | Negative           | 4.6                                | Positive                    | 0.22                |                              |
| 1043-TDA-0155 | 1.16                 | Negative                                    | Negative           | 3.9                                | Positive                    | 0.93                |                              |
| 1043-TDA-0156 | 0.92                 | Negative                                    | Negative           | 3.8                                | Positive                    | 6.12                | Positive                     |
| 1043-TDA-0158 | 0.97                 | Negative                                    | Negative           | 12.2                               | Positive                    | 0.9                 |                              |
| 1043-TDA-0159 | 1.25                 | Negative                                    | Negative           | 3.8                                | Positive                    | 6.74                | Positive                     |
| 1043-TDA-0160 | 0.95                 | Negative                                    | Negative           | 5.2                                | Positive                    | 7.61                | Positive                     |
| 1043-TDA-0161 | 0.72                 | Negative                                    | Negative           | 4.5                                | Positive                    | 7.61                | Positive                     |
| 1043-TDA-0164 | 1.01                 | Negative                                    | Negative           | 16.9                               | Positive                    | 6.3                 | Positive                     |
| 1043-TDA-0165 | 1.09                 | Negative                                    | Negative           | 4.1                                | Positive                    | 4.51                | Positive                     |
| 1043-TDA-0167 | 1.46                 | Negative                                    | Negative           | 10.7                               | Positive                    | 2.12                | Positive                     |
| 1043-TDA-0168 | 0.96                 | Negative                                    | Negative           | 7.8                                | Positive                    | 3.68                | Positive                     |
| 1043-TDA-0169 | 1.08                 | Negative                                    | Negative           | 10.2                               | Positive                    | 0.62                |                              |
| 1043-TDA-0170 | 1.38                 | Negative                                    | Negative           | 10.0                               | Positive                    | 0.31                |                              |
| 1043-TDA-0171 | 1.03                 | Negative                                    | Negative           | 11.2                               | Positive                    | 0.22                |                              |
| 1043-TDA-0172 | 1.22                 | Negative                                    | Negative           | 6.7                                | Positive                    | 0.44                |                              |
|             | 0.96                 | Negative                                    | Negative           | 3.7                                | Positive                    | 1.59                | Positive                     |

(continued on next page)
For rapid screening purpose, the emphasis of the test performance is on sensitivity. Once a positive sample is identified, a potentially infected patient can be immediately put into quarantine while the sample may be sent for more accurate laboratory test. When dealing with highly contagious and high risk emerging infectious diseases, such as the case of Zika and the current ongoing COVID-19 outbreak, it is essential to identify all potentially positive patients as rapidly as possible to prevent the positive patients from unknowingly spreading the diseases to others.

### 3.4. The high positive rate detected among Zika asymptomatic donors from endemic regions using D2Dx™ test and potential implications in herd immunity development

In this study, we also tested 38 samples collected in Dominican Republic in September 2016, a time that was near the end of the Zika epidemic in the country. These samples were collected from donors that had not shown Zika-related symptoms. These samples were tested negative by both Aptima RT-PCR test and InBios anti-Zika IgM test. However, a significant number of these samples were tested positive (61%) using the DiaPro anti-Zika IgM test (Table 6). Our study revealed a surprisingly high positive rate of 63% from this group of samples. We believe the positive samples from the Zika-asymptomatic cohort are most likely from patients who have been infected with Zika during the outbreak between January to August 2016, but had not had clinical symptoms. This hypothesis requires additional investigation. InBios anti-Zika IgM test is a test that detects the specific anti-Zika IgM antibodies. IgM antibodies are produced at very early stage of infection, and the antibody isotype switch from IgM to IgG usually occurs with the progression of the humoral immune response development [18,19]. The D2Dx™ test, while detecting the overall humoral immune response to a viral infection, does not distinguish specific immune molecules and does not detect specific antibodies or antibody isotypes such as IgM or IgG. It is known that anti-virus IgG antibodies can be present in the body for weeks, months or years after the infection is completely resolved and the patient is fully recovered [20]. It has also been suggested that the end of Zika endemics and epidemics is achieved through the establishment of herd immunity [21,22]. This means, only after the majority of a population in an endemic and epidemic region is infected, the epidemics will stop. With all these confounding factors, it is not surprising that a high positive rate was detected from blood samples collected from an epidemic region even after the epidemic spread has stopped.

### 4. Conclusions

In this study, we have demonstrated the development and preliminary clinical assessment of a rapid blood test for virus infection detection and diagnosis. Data shows that the new test can have significantly higher sensitivity than the traditional serology test, while maintain similar specificity. Although our current study was focused on Zika, the technology platform can be easily adapted for other virus infectious diseases. The development of the AuNP pseudo virus particle is an extremely simple process as shown in our study, by mixing a virus lysate solution with a citrate AuNP solution. The AuNP pseudo virus reagent can be made in situ for immediate testing or pre-made for later testing. The test involves a single step of mixing the AuNP pseudo virus solution with an untreated, undiluted blood plasma sample, with results obtained in minutes. Finally, we want to mention here that although all of the results reported here are based on average particle size measurement of the assay solution using dynamic light scattering, we have preliminary evidence showing that the results of this new test may also be read using a simple colorimeter, which costs no more than a few hundred US dollars per unit; or on a high throughput automatic microplate reader platform. It is well known that AuNPs, upon aggregate formation, change color due to their surface plasmon resonance wavelength change, and this change can be monitored using a small colorimeter or a UV–Vis spectrophotometer microplate reader. We will report these additional developments in due course.

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### Declaration of competing interest

The authors declare the following competing financial interest(s): Q.H. is an owner and officer of Nano Discovery Inc. Nano Discovery Inc. Licensed and commercialized the assay technology reported in the manuscript.

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### Table 6 (continued)

| Sample ID | InBios anti-Zika IgM | Interpretation of InBios anti-Zika IgM test | Aptima Zika RT-PCR | D2Dx test score | *Interpretation of D2Dx test | DiaPro anti-Zika IgG | Interpretation of DiaPro anti-Zika IgG |
|-----------|----------------------|-------------------------------------------|-------------------|----------------|--------------------------|-------------------|-------------------------------------|
| 1043-TDA-0173 | Negative | Negative | 6.7 | Positive | 0.4 | Positive rate: 24/38 – 63% | Positive rate: 23/38 – 61% |

*For D2Dx™ test interpretation, a test score of 5.0 is used as the clinical cutoff value, equal or above 5.0: positive; lower than 5.0: negative.
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