Electronic Supplementary Material (ESI) for ChemComm.

• Supplementary Information

Ultrafast and One-step Assay for Visual Detection of RNA Virus

Cuiping Ma[a], Hao Jing[a], Pansong Zhang[a], Lingzhi Han[c], Meiling Zhang[c], Fuxin Wang[a], Shuyan Niu[c] and Chao Shi[*,b]

[a]*College of Marine Science and Biological Engineering, Qingdao University of Science and Technology, Qingdao, 266042, PR China.*

[b]*College of Life Sciences, Qingdao University, Qingdao, 266071, PR China.*

[c]*Key Laboratory of Sensor Analysis of Tumor Marker, Ministry of Education, College of Chemistry and Molecular Engineering, Qingdao University of Science and Technology, Qingdao, 266042, PR China*

.

Correspondence and requests for materials should be addressed to (sc169@163.com).

Materials and methods:

Materials

All primers (Table S1 and Table S2) were designed and synthesized by NUPACK software (http://www.nupack.org/) and Sangon Biotech (Shanghai, China), respectively. The *Bst* 2.0 WarmStartTM DNA polymerase (8 U/μL) and *Bsr* I endonuclease (10 U/μL) were purchased from New England Biolabs. 20 × Eva Green and 10000 × SYBR Green I were purchased from Bridgen (Beijing, China) and Solarbio (Beijing, China), respectively. All chemicals were of analytical grade unless indicated otherwise.

Methods

The optimized ASEA reaction was carried out in 10 μL mixture containing 1.0×10^{-6} M P1 and P2, 1.0×10^{-7} M P3, 5.0×10^{-4} M dNTPs, 0.5×Eva Green, 0.1 μL *Bst* 2.0 WarmStart™ DNA polymerase, 0.2 μL *Bsr* I and 1 × ThermoPol buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, pH8.8 @ 25℃). The reaction was incubated at 65℃ for 25 min. Control (NTC) without target was used as a negative control. The ASEA reaction was performed in a CFX96™ Real-Time PCR detection system (Bio-Rad) at 1-min intervals. After the amplification reaction was finished, a final concentration of 40 × SYBR Green I placed on the lid in advance was mixed to reaction system for visual detection by spinning with hand.

Supplementary Figures

Comparison of ASEA and SEA-EXPAR

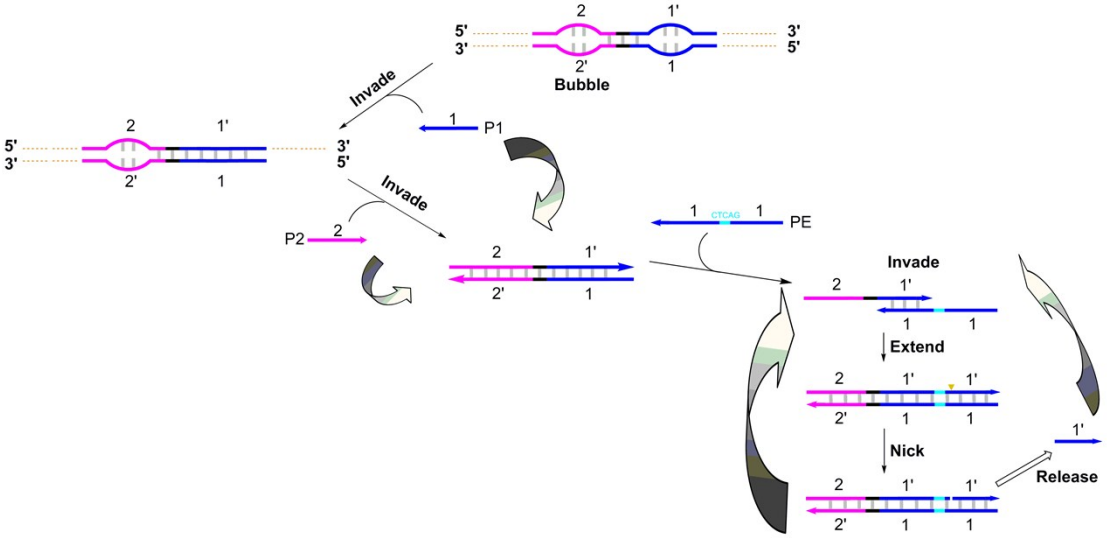

Figure S1 The schematic diagram of SEA-EXPAR. Different domains of DNA were marked with the numbers. Numbers marked with a prime symbol (') were complementary to the corresponding unmarked number. All sequences used in this work were listed in Table S1.

In 2003, the exponential amplification reaction (EXPAR) that is an exponential isothermal amplification reaction was reported[14]. So, we tried to combine EXPAR with SEA to improve the sensitivity of SEA, designated SEA-EXPAR. The experimental principle was shown in Figure S1. The different concentrations of targets were respectively used to trigger ASEA and SEA-EXPAR reactions. The ASEA and SEA-EXPAR reactions were performed in a CFX96^{TM} Real-Time PCR detection system (Bio-Rad) at 1-min intervals. When 1.0×10^{-15} M Zika virus RNA was respectively used to trigger ASEA and SEA-EXPAR reactions under their optimum conditions, the fluorescence intensity of ASEA began to significantly increase at 18 min, while SEA-EXPAR required about 32 min (Figure S2A). We therefore could conclude that ASEA had a higher reaction rate than that of SEA-EXPAR, which was very encouraging to point-of-care testing (POCT) field for its rapidity. In addition, we further detected different concentrations of Zika virus RNA by ASEA and SEA-EXPAR methods. As shown in Figure S2B, both ASEA and SEA-EXPAR methods were capable of detecting different concentrations of Zika virus RNA ranging

from 1.0×10^{-11} M to 1.0×10^{-15} M. However, the fluorescence intensity of the negative control of ASEA was lower than that of SEA-EXPAR, which was consistent with Figure S2A. Thus, ASEA method was chosen to perform the detection of Zika virus RNA in this work owing to its rapidity and high specificity.

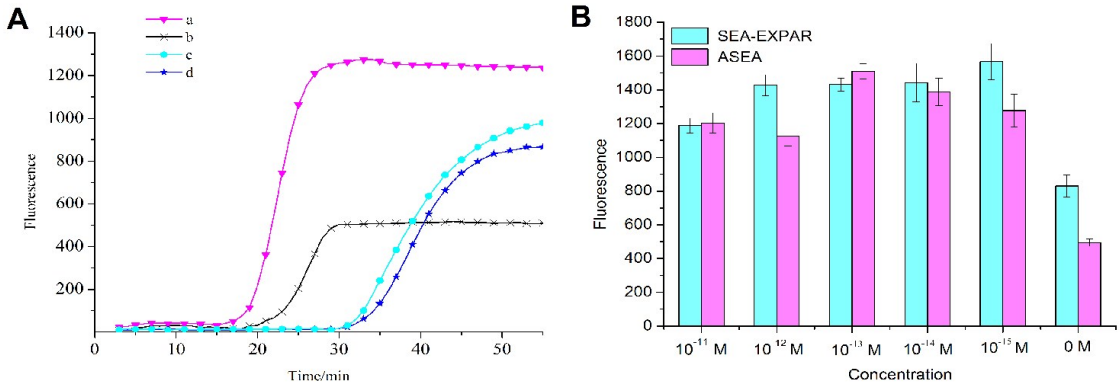

Figure S2 Comparison of ASEA and SEA-EXPAR. A. The real-time fluorescence curves of 1.0×10^{-15} M Zika virus RNA by ASEA and SEA-EXPAR methods. NTC was no target control. a 1.0×10^{-15} M Zika virus RNA for ASEA b NTC of ASEA c 1.0×10^{-15} M Zika virus RNA for SEA-EXPAR d NTC of SEA-EXPAR B. The detection of different concentrations of Zika virus RNA by ASEA and SEA-EXPAR methods. 10-fold dilution of Zika virus RNA was respectively used to trigger ASEA and SEA-EXPAR, and the reaction was respectively incubated for 25 and 45 min. The ASEA was carried out under the optimum conditions. The EXPAR was performed in 10 μL containing 1.0×10^{-6} M P1 and P2, 1.0×10^{-7} M P3, 5.0×10^{-4} M dNTPs, 0.5×Eva Green, 0.8 U *Bst* 2.0 WarmStart™ DNA polymerase, 4 U *Nt.BstNB* I, 1×ThermoPol buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, pH8.8 @ 25℃), 0.5×Buffer 3.1 (25 mM Tris-HCl, 50 mM NaCl, 5 mM $MgSO_4$, 0.5 mM dithiothreitol, pH 7.9 @ 25℃) and a final concentration of 0.25×Eva green.

Feasibility of ASEA method

The results of real-time fluorescence and visual detection of *Vibrio parahaemolyticus* (*V. parahemolyticus*) genomic DNA by ASEA method were shown in Figure S3. When 1.0×10^{-13} M and 1.0×10^{-14} M *V. parahemolyticus* genomic DNA were respectively added to reaction system and incubated at 65℃, the corresponding fluorescence intensities significantly increased compared with their no target controls (NTC), and their fluorescence curves could be well distinguished. DNA visual detection was also performed according to experimental protocol described in the Experimental Section and the colorimetric result was shown in Figure S3 inset. The positive reactions showed significant changes in color compared with the negative reaction.

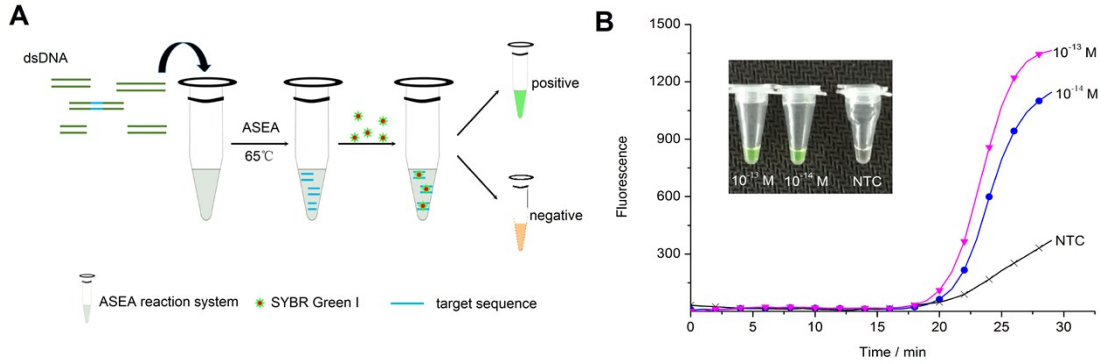

Figure S3 The sensitive, isothermal, and one-step biosensor for *V.parahaemolyticus* genomic DNA detection. A. The schematic illustration of utilizing isothermal ASEA method to detect *V.parahaemolyticus* genomic DNA. B. The real-time fluorescence curve for different concentrations of *V.parahaemolyticus* genomic DNA. NTC was no target control. Inset, the visual detection of *V.parahaemolyticus* genomic DNA. The positive was green and the negative was orange.

Detecting different concentrations of Zika virus RNA by ASEA method

We further performed different concentrations of RNA detection to investigate the feasibility of ASEA. We found that all chosen concentrations could activate ASEA reactions with as little as 1.0×10^{-15} M (Figure S4A). To obtain visual detection, a final concentration of 40×SYBR Green I was placed inside the lid of the reaction tube beforehand, and then mixed to the above-described reaction systems with no need of decapping. The colorimetric results were shown in Figure S4B. The color of the positive samples was changing from orange to green, while the color of the negative result was still orange. Therefore, all chosen concentrations of Zika virus RNA with as little as 1.0×10^{-15} M could be well distinguished from the negative control not only by fluorescence but also colorimetric method.

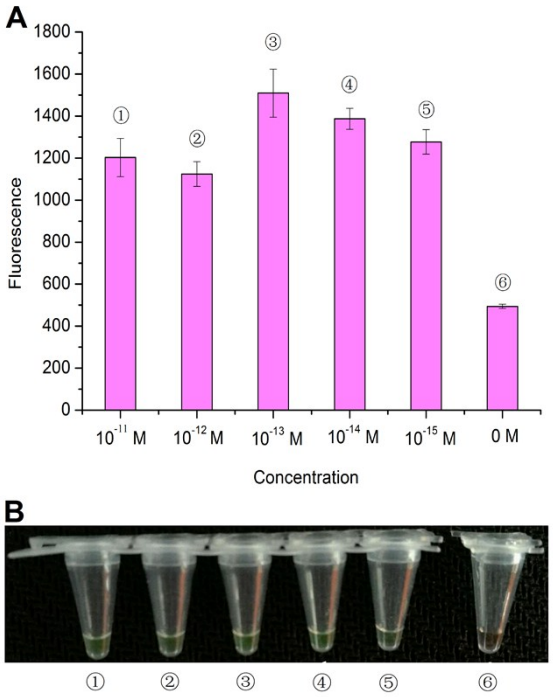

Figure S4 Detection of different concentrations of Zika virus. A. Serial 10-fold dilutions of Zika virus RNA were used to trigger ASEA method and incubated for 25 min, followed by fluorescence detection. Error bars represented SD from three replicates. B. Colorimetric detection of different concentrations of Zika virus RNA. The concentrations of Zika virus RNA were ① 1.0×10^{-11} M, ② 1.0×10^{-12} M, ③ 1.0×10^{-13} M, ④ 1.0×10^{-14} M, ⑤ 1.0×10^{-15} M and ⑥ 0 M, respectively.

Optimization of assay conditions for Zika virus RNA detection

To investigate the effect of the *Bst* 2.0 WarmStart™ DNA polymerase used on ASEA, the real-time fluorescence curve produced by 1.0×10^{-13} M Zika virus RNA was measured by using 0.1 μL ,0.2 μL,0.3 μL, and 0.4 μL *Bst* 2.0 WarmStart™ DNA polymerase, respectively. As shown in Figure S5, the fluorescence intensities of NTC were increased with the increase of *Bst* 2.0 WarmStart™ DNA polymerase, which was not beneficial to distinguish the positive sample and NTC. Notably, there was a great change of fluorescence intensity between the positive sample and NTC, when 0.1 μL DNA polymerase was added to the 10.0 μL reaction system. Therefore, 0.1 μL DNA polymerase was used in the following experiments.

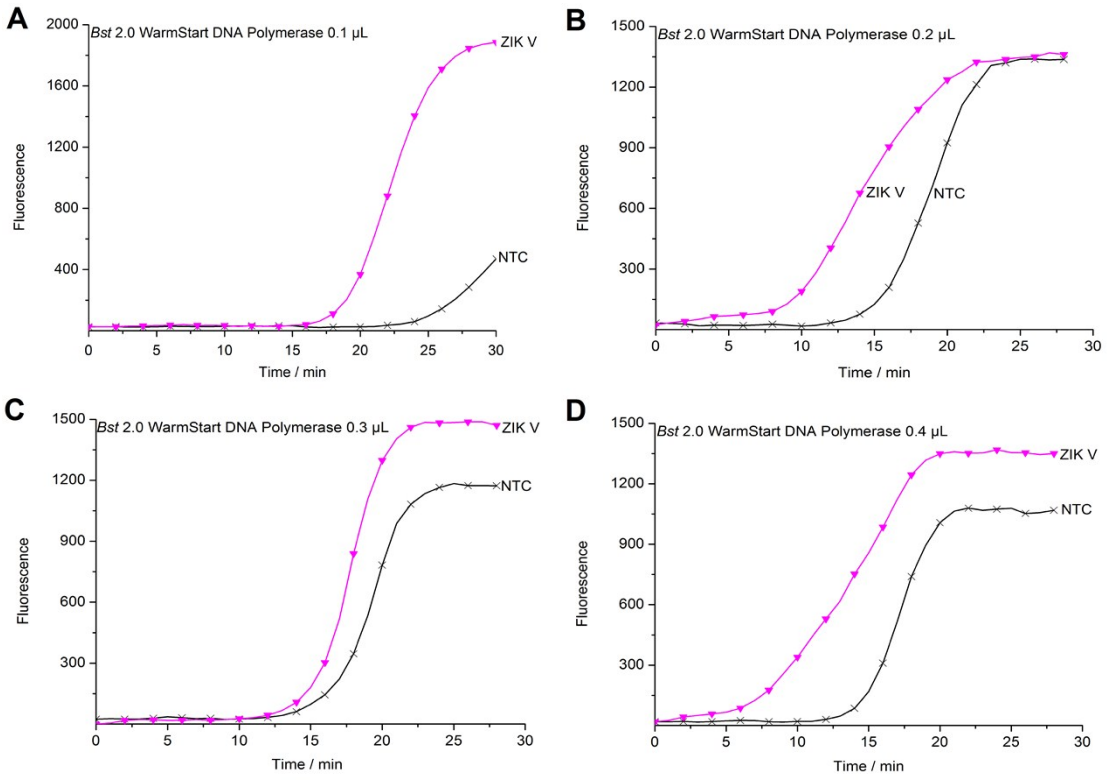

Figure S5 The effect of the *Bst* 2.0 WarmStart™ DNA polymerase on ASEA method. The real-time fluorescence curves were initiated by 1.0×10^{-13} M Zika virus RNA and were carried out according to the experimental procedure described in the Experimental Section.

The effect of the *Bsr* I endonuclease on ASEA for Zika virus RNA detection was also investigated. Just like Figure S5, 1.0×10^{-13} M Zika virus RNA was used as target. 0.1 μL, 0.2 μL, 0.3 μL, and 0.4 μL *Bsr* I endonuclease were respectively added to 10.0 μL reaction systems. As

shown in Figure S6, the positive samples and their NTC couldn't be well distinguished, when 0.1 μL, 0.3 μL and 0.4 μL *Bsr* I endonuclease were added to the reaction system, respectively. On the contrary, 0.2 μL *Bsr* I endonuclease was added to the reaction system and the positive sample was well distinguished from NTC. Therefore, 0.2 μL *Bsr* I endonuclease was selected for the following experiments.

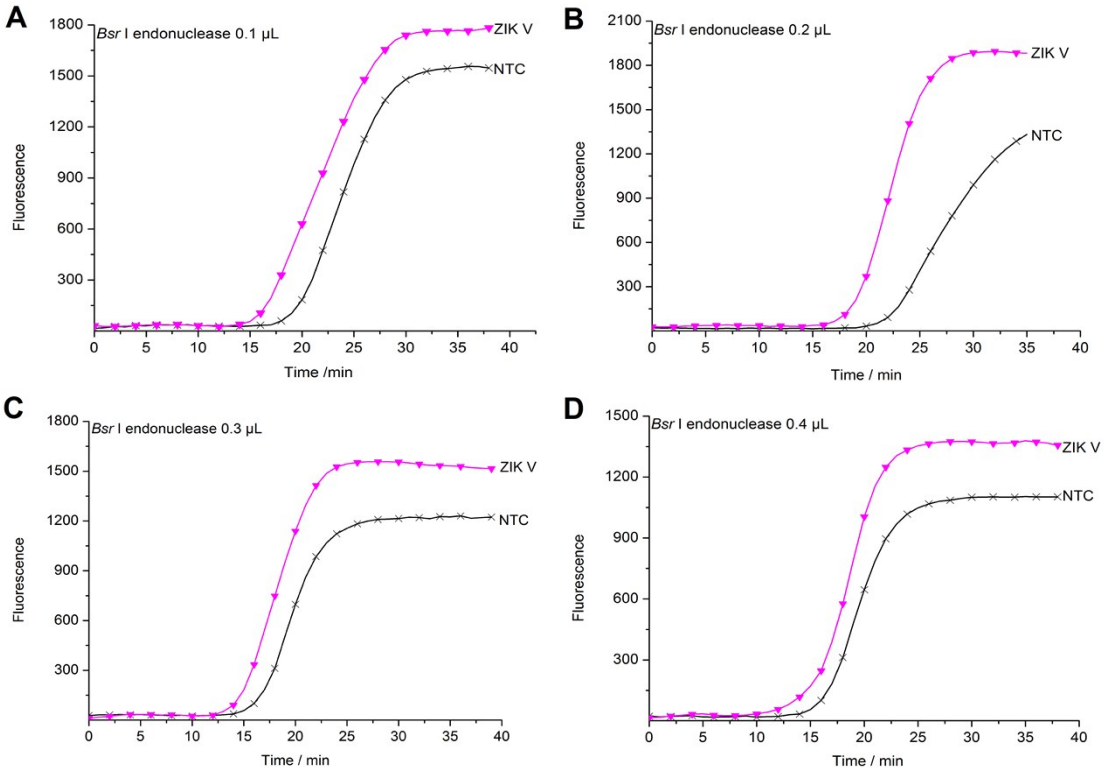

Figure S6 The effect of the *Bsr* I endonuclease on ASEA method. The real-time fluorescence curves were initiated by 1.0×10^{-13} M Zika virus RNA and were carried out according to the experimental procedure described in the Experimental Section.

Specificity of ASEA method

To further demonstrate the specificity of ASEA, Dengue virus (GenBank: AF326573), Japanese Encephalitis virus (GenBank: EF623988), and Zika virus including Asian (GenBank: KX266255) and African strains (GenBank: AY632535, strain MR766) genomic sequence alignments were performed using CLUSTALW for multiple alignments by BioEdit software because that they share substantial similarity genome regions (Figure S7). The boxed regions were selected to design specificity primer sets of ASEA corresponding to different targeted sequences (Table S2).

Figure S7 Sequence alignments of Zika virus (GenBank: KX266255, strain SMGC; GenBank: AY632535, strain MR766), Dengue virus (GenBank: AF326573) and Japanese Encephalitis virus (GenBank: EF623988) genomic regions.

The specificity of ASEA method was investigated with synthetic DNA corresponding to target sequences from Zika African lineage (GenBank: AY632535), Dengue virus (GenBank: AF326573) and Japanese Encephalitis virus (GenBank: EF623998), which have high homology with Zika viral target (GenBank: KX266255, strain SMGC). As shown in Figure S8, ASEA primer sets for specificity to their targeted sequences were successful to activate the ASEA reactions with high fluorescence intensity. Again, we saw no or a little fluorescence increase corresponding to their non-targeted sequences, demonstrating robust sequence specificity of ASEA method in fluorescent detection.

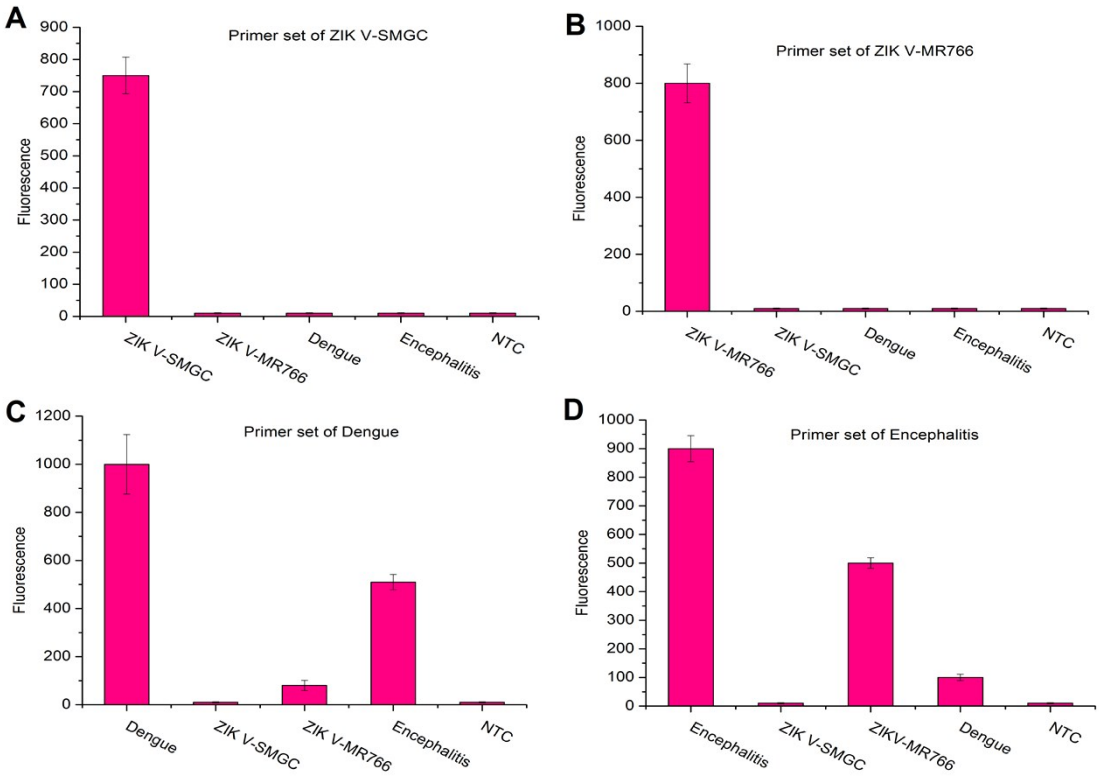

Figure S8 The specificity of ASEA method. The target concentration corresponding to different primer sets was 1.0×10^{-13} M . The ASEA reaction was incubated under their optimum temperatures for 25 min. All sequences of primer sets were shown in Table S2. Error bars represented SD using three replicates. A. 65℃ B. 65℃ C. 61℃ D. 62℃

Supplementary Table

Table S1. Sequences of primer sets for ASEA and SEA-EXPAR methods

Primer set	Sequence (5' — 3')
ASEA method	
P1	5' —AAGTCGACGATTTCTGTTCCAGAG— 3'
P2	5' —GACAACAGCAGTCAATGTCACC— 3'
P3	5' —AAGTCGACGATTTCTGTTCTCCAGTAAGTCGAC GATTTCTGTTCG— 3'
SEA-EXPAR method	
P1	5' —AAGTCGACGATTTCTGTTCCAGAG— 3'
P2	5' —GACAACAGCAGTCAATGTCACC— 3'
P3	5' —AAGTCGACGATTTCTGTTCCGCTTGACTCAAGTC GACGATTTCTGTTCC— 3'

Table S2. Sequences of four primer sets for the specificity of ASEA method

Primer set	Sequence (5' — 3')
ZIK V-SMGC	
P1	5' —AAGTCGACGATTTCTGTTCCAGAG— 3'
P2	5' —GACAACAGCAGTCAATGTCACC— 3'
P3	5' —AAGTCGACGATTTCTGTTCTCCAGTAAGTCGAC GATTTCTGTTCG— 3'
ZIK V-MR766	
P1	5' —GACAACAGCAGTCAACGTCAC— 3'
P2	5' —TCAAATCAACGATTTCTGTCCCAGAA— 3'
P3	5' —TCAAATCAACGATTTCTGTCCTCCAGTTCAAAT CAACGATTTCTGTCCG— 3'
Dengue	
P1	5' —GACCCCAGCTGTGAAATCAGAA— 3'
P2	5' —GAGGTCTACAATCTCTCTTCCTGTG— 3'
P3	5' —GAGGTCTACAATCTCTCTTCTCCAGTGAGGTCTA CAATCTCTCTTCG— 3'
Encephalitis	
P1	5' —AACTTCAGCAGTGCAGAGAGAG— 3'
P2	5' —TCACATCCACTATTTCATTCCCTTGG— 3'
P3	5' —TCACATCCACTATTTCATTCCTCCAGTTCACATC CACTATTTCATTCCG— 3'