1   *Type of the Paper (Article)*

2   Establishment of micro droplet digital polymerase

3   chain reaction and real-time fluorescence

4   quantitative polymerase chain reaction

5   technologies for detecting Zika virus

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22   **Abstract:** Establishment of diagnostic methods with low detection limits plays a critical role
23   in the maintenance of early diagnosis, prevention of serious neurological complications, and
24   control of the spread of ZIKA. In this study, we established the micro-droplet digital
25   polymerase chain reaction (ddPCR) and real-time fluorescent quantification PCR (qPCR)
26   protocols for the detection of Zika virus based on the NS5 gene. For the Zika standard

27   plasmid, the standard curve of R^2 was 0.999, and the amplification efficiency was 92.203%, as
28   determined by qPCR. Both ddPCR and qPCR were positive for cell culture of Zika nucleic
29   acid.The minimum detection limit of ddPCR is 1–2 times lower than qPCR. Moreover, all
30   tests of Dengue virus (1–4 serotypes) were negative in cell culture. Overall, these results
31   suggested than ddPCR may have a lower limit of detection than qPCR.

32   **Keywords:** Zika virus; nucleic acid detection; micro-droplet digital polymerase chain reaction;

33   real-time fluorescence quantitative polymerase chain reaction

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35   **1. Introduction**

36   Zika virus (ZIKA) is a single-stranded RNA virus belonging to the family *Flaviviride* genus

37   *Flavivirus*. It was first isolated from a forest in Uganda in 1947 [1] and encodes three structural

38   proteins (capsid protein C, precursor protein pr M, and envelope protein E) and seven

39   nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [2].

40   The NS5 gene is the largest gene in the ZIKA genome and encodes NS5 protein, which

41   has the highest relative molecular weight of all proteins encoded by ZIKA. NS5 contains an

42   RNA-dependent RNA polymerase active site at the N terminal and harbors a methylation site,

43   which is associated with the viral cap reaction of the viral RNA. The ZIKA genome has only

44   one open reading frame (ORF). NS5 is located at the 3′ end of the ORF and is a nucleic acid

45   detection target commonly used by the flavivirus [3]. Currently, several laboratories have

46   established nucleic acid detection technologies for ZIKA; the main targets of detection are the

47   E and NS5 genes [4]. The E gene has undergone several adaptive changes, whereas the NS5

48   gene is relativelystable. Therefore, the conserved sequence of NS5 gene may be more reliable

49   as a detection target.

50   ZIKA is transmitted through mosquito bites, particularly those from *Aedes aegypti* and *A.*

51   *albopictus*[5], and can also spread between independent mosquitoes [6]. More importantly, the

52   virus can be transmitted from mother to fetus via amniotic fluid and fetal tissues [7] and can

53   be transmitted through sexual activity [8]. In humans, ZIKA infection is characterized by mild

54   fever (37.8–38.5℃), joint pain (particularly pain in the hands and feet), myalgia, headache,

55   orbital pain, conjunctivitis, and skin rash and can also cause microcephaly and Guillain-Barré

56   syndrome [9]. However, only 20% of infected individuals exhibit symptoms [10],and ZIKA

57   infection can be a benign, self-limiting disease [11]. As a result, the incidence of ZIKA

58   infection may be underestimated in epidemics or returning travelers. For the detection of

59   ZIKA, Giada Rossini etal [12] showed that the blood test period is longer after onset of the

60   disease than urine or whole blood tests. ZIKA can be detected in whole blood in about 3–26

61   days and in the plasma in 3–10 days [13].

62   Accordingly, in this study, we aimed to establish micro-droplet digital polymerase chain

63   reaction (ddPCR) and real-time fluorescent quantification PCR (qPCR) protocols for the

64   detection of Zika virus based on the NS5 gene.

65   2. **Materials and methods**

66   *2.1 Sample processing*

67   Asian Zika virus Z16006 strain was provided by the Institute of Microbiology in the Center

68   for Disease Control and Prevention of Guangdong Province, China (GenBank no.

69   KU955589.1). Four serotypes of Dengue virus (DV1 Hawaii strain, DV2 NGC strain, DV3 H87

70   strain, and DV4 H241 strain) were preserved in our laboratory. The above viruses were all

71   amplified and cultured in C6/36 cells. After observing the cytopathic effect, the cells were

72   repeatedly frozen and thawed three times, centrifuged and frozen at -80℃. Clinically positive

73   ZIKA blood samples were obtained from the Centerfor Disease Control and Prevention of

74   Jiangmen,Guangdong Province, China.

75   *2.2 Instruments and reagents*

76   The micro-ddPCR instrument, amplification premixed reagent, Droplet PCR Supermix, and

77   micro-drop reagent were all from Bio-Rad (Hercules, CA, USA). Reverse transcription was

78   performed using a TAKARA Prime Script RT reagent Kit (Perfect Real Time kit; TaKaRa,

79   Shiga, Japan), Bestar qPCR Master Mix (TaqMan Probe) (DBI Bioscience, Germany), and a

80   QuantStudio 6 Flex Real-Time PCR System(ThermoFIsher, USA).

81   *2.3 Design of primers and probes*

82   The sequences of 30 strains of ZIKA were downloaded from GenBank and compared. After

83   identifying conserved sequences, primers and probes were designed using Primer 5.0with the

84   Z16006 strain as the reference strain. The primers and probes were designed using Oligo 7.

85   *2.4 RNA extraction and reverse transcription*

86   Samples were brought to room temperature before use. Viral RNA was extracted using the

87   QIAamp® (Qiagen, Hilden, Germany), with a final RNA volume of 50 μL. The RNA was

88   separated into aliquots and preserved at -80℃.

89   For reverse transcription, the components were prepared on ice, as follows: 2 μL

90   PrimeScript RT Enzyme Mix I, 0.5 μL Oligo dT Primer, 2 μL Random 6-mers, 4 μL total RNA,

91   and 1 μL RNase-free dH$_2$O. The reaction conditions were as follows: reverse transcription at

92   37℃ for 15 min, reverse transcriptase inactivation at 85℃ for 5 s, and a final hold at 4℃.

93   *2.5 ddPCR*

94   For ddPCR, the 20μL reaction system contained 10 μL ddPCR Supermix (no dUTP), 6 μL

95   primer probe premix (initial concentration of 10 μM upstream primer and 0.4 μL downstream

96   primer, probe 0.2 μL, deionized water 5 μL), and 4 μL nucleic acid extract. After mixing, 20

97   μL of the sample reaction system was added to the middle of a DGB cartridge. Next, 70 μL oil

98   was added to the bottom row of each lane to avoid the formation of bubbles, and the wells

99   were covered. The reaction system and droplet-forming cartridge were placed in a droplet

100   generator and subjected to micro-droplet treatment.

101   Droplets were generated in the top row of the wells, and the suction volume was adjusted

102   to 40 μL. Samples were then slowly transferred to 96-well plates, and a preheated PX1 heat

103   sealing device was used as a sealing film (with the red line up) at 180°C for 5 s. The PCR

104   conditions were as follows: predenaturation at 95°C for 10 min, temperature change rate of

105   2°C/s; and 40 cycles of 94°C for 30 s and 55°C for 1 min, at 2°C/1°C/s. The 96-well plate

106   containing the PCR-amplified products was then placed on a QX200 micro-drop reader and

107   analyzed using Quanta Soft software.

108   *2.6 Real-time fluorescent qPCR*

109   For qPCR, reactions contained Bestar qPCR Master Mix (10 μL), upstream and downstream

110   primers (0.4 μL each), probe (0.2 μL), template DNA (4 μL), and ddH$_2$O (5 μL). The

111   amplification conditions for qPCR were as follows: 95℃ denaturation for 2 min, follows by 40

112   cycles of 95℃ for 10 s and 60℃ for 30 s.

113   3. **Results**

114   *3.1 Design and evaluation of primers and probes*

115   We downloaded 30 popular ZIKA strains, compared them, and found conserved sequences.

116   Primers were designed according to the NS5 gene, keeping the GC content and Tm values as

117   similar as possible. The length of the target fragment was within 100 bp without hairpin

118   structures to avoid the formation of stable dimers and mismatches at the C-terminus. The

119   designed primers are shown in Table 1.

120   **Table 1**. Primers and probe for ZIKA detection

Primer/probe	Sequence
ZK-F	5′-GGCRTTRGCCATCAGTCG-3′
ZK-R	5′-ATGGAGCATCCGKGAGACT-3′
Probe	5′-FAM-TGGCAGCTYCTTTATTTCCACARAAG-BHQ1-3′

121

122   *3.2 Evaluation of the sensitivity of detection*

123   Ten-fold dilutions of ZIKA plasmid standards (3.2×10^{10} copies/µL) were performed.

124   Detection by qPCR showed that the standard curve R^2 value was 0.999, and the amplification

125   efficiency was 92.203%. The relationship between the Ct value and the copy number was as

126   follows: $Y = -3.524X + 38.045$, based on the standard curve (Figure 1).

6

Standard Curve

127
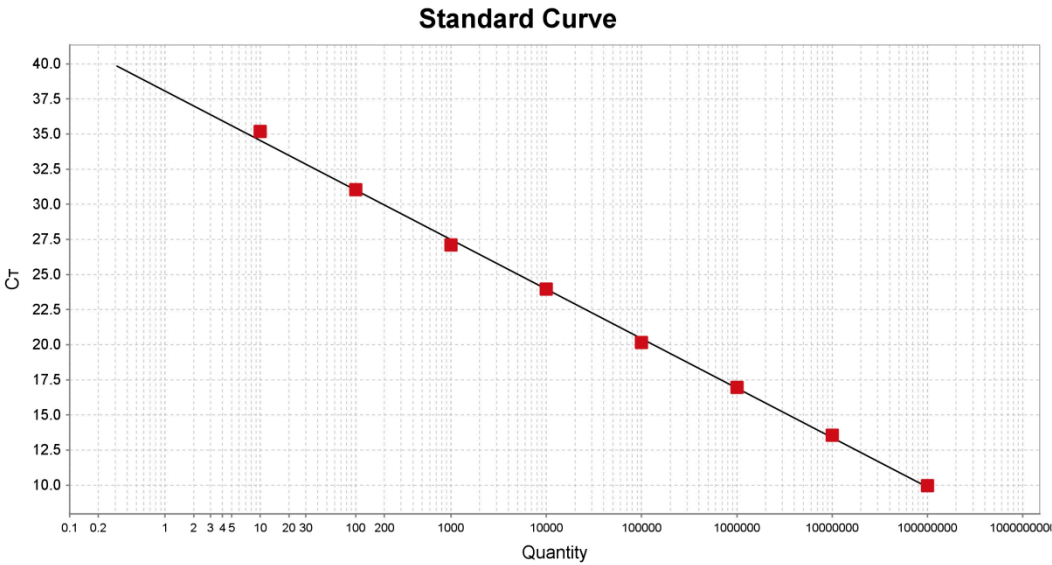
128　**Figure 1.** The standard curve for fluorescent quantitative PCR. The concentration of each

129　point on the standard curve increased 10-fold from left to right.

130　　The initial concentration of 7.8×10^6 copies/µL was gradient diluted 5-fold from the cell

131　culture ZIKA solution, and qPCR was used to test each sample in order to evaluate the

132　detection method. The results are shown in Figure 2; a detection limit of about 100 copies/µL

133　was obtained.

134
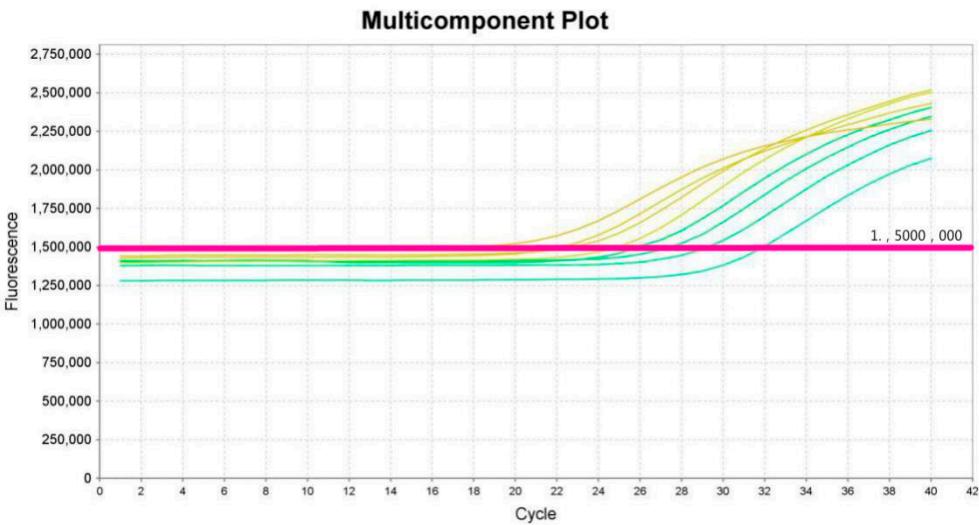
135　**Figure 2.** Results of 5-fold dilutions of ZIKA cell culture by qPCR.

7

136   Next, we used ddPCR to detect ZIKA from cell cultures, as described above for qPCR. As

137   shown in Figure 3A, qPCR detected ZIKA with clear differences among sample

138   concentrations. In Figure 3B, the threshold was set to 1000, with positive nucleic acid droplets

139   showing values above 1000 and negative nucleic acid droplets showing values below 1000. As

140   indicated in Figure 3C, almost all samples generated more than 10,000 droplets, suggesting

141   that ddPCR may be more sensitive, with a detection limit of 1 copy/µL.

142
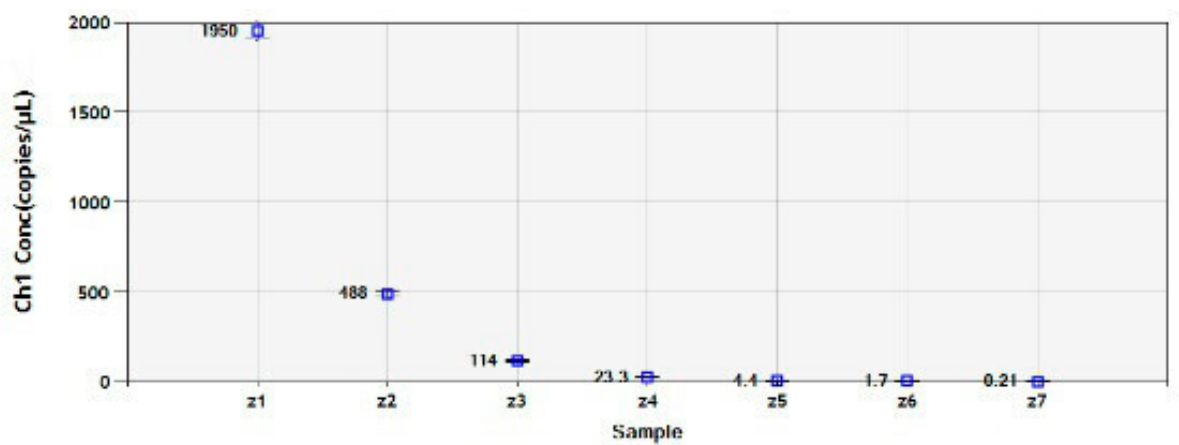
143   **Figure 3A**. Nucleic acid concentration.

144
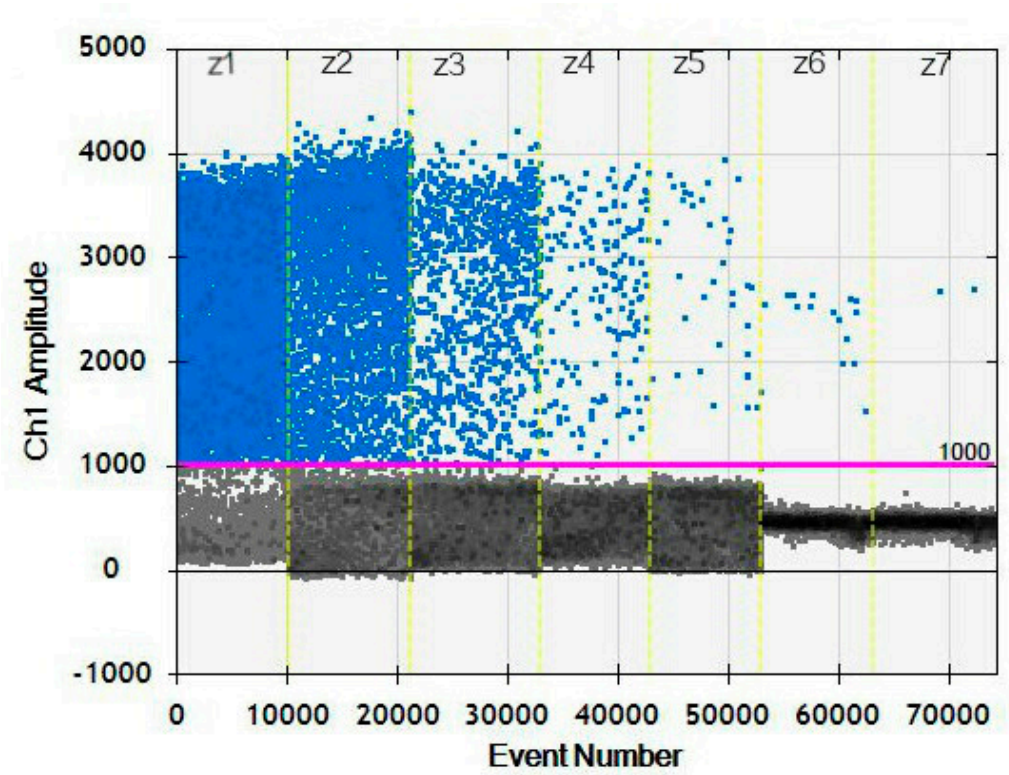
Figure 3B. Scatter plot showing event numbers.

146
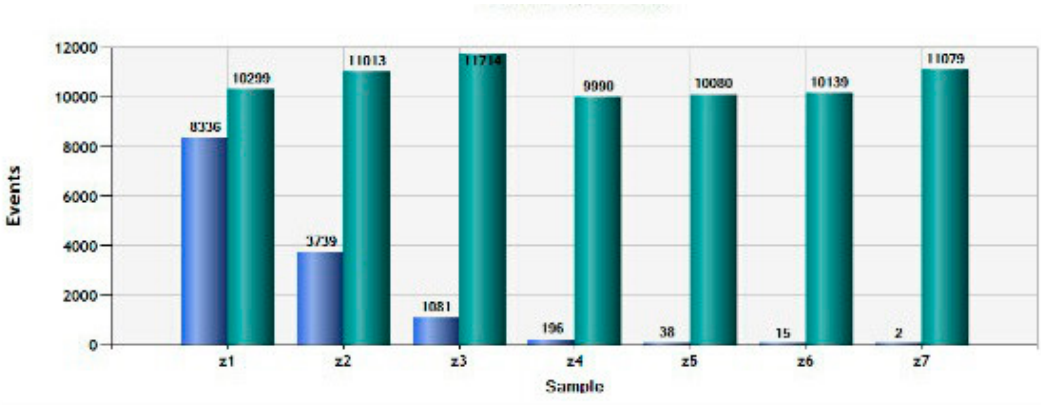
**Figure 3C.** Histogram showing event numbers.

Figure 3. Results of ddPCR for 5-fold dilutions of cell culture.

*3.3 Evaluation of the accuracy of qPCR and ddPCR*

Next, we evaluated the accuracy of the assays using four Dengue virus serotypes. All of

qPCR results were negative. Figure 4 shows the results of ddPCR. As shown in Figure 4A,

9

152   nucleic acid concentrations detected by the absolute quantification method were all zero.

153   Moreover, only negative micro-droplets were observed (Figure 4B), and histogram analysis

154   showed that the total number of droplets was more than 10,000, of which none were positive
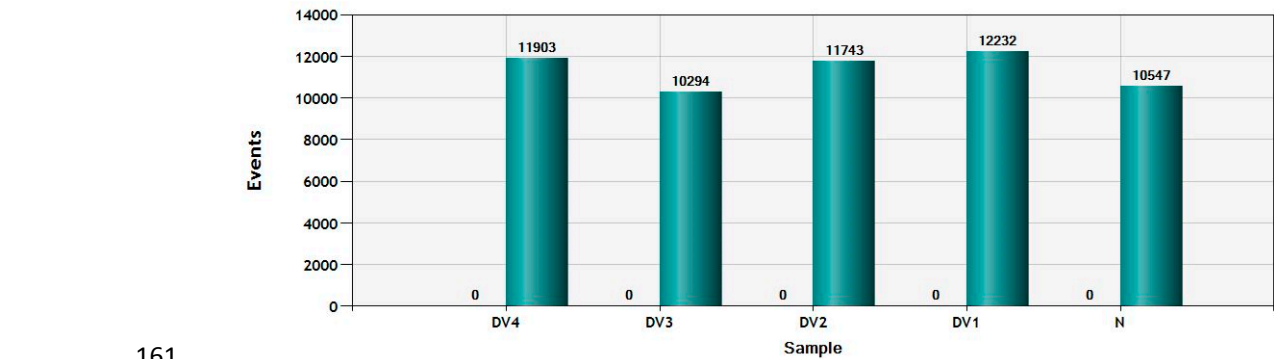
155   (Figure 4C).

156
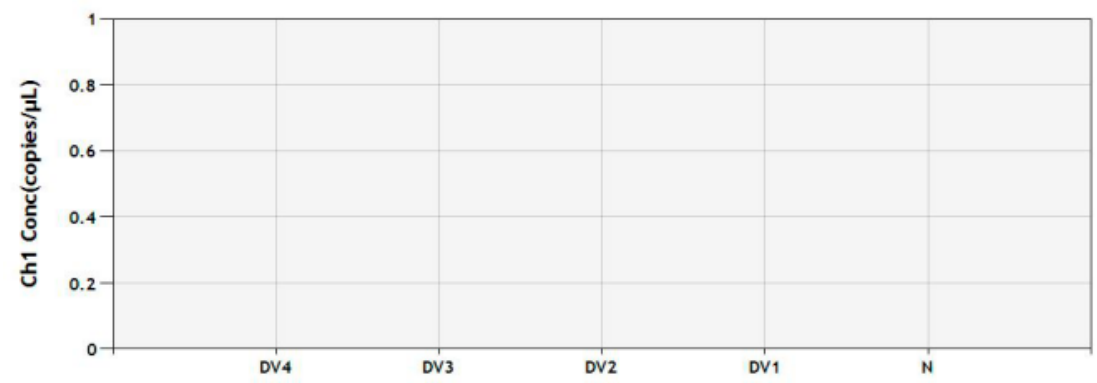
157   **Figure 4A.** Absolute quantitative detection.

158
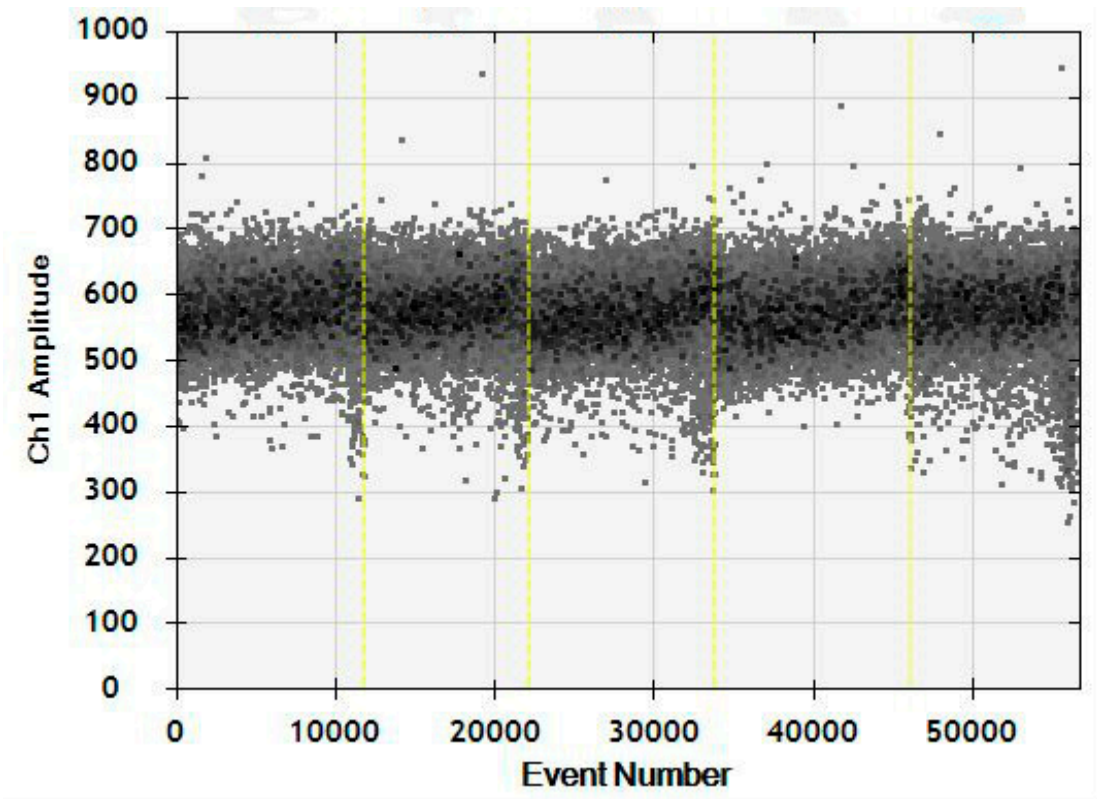
159   **Figure 4B.** Scatter plot of the total number of droplets (from left to right: DV4, DV3, DV2,

160   DV1, N; N is the negative control).

161

162   **Figure 4C.** Total number of micro-droplets, as shown in a histogram (positive on left and total

163   on right).

164   *3.4 qPCR detection of Dengue virus samples in clinically positive blood samples*

165   The lowest Ct value of clinically positive blood samples in qPCR analysis was 38.868 after

166   5-fold dilutions; this exceeded the detection range of qPCR (Ct values of 15–35; Figure 5).

167
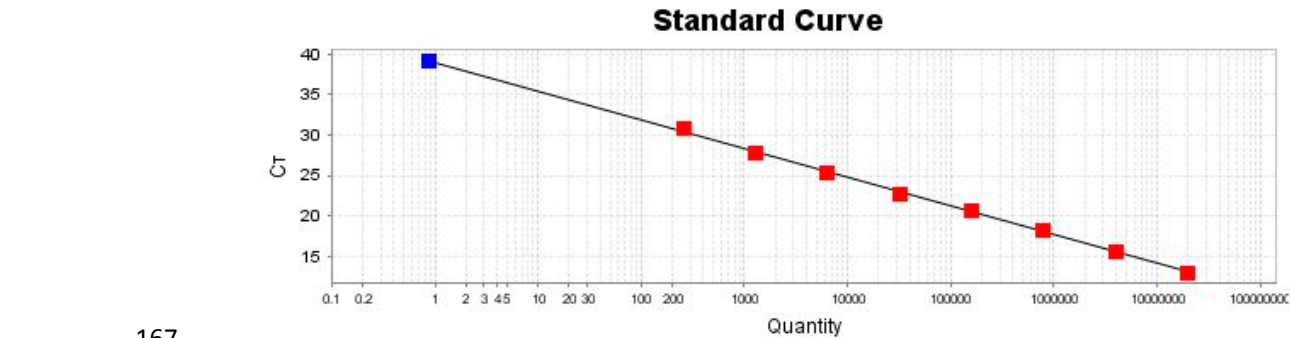
168   **Figure 5.** Standard curve of qPCR. R^2 = 0.998, amplification efficiency = 91.226%, Y = -3.552X +

169   39.06. The red dots are plasmid standards, and the blue dots are positive blood samples.

170   DdPCR analysis showed that the sample concentration was 14.2 copies/μL (Figure 6).

171   Figure 6A shows the scatter plot of the events, and Figure 6B shows the concentrations based

172   on absolute quantification. Finally, the histogram of the event number showed that the total

11

173   event number was 10923, with 131 positive events (Figure 6C).
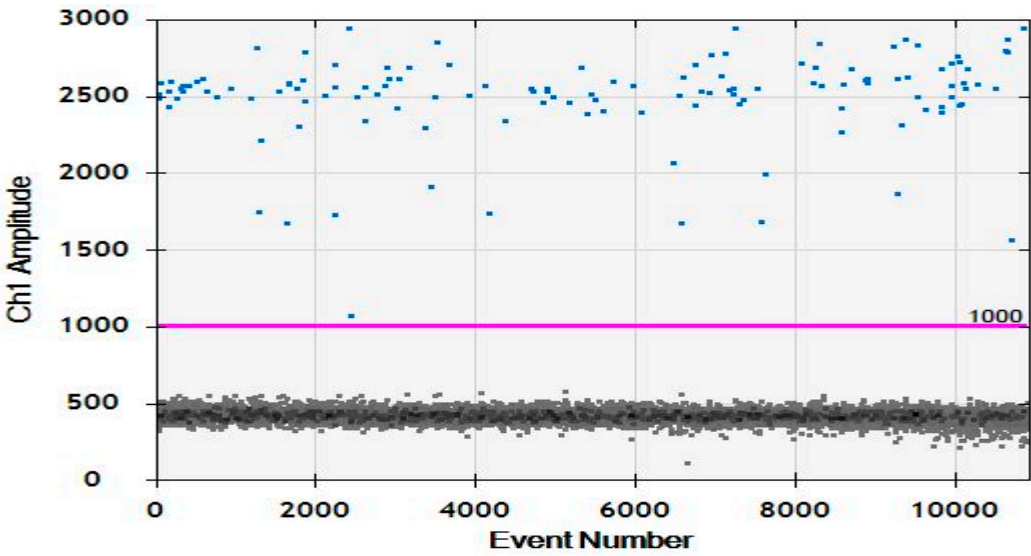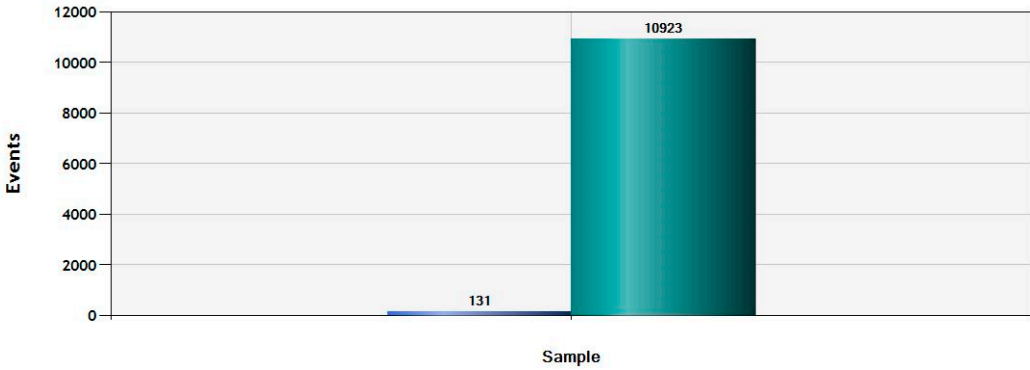

174

175   **Figure 6A.** Scatter plot showing the event numbers.
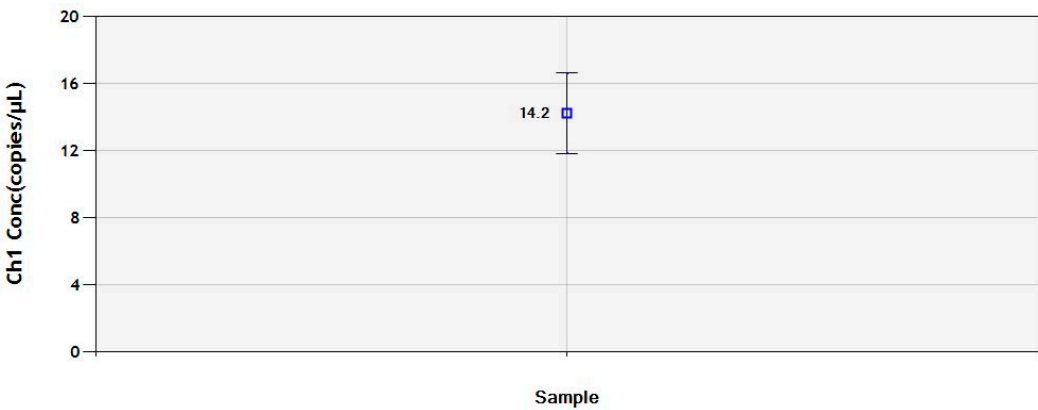
176

177   **Figure 6B.** Concentrations determined by absolute quantification.

178

179   **Figure 6C.** Histogram showing event numbers.

180   **Figure 6.** Limit of detection of ZIKA in blood samples by ddPCR.

181   **4. Discussion**

182   Approximately 20% of individuals infected with ZIKA may develop symptoms similar to

183   other flaviviruses [11], presenting a great challenge to clinical diagnosis. There are two types

184   of clinical diagnostic tests for ZIKA, serological and molecular detection. IgM specific

185   neutralizing antibodies and antibody capture enzyme-linked immunosorbent assays can be

186   used for qualitative detection of ZIKA IgM antibodies, but may crossreact with other

187   flaviviruses, making the results difficult to explain. Therefore, molecular biology diagnosis

188   has become a commonly used means of laboratory testing.

189   In molecular biology, traditional PCR can only roughly detect the amount of

190   amplification after the reaction has ended and cannot be used to quantitatively detect nucleic

191   acids in the sample. Real-time fluorescence qPCR technology utilizes changes in the

192   fluorescence intensity of chemical substances in the reaction system to realize quantitative

193   detection of nucleic acids. Xu et [1]al [14] used one-step SYBR Green real-time PCR for the

194   detection of ZIKA with a detection limit of at least 1.0 PFU/mL (1 PFU is approximately equal

195   to 2×10^5 RNA genome copies). However, this method allows the simultaneous detection of

196   both specific and nonspecific PCR products and therefore produces false positives. Calvert et

197   al. [15] used reverse transcriptase loop-mediated isothermal amplification to detect RNA from

198   ZIKA as low as 1.2 copies/µL; however, they observed a very high false-negative rate. Our

199   laboratory uses a probe-based real-time PCR method with a detection limit of approximately

200   100 copies/µL.

201   Micro-ddPCR detects nucleic acid molecules without relying on external standard curves

202   based on the Poisson distribution principle. With the advantage of absolute quantitative, this

203   method permits better accuracy at low concentrations without the need for preparing

204   standard curves [16].

205   In this study, we designed specific primers and probes based on the NS5 gene of ZIKA and

206   established a method for ZIKA nucleic acid detection using ddPCR and qPCR. Our results

207   showed that good specificity could be obtained by designing primers and probes based on

208   the NS5 gene and that these primers/probes could be used for detection the differential ZIKA

209   and four serotypes of Dengue virus. Moreover, our findings demonstrated that ddPCR had

210   good sensitivity but was inaccurate for samples with high concentrations. Thus, ddPCR may

211   be more suitable for low viral loads. Thus, ZIKA, as well as four serotypes of Dengue virus,

212   can be detected by analyzing the NS5 gene at concentrations of about $1–10^5$ copies/µL.

213   In summary, in clinical samples with low concentrations of ZIKA, such as those at 1–3 days

214   after infection, micro-droplet digital ddPCR may show the best diagnostic accuracy and

215   sensitivity, whereas in routine analyses laboratory of viral nucleic acid detection, including

216   analysis of clinical samples at more than 3 days after infection, fluorescence qPCR can be

217   used.

222   **Author Contributions :** Yuan Hui, Bao Zhang, Weiwei Xiao and Qinghua Wu conceived and designed

223   the experiments, Wei Zhao and Junhe Liang contributed reagents/materials/analysis tools; Zhiming Wu,

224   Zhiran Qin and Li Zhu performed the experiments, designed the experiments; Xujuan Li, Hanmin Han

225   and Shiyu Feng performed part of the experiments, analyzed the data and wrote the paper.

226   **Competing interests :** The authors declare no conflicts of interest.

227   **References**

228   [1] Dick, G. W. A. (1952). Zika Virus (I). Isolations and serological specificity. Transactions of the Royal
229   Society of Tropical Medicine and Hygiene, 46(5), 509–520. https://doi.org/10.1016/0035-9203(52)90042-4

230   [2] Fontes-Garfias, C. R., Shan, C., Luo, H., Muruato, A. E., Medeiros, D. B. A., Mays, E., … Shi, P. Y.
231   (2017). Functional Analysis of Glycosylation of Zika Virus Envelope Protein. Cell Reports, 21(5),
232   1180–1190. https://doi.org/10.1016/j.celrep.2017.10.016

233   [3] Faye, O., Freire, C. C. M., Iamarino, A., Faye, O., de Oliveira, J. V. C., Diallo, M., … Sall, A. A. (2014).
234   Molecular evolution of Zika virus during its emergence in the 20(th) century. PLoS Neglected Tropical
235   Diseases, 8(1). https://doi.org/10.1371/journal.pntd.0002636

236   [4] Gourinat, A. C., O'Connor, O., Calvez, E., Goarant, C., & Dupont-Rouzeyrol, M. (2015). Detection of
237   zika virus in urine. Emerging Infectious Diseases, 21(1), 84–86. https://doi.org/10.3201/eid2101.140894

238   [5] Weaver, S. C., Costa, F., Garcia-Blanco, M. A., Ko, A. I., Ribeiro, G. S., Saade, G., … Vasilakis, N.
239   (2016). Zika virus: History, emergence, biology, and prospects for control. Antiviral Research.
240   https://doi.org/10.1016/j.antiviral.2016.03.010

241   [6] Chouin-Carneiro, T., Vega-Rua, A., Vazeille, M., Yebakima, A., Girod, R., Goindin, D., … Failloux, A.
242   B. (2016). Differential Susceptibilities of Aedes aegypti and Aedes albopictus from the Americas to Zika
243   Virus. *PLoS Neglected Tropical Diseases*, *10*(3). https://doi.org/10.1371/journal.pntd.0004543

244   [7] Dupont-Rouzeyrol, M., Biron, A., OConnor, O., Huguon, E., & Descloux, E. (2016). Erratum:
245   Infectious Zika viral particles in breastmilk (The Lancet (2016) 387 (1051)). The Lancet.
246   https://doi.org/10.1016/S0140-6736(16)00663-2

247   [8] Calvet, G., Aguiar, R. S., Melo, A. S. O., Sampaio, S. A., de Filippis, I., Fabri, A., … de

248   Filippis, A. M. B. (2016). Detection and sequencing of Zika virus from amniotic fluid of fetuses with
249   microcephaly in Brazil: a case study. The Lancet Infectious Diseases, 16(6), 653–660.
250   https://doi.org/10.1016/S1473-3099(16)00095-5

251   [9] Chouin-Carneiro, T., Vega-Rua, A., Vazeille, M., Yebakima, A., Girod, R., Goindin, D., … Failloux, A.
252   B. (2016). Differential Susceptibilities of Aedes aegypti and Aedes albopictus from the Americas to Zika
253   Virus. PLoS Neglected Tropical Diseases, 10(3). https://doi.org/10.1371/journal.pntd.0004543

254 [10] Fellner, C. (2016). Zika Virus: Anatomy of a Global Health Crisis. P & T : A Peer-Reviewed Journal
255 for   Formulary   Management,   41(4),   242–53.   Retrieved   from
256 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4811255&tool=pmcentrez&rendertype=abst
257 ract

258 [11] Duffy, M. R., Chen, T.-H., Hancock, W. T., Powers, A. M., Kool, J. L., Lanciotti, R. S., … Hayes, E. B.
259 (2009). Zika virus outbreak on Yap Island, Federated States of Micronesia. *The New England Journal of*
260 *Medicine*, *360*(24), 2536–2543. https://doi.org/10.1056/NEJMoa0805715

261 [12] Rossini, G., Gaibani, P., Vocale, C., Cagarelli, R., & Landini, M. P. (2017). Comparison of zika virus
262 (zikv) rna detection in plasma, whole blood and urineÂ - case series of travel-associated zikvÂ infection
263 imported to italy, 2016. Journal of Infect, 75(3), 242-245.https://doi.org/10.1016/j.jinf.2017.05.021

264 [13] Hayes, E. B. (2009). Zika virus outside Africa. *Emerging Infectious Diseases*.
265 https://doi.org/10.3201/eid1509.090442

266 [14] Xu, M. Y., Liu, S. Q., Deng, C. L., Zhang, Q. Y., & Zhang, B. (2016). Detection of Zika virus by SYBR
267 green   one-step   real-time   RT-PCR.   Journal   of   Virological   Methods,   236,   93–97.
268 https://doi.org/10.1016/j.jviromet.2016.07.014

269 [15] Calvert, A. E., Biggerstaff, B. J., Tanner, N. A., Lauterbach, M., & Lanciotti, R. S. (2017). Rapid
270 colorimetric detection of Zika virus from serum and urine specimens by reverse transcription
271 loop-mediated   isothermal   amplification   (RT-LAMP).   PLoS   ONE,   12(9).
272 https://doi.org/10.1371/journal.pone.0185340

273 [16] Strain, M. C., Lada, S. M., Luong, T., Rought, S. E., Gianella, S., Terry, V. H., … Richman, D. D. (2013).
274 Highly Precise Measurement of HIV DNA by Droplet Digital PCR. *PLoS ONE*, *8*(4).
275 https://doi.org/10.1371/journal.pone.0055943