

PRIMER AND PROBE SET FOR DETECTING ZIKA VIRUS AND KIT THEREOF

FIELD OF THE INVENTION

5 **[0001]** The present invention relates to a nucleic acid detection method for Zika virus, and in particular, to a primer and probe set for detecting Zika virus and a kit thereof.

BACKGROUND OF THE INVENTION

10 **[0002]** Zika virus (ZIKV) is a single-stranded RNA, mosquito-borne flavivirus, it was first isolated in Uganda's Zika forest in 1947 from a rhesus monkey (Dick *et al*, Trans R Soc Trop Med Hyg 1965; 46:509-20). The first ZIKV epidemic was documented in Yap, Federated States of Micronesia at 2007, it was estimated that 73% of the babies that were three years old or younger had been infected by ZIKV. In May 2015, World Health Organization (WHO) reported the first local transmission of ZIKV in Americas, 16 patients from Brazil were reported ZIKV-positive.

15 **[0003]** Only one in five people infected with ZIKV will show symptoms like mild fever, headache, conjunctivitis, myalgia, and cutaneous maculopapular rash. Due the mild symptoms of ZIKV infection, patients diagnosed with ZIKV were usually told to rest and increase fluid intake for a week or two until the symptoms disappear (Duffy *et al*, N Engl J Med 2009; 360:2536-43). In 2015, an outbreak in Brazil happened where ZIKV RNA was identified from a significant numbers of infants born with microcephaly and from fetal losses in women who were infected during pregnancy (Zanluca *et al*, Mem Inst Oswaldo Cruz 2015; 110:569-72). Other neurological complications associated with ZIKV are still in investigation, although there was a scientific consensus on the relation between Zika virus infection and Guillain-Barré syndrome (Araujo *et al*, Arq Neuropsiquiatr 2016; 74(3):253-5).

25 **[0004]** Zika virus infection is diagnosed using polymerase chain reaction (PCR) through virus isolated from blood samples (CDC, Triplex Real-time RT-PCR Assay, Atlanta, GA: US Department of Health and Human Services 2016). Current diagnosis using serology methods were difficult as the virus might cross-react with other flavivirus such as Dengue, West Nile, and Yellow Fever (Lyle *et al*, N Engl J Med 2016; 374:1552-1563).

30 **[0005]** There is still a need for a nucleic acid detection method capable of specifically detecting Zika virus.

SUMMARY OF THE INVENTION

[0006] In one aspect, the present invention provides a primer and probe set for detecting Zika virus, comprising a forward primer of SEQ ID NO: 1, a reverse primer of SEQ ID NO: 2, and a probe of SEQ ID NO: 3.

5 [0007] In another aspect, the present invention provides a kit for detecting Zika virus, comprising said primer and probe set.

[0008] The forward primer is an oligonucleotide having the sequence 5'-ACCTCCGAC TGATGGCCAATGCC-3' (SEQ ID NO: 1). The reverse primer is an oligonucleotide having the sequence 5'-GGGTCTTGTCTTCCATGTGG-3' (SEQ ID NO: 2). The probe is an
10 oligonucleotide having the sequence 5'-CCTGGTCAATCCATGGAAAGGGAGAATGG-3' (SEQ ID NO: 3).

[0009] In certain preferred embodiments of the present invention, a first fluorescence group and a first fluorescence quenching group are bound to the 5' end and the 3' end of the probe, respectively.

15 [0010] According to the present invention, the primer and probe set may be used for detecting an RNA sample from the blood serum.

[0011] In one embodiment of the present invention, the primer and probe set is used in RT-qPCR.

[0012] According to certain preferred embodiments of the present invention, the kit further
20 comprises a primer and probe set for internal control. In one preferred embodiment of the present invention, the kit further comprises a positive control sample and a negative control sample.

[0013] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] The foregoing summary, as well as the following detailed description of the invention, will be better understood when read in conjunction with the appended drawing. In the drawings:

30 [0015] Fig. 1 shows the detection of Zika virus RNA using a primer and probe of the present invention, and the interference to the fluorescent group (FAM) of the primer and probe specific to Zika virus by the fluorescent group (HEX) of the primer and probe for detecting internal

control gene MS2. “MS2”: MS2 RNA + MS2 primer and probe + primer and probe specific to Zika virus of the present invention; “Zika”: Zika virus RNA + MS2 primer and probe + primer and probe specific to Zika virus of the present invention; and “Mix”: MS2 RNA + Zika virus RNA + MS2 primer and probe + primer and probe specific to Zika virus of the present invention.

DESCRIPTION OF THE INVENTION

[0016] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by a person skilled in the art to which this invention belongs. The following detailed description is exemplary and explanatory only and is not to be construed as limiting the invention.

[0017] In one aspect, the present invention provides a primer and probe set for detecting Zika virus, comprising: a forward primer of SEQ ID NO: 1, a reverse primer of SEQ ID NO: 2, and a probe of SEQ ID NO: 3.

[0018] In another aspect, the present invention provides a kit for detecting Zika virus, comprising the aforementioned primer and probe set.

[0019] The forward primer is an oligonucleotide having the sequence 5'-ACCTCCGAC TGATGGCCAATGCC-3' (SEQ ID NO: 1). The reverse primer is an oligonucleotide having the sequence 5'-GGGTCTTGTCTTCCATGTGG-3' (SEQ ID NO: 2). The probe is an oligonucleotide having the sequence 5'-CCTGGTCAATCCATGGAAAGGGAGAATGG-3' (SEQ ID NO: 3).

[0020] In certain preferred embodiments of the present invention, a first fluorescence group and a first fluorescence quenching group are bound to the 5' end and the 3' end of the probe, respectively.

[0021] In one embodiment of the present invention, the first fluorescence group is FAM, and the first fluorescence quenching group is TAMRA. However, the present invention is not limited thereto.

[0022] According to the present invention, the primer and probe set may be used for detecting an RNA sample from the blood serum. In one embodiment of the present invention, the RNA sample is extracted from the blood serum sample of a subject via a conventional method, and then detected using the primer and probe set.

[0023] In one embodiment of the present invention, the primer and probe set is used in RT-qPCR.

[0024] According to certain preferred embodiment of the present invention, the kit further comprises a primer and probe set for internal control.

[0025] In one preferred embodiment of the present invention, the kit further comprises a positive control sample and a negative control sample. For example, the positive control sample may be a whole genome synthetic RNA of Zika virus, and the negative control sample may be double distilled water.

[0026] In certain embodiments of the present invention, the primer and probe set for internal control is able to specifically recognize MS2 bacteriophages, the primer and probe set including the following primers and probe: a forward primer of SEQ ID NO: 4, a reverse primer of SEQ ID NO: 5, and a probe of SEQ ID NO: 6.

[0027] According to one embodiment of the present invention, a second fluorescence group and a second fluorescence quenching group are bound to the 5' end and the 3' end of the probe of SEQ ID NO: 6, respectively. For example, the second fluorescence group is HEX, and the second fluorescence quenching group is TAMRA.

[0028] The primer and probe set for detecting Zika virus or the kit for detecting Zika virus of the present invention can be used in one step quantitative real time polymerase chain reaction (RT-qPCR), so as to detect Zika virus. The one step RT-qPCR mainly utilizes fluorescein dye and polymerase reaction to monitor the amount of reaction product in real time. The one step RT-qPCR allows direct performing RT-qPCR on the RNA of Zika virus in the blood serum with reverse transcription step done simultaneously. Comparing to common RT-qPCR, the method described herein has the advantages of being faster, easier in operation and more sensitive.

[0029] The present invention is further illustrated by the following examples, which are provided for the purpose of illustration rather than limitation.

[0030] Experimental conditions and methods not specified in the following examples generally follow conventional conditions, such as those described in Molecular Cloning: A Laboratory Manual (Sambrook *et al.*), or follow the instructions provided by manufacturers.

[0031] **Examples**

[0032] **Example 1: One step real time quantification of Zika virus by fluorescence detection method.**

[0033] **1.1 Materials**

[0034] Solutions of primers and probes (each reaction tube, if added), a forward primer of SEQ ID NO: 1 (10 μ M, 1.0 μ L) , a reverse primer of SEQ ID NO: 2 (10 μ M, 1.0 μ L), a probe of SEQ ID NO: 3 (10 μ M, 0.5 μ L)), a forward primer of SEQ ID NO: 4 (10 μ M, 1.0 μ L), a reverse primer of SEQ ID NO: 5 (10 μ M, 1.0 μ L) and a probe of SEQ ID NO: 6 (10 μ M, 0.5 μ L), 10.0 μ L of TaqMan® RNA-to-Ct™ 1-Step Mix reaction solution, 0.5 μ L of reverse transcriptase and 3.5 μ L ddH₂O were used. The positive control sample is a whole genome, synthetic RNA of Zika virus. The negative control sample (non-template control, NTC) is double distilled water. The internal control is MS2 bacteriophage whole genome RNA. FAM and TAMRA were bound to the 5' end and the 3' end of the probe of SEQ ID NO: 3, respectively. HEX and TAMRA were bound to the 5' end and the 3' end of the probe of SEQ ID NO: 6, respectively.

[0035] 1.2 Methods

[0036] The preparation of positive control sample of Zika virus RNA is described as follows: Whole genome synthetic Zika virus RNA was extracted and purified. RNA concentration was measured by the spectrophotometer for absorbance at 260 nm (A₂₆₀), and the RNA copy number was calculated. The RNA sample was diluted by sterile water to 10⁸-10⁵ replicates/mL replicons/mL as a standard sample. 5.0 μ L of the negative control samples and the Zika virus positive control samples, at different concentrations, were respectively added with 10.0 μ L of polymerase reaction solution and the various primer and probe solutions. Then, the total reaction volume of each reaction tube was adjusted to 20 μ L by adding double distilled water (ddH₂O). Experiments were made in triplicate for each concentration. The fluorescence detection is performed by a RT-qPCR machine, wherein the instrumental condition is set as follows: a preparatory procedure for 1 cycle at 48 °C for 15 minutes and 95°C for 10 minutes. Then a 45-cycle amplification reaction is performed at 95°C for 15 seconds and 60°C for 1 minute. Finally, maintaining at 95 °C for 15 seconds and 60°C for 1 minute, and a melting curve test is performed at an increasing rate of 0.15°C per second to 95°C.

[0037] 1.3 Results

[0038] Experiments were made in triplicate, and the results are shown in Fig. 1. The obtained Ct values for each group were subjected to statistical analysis. The difference between groups (inter-assay; CV%< 4) and the difference within group (intra-assay; CV%< 2) were small. In Fig. 1, the sample of “MS2” group includes MS2 RNA + MS2 primer and probe + primer and probe specific to Zika virus of the present invention. The sample of “Zika” group includes Zika virus RNA + MS2 primer and probe + primer and probe specific to Zika virus of the present

invention. The sample of “Mix” group includes MS2 RNA + Zika virus RNA + MS2 primer and probe + primer and probe specific to Zika virus of the present invention. The results of “MS2” group show that the primer and probe specific to Zika virus of the present invention does not specifically recognize MS2 RNA. The results of “Zika” group show that the MS2 primer and probe does not affect the primer and probe specific to Zika virus of the present invention. The results of “Mix” group show that the fluorescence (HEX) of MS2 probe does not interfere the fluorescence (FAM) of Zika virus probe.

[0039] Example 2: Computer analysis for specificity to Zika virus

[0040] The primer and probe of the present invention is analyzed by Clustal Omega software which performs a comparison analysis of the primer and probe with known Zika virus strains in human and mosquitoes. All of the results showed 100% match (results not shown), indicating that the primer and probe of the present invention are highly specific to different Zika virus strains. Further, perform BLASTN analysis on the primer and probe of the present invention and the genes of eight virus strains similar to Zika virus (including West Nile virus, Yellow fever virus, Dengue virus, and Chikungunya virus), and the results showed that the primer and probe of the present invention do not specifically bind to them (results not shown).

[0041] In addition, through the analysis of Clustal Omega software, the specific recognition of different Zika strains by the primer and probe set of the present invention and the primer and probe sets disclosed in the prior art were compared. The results, shown in table 1, indicate that the primer and probe set of the present invention is capable of recognizing the widest range of Zika virus strains.

[0042] Table 1. Comparison between the primer and probe set of the present invention and those of prior art

primer and probe	100% sequence pairing		references
	human	mosquitoes	
Lanciotti-1 F	124/128	14/30	[1]
Lanciotti-1 R	125/128	11/30	[1]
Lanciotti-1 P	118/128	12/30	[1]
Lanciotti-2 F	126/128	14/30	[1]
Lanciotti-2 R	120/128	11/30	[1]
Lanciotti-2 P	3/128	11/30	[1]
Tappe-F	0/128	15/30	[2]
Tappe-R	0/128	6/30	[2]
Tappe-P	0/128	1/30	[2]

Pyke-1 F	121/128	14/30	[3]
Pyke-1 R	105/128	11/30	[3]
Pyke-1 P	123/128	11/30	[3]
Pyke-2 F	122/128	11/30	[3]
Pyke-2 R	127/128	29/30	[3]
Pyke-2 P	98/128	11/30	[3]
PAHO-F	126/128	30/30	[4]
PAHO-R	113/128	14/30	[4]
PAHO-P	0/128	0/30	[4]
Goebel-F	27/128	15/30	[5]
Goebel-R	118/128	13/30	[5]
Goebel-P	126/128	20/30	[5]
Corman-1 F	122/128	11/30	[6]
Corman-1 R	116/128	11/30	[6]
Corman-1 P	0/128	0/30	[6]
Corman-2 F	116/128	11/30	[6]
Corman-2 R	123/128	14/30	[6]
Corman-2 P	0/128	0/29	[6]
SEQ ID NO: 1	127/128	19/30	-
SEQ ID NO: 2	126/128	11/30	-
SEQ ID NO: 3	128/128	25/29	-

F: forward primer; R: reverse primer; P: probe

[0043] References:

- [1] Lanciotti et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap state, Micronesia, *Emerg Infect Dis*, 14(8):1232–9; 2008
- [2] Tappe Det al. First case of laboratory-confirmed Zika virus infection imported into Europe. *Euro Surveill*, 19(4):20685; 2014
- [3] Pyke AT et al. Imported Zika virus infection from the Cook Islands into Australia. *PLoS Curr*, 6:6; 2014
- [4] Waggoner et al. Zika Virus: Diagnostics for an emerging pandemic threat. *J Clin Microbiol*, 54(4); 2016
- [5] Goebel et al. A sensitive virus yield assay for evaluation of Antivirals against Zika Virus. *J Viro Met*, 238 (13-20); 2016
- [6] Corman et al. Assay optimization for molecular detection of Zika virus. *Bull World Health Organ*, 94:879–892; 2016

[0044] It is to be understood by those skilled in the art that the foregoing embodiments may be modified without departing from the broad inventive concept thereof. It is therefore to be understood that the present invention is not to be limited to the particular embodiments disclosed, but is intended to cover such modifications as fall within the true spirit and scope of the present invention as defined by the claims.

5

SEQUENCE LISTING

5 <110> MERIBANK BIOTECH CO., LTD.

<120> PRIMER AND PROBE SET FOR DETECTING ZIKA VIRUS AND KIT THEREOF

<130> MRD0007SG

10 <160> 6

<170> PatentIn version 3.5

15 <210> 1

<211> 23

<212> DNA

<213> Artificial sequence

20 <220>

<223> Forward primer

<400> 1

acctccgact gatggccaat gcc

23

25

<210> 2

<211> 20

<212> DNA

30 <213> Artificial sequence

<220>

<223> Reverse primer

35 <400> 2

gggtcttgtc ttccatgtgg

20

40 <210> 3

<211> 29

<212> DNA

<213> Artificial sequence

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<223> Primer/probe

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50 29

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 <213> Artificial sequence
 30
 <220>
 <223> Primer/probe
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 35 acgccggcca ttcaaacaatg agg
 23

40

45

CLAIMS

I/we claim:

1. A primer and probe set for detecting Zika virus, comprising: a forward primer of SEQ ID NO: 1, a reverse primer of SEQ ID NO: 2, and a probe of SEQ ID NO: 3.
- 5 2. The primer and probe set of claim 1, wherein a first fluorescence group and a first fluorescence quenching group are bound to the 5' end and the 3' end of the probe, respectively.
3. The primer and probe set of claim 2, wherein the first fluorescence group is FAM.
4. The primer and probe set of claim 2, wherein the first fluorescence quenching group is TAMRA.
5. The primer and probe set of claim 1 for detecting an RNA sample from the blood.
- 10 6. The primer and probe set of claim 1, which is used in RT-qPCR.
7. A kit for detecting Zika virus, comprising a primer and probe set according to any of claims 1 to 6.
8. The kit of claim 7, further comprising a primer and probe set for internal control.
9. The kit of claim 8, wherein the primer and probe set for internal control comprises: a forward
15 primer of SEQ ID NO: 4, a reverse primer of SEQ ID NO: 5, and a probe of SEQ ID NO: 6.
10. The kit of claim 9, wherein a second fluorescence group and a second fluorescence quenching group are bound to the 5' end and the 3' end of the probe of SEQ ID NO: 6, respectively.
11. The kit of claim 10, wherein the second fluorescence group is HEX.
12. The kit of claim 10, wherein the second fluorescence quenching group is TAMRA.
- 20 13. The kit of claim 7, further comprising a positive control sample and a negative control sample.