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A method of layer-by-layer gold nanoparticle hybridization in a quartz crystal microbalance DNA sensing system used to detect dengue virus

Sz-Hau Chen, Yao-Chen Chuang, Yi-Chen Lu, Hsiu-Chao Lin, Yun-Liang Yang and Chih-Sheng Lin¹

Department of Biological Science and Technology, National Chiao Tung University, Hsinchu 30068, Taiwan

E-mail: lincs@mail.nctu.edu.tw

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Abstract

Dengue virus (DENV) is nowadays the most important arthropod-spread virus affecting humans existing in more than 100 countries worldwide. A rapid and sensitive detection method for the early diagnosis of infectious dengue virus urgently needs to be developed. In the present study, a circulating-flow quartz crystal microbalance (QCM) biosensing method combining oligonucleotide-functionalized gold nanoparticles (i.e. AuNP probes) used to detect DENV has been established. In the DNA-QCM method, two kinds of specific AuNP probes were linked by the target sequences onto the QCM chip to amplify the detection signal, i.e. oscillatory frequency change (ΔF) of the QCM sensor. The target sequences amplified from the DENV genome act as a bridge for the layer-by-layer AuNP probes' hybridization in the method. Besides being amplifiers of the detection signal, the specific AuNP probes used in the DNA-QCM method also play the role of verifiers to specifically recognize their target sequences in the detection. The effect of four AuNP sizes on the layer-by-layer hybridization has been evaluated and it is found that 13 nm AuNPs collocated with 13 nm AuNPs showed the best hybridization efficiency. According to the nanoparticle application, the DNA-QCM biosensing method was able to detect dengue viral RNA in virus-contaminated serum as plaque titers being 2 PFU ml⁻¹ and a linear correlation ($R^2 = 0.987$) of ΔF versus virus titration from 2×10^0 to 2×10^6 PFU ml⁻¹ was found. The sensitivity and specificity of the present DNA-QCM method with nanoparticle technology showed it to be comparable to the fluorescent real-time PCR methods. Moreover, the method described herein was shown to not require expensive equipment, was label-free and highly sensitive.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Dengue is the most important arbovirolosis in terms of numbers of humans affected. It constitutes a serious public health problem in many subtropical and tropical regions where environmental conditions allow the proliferation of insect

vectors. *Aedes aegypti* is the main vector of dengue virus (DENV) and is present in most countries between Asia, Africa and Central and South America, with more than 2.5 billion people at risk of infection and dengue transmission is endemic in more than 100 countries [1, 2]. DENVs cause infections which lead to clinical symptoms ranging from self-limited, acute, febrile disease called dengue fever (DF) to life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [3, 4]. An estimated 500 000 cases of

¹ Address for correspondence: Department of Biological Science and Technology, National Chiao Tung University, No. 75 Po-Ai Street, Hsinchu 30068, Taiwan.

DHF require hospitalization each year, of which a very large proportion are children. At least 2.5% of cases die, although case fatality could be twice as high.

Effective vaccines or drugs are still unavailable to prevent or cure the disease caused by DENV. Therefore, a rapid and reliable method for DENV detection is an important task, which rapidly focuses the medical treatment for doctors to save the patients' life as early as possible. Especially in the acute phase of the dengue infection before any specific antibody response directed against the DENV is detectable, only virus detection, either by cDNA detection and/or virus isolation [5], will be the matter of choice for the diagnostic. However, virion isolation consumes a lot of time for cultivation and identification, usually needing three to four working days [6]; only cDNA detection can give quick and reliable results within a few hours via polymerase chain reaction (PCR). Traditional amplification methods like nested or single-tube multiplex PCR [7, 8] are being more and more replaced by real-time, automated RT-PCR assays like the TaqMan or light cycler technique [5, 9, 10]. However, these methods need expensive instruments and professionals to operate and analyze them. There is great urgency to develop a novel detection system with high sensitivity, short operation time and handy processing with easy interpretation.

Piezoelectric biosensors, known as quartz crystal microbalances (QCM), combine high sensitivity to mass on the surface of the quartz crystal with the high specificity of a biosensor. Wu and co-workers [11] are the pioneers in using immuno-QCM biosensors to detect DENV in clinical samples. The authors used monoclonal antibodies to recognize the DENV envelope protein (E) and non-structure protein 1 (NS-1) and detect the mass increase on the antibody-modified QCM. Recently, QCM sensors have been extensively applied as transducers in hybridization based on DNA biosensors for the detection of gene mutation [12, 13], genetically modified organisms [14] and foodborne pathogens [15–17]. Further, gold nanoparticles (AuNPs) were integrated into QCM as effective amplifiers in the DNA detection, because AuNPs have relatively large mass compared with oligonucleotides [18, 19].

Although AuNPs have been used in biotechnology over the last few decades as immunocytochemical probes and immuno-conjugates, recent advances in DNA-functionalized AuNPs have paved the way for the development of a series of new and practical bioassay or biosensing systems [20]. Several papers have revealed that the oligonucleotide-functionalized AuNPs can be used as amplifiers in a microgravimetric DNA sensor [21–23], but rarely has detection for pathogenic viruses been demonstrated because a lot of procedures, including pretreatment of detection sample, specific probe design and optimal detection processes, need to be addressed to develop a DNA-based sensing QCM or cantilever system.

In the present study, we have developed a real-time and circulating-flow QCM combined with oligonucleotide-capped AuNPs (i.e. AuNP probes) to specifically detect the DNA fragment which was amplified from the DENV genome by the layer-by-layer hybridization of AuNP probes. Using this method, as low as 2 plaque forming units (PFU)/ml of DENV serotype 2 (DENV2) could be detected. The AuNP probes

in the DNA-QCM sensor were used not only as amplifiers to enhance the detection sensitivity but also as verifiers to increase the specificity of the DNA hybridization.

2. Experimental details

2.1. Chemicals and reagents

The chemicals and reagents used in the study were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich (St Louis, MO, USA). These included 30% hydrogen peroxide (H₂O₂; Merck), 98% sulfuric acid (H₂SO₄; Sigma-Aldrich), sodium chloride (NaCl; Sigma-Aldrich), sodium phosphate (Na₂HPO₄; Merck), hydrogen chloride (HCl; Merck), sodium citrate (C₆H₅Na₃O₇·2H₂O; Merck), chloroauric acid (HAuCl₄; Sigma-Aldrich) and sodium hydroxide (NaOH; Sigma-Aldrich).

2.2. Oligonucleotide primers, probes and target design

All of the oligonucleotides were designed using Primer Express software (Applied Biosystems, Foster, CA, USA), and synthesized by Applied Biosystems. The characteristics of the synthesized oligonucleotides are listed in table 1, which includes single-stranded DNA of probes (P), target (T) and primers for reverse transcription (RT) and PCR. For the detection of the gene sequence encoding envelope (E) protein of DENV2 (GenBank DQ341195), the specific probe sequences DENV2-P1 and DENV2-P2 that were modified with a thiol-linkered tag (HS-(CH₂)₆-SH) and with additional 12-deoxythymidine-5'-monophosphate (12-dT) at the 3' terminus and 5' terminus were synthesized, respectively. The synthesized target oligonucleotide is the sequence that is complementary to the probe oligonucleotides. In order to amplify the E gene fragment from all four dengue serotypes, DENV serotype 1 (DENV1), DENV2, DENV serotype 3 (DENV3) and DENV serotype 4 (DENV4) a pair of universal primers (DENV-UF and DENV-UR) were designed at the conserve domains of the E gene among the dengue serotypes. A target DNA fragment with a length of 130 bp could be obtained by RT-PCR amplification with DENV-URT as the RT primer, and DENV-UF and DENV-UR as the PCR primer pair. The internal sequences of amplified DNA fragments (i.e. target sequences) from four DENV serotypes, DENV1–4, are diverse; therefore, the specific probe sequences designed according to this diverse domain could be used to differentiate their complementary target sequences from the four DENV serotypes via DNA hybridization.

2.3. DENV preparation

The DENV was transfected and amplified in mosquito cells, *Aedes albopictus* C6/36 (ATCC number CRL 1660™) that were obtained from the Food Industry Research and Development Institute (HsinChu, Taiwan). C6/36 cells were grown in minimum essential medium (MEM) (Gibco, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 27 °C in a humidified incubator with 5% CO₂. The parental PL046

Table 1. Sequences of the probe, target and primer oligonucleotides for the dengue virus serotype-2 (DENV2) detection used in this study.

Probe oligonucleotides ^a :	
DENV2-P1	5'-GTATCCAATTTGATCCTTGTGTGTCGCTC(T) ₁₂ -(CH ₂) ₆ -SH-3' (42 mer)
DENV2-P2	5'-HS-(CH ₂) ₆ -(T) ₁₂ GATTCTGAAGGTGACCAATGTCTCTTTCT-3' (42 mer)
Synthesized target oligonucleotide ^b :	
DENV2-T	5'-GAGCGGACACACAAGGATCAAATTGGATACAGAAAGAGACATTGGTGACTTTCAAAAATC-3' (60 mer)
PCR primer pair ^c :	
DENV-UF	5'-TGGTGCACAGACAATGGTT-3' (19 mer)
DENV-UR	5'-ACATCCTGTTTCTTCGCATG-3' (20 mer)
RT primer ^d :	
DENV-URT	5'-GCCATTCTCTTCGCTCCCCT-3' (20 mer)

^a Probe (P) oligonucleotides were designed according to the gene region of the DENV E protein and used to detect the sequence of DENV2. P1 was used to immobilize on the gold surface of the QCM chip and cap onto the AuNP surface to purposely enhance the detection signal of the QCM sensor; P2 was used to cap onto the AuNPs.

^b The sequence of synthesized target (T) oligonucleotide is complementary to the P1 at its 5' terminus and to the P2 at its 3' terminus.

^c Universal primer pair (UF and UR) was used to amplify a 130 bp DNA fragment within the gene encoding DENV E protein.

^d The universal RT (URT) primer was used to reverse-transcribe DENV mRNA to cDNA.

DENV, DENV2, was originally obtained from Professor Huan-Yao Lei (National Cheng Kung University, Tainan, Taiwan). The stock of PL046 DENV was amplified in C6/36 cells, concentrated via ultracentrifugation, and titrated by plaque assays as previously described [24, 25]. The virus titers were adjusted to 2×10^6 PFU ml⁻¹ in MEM with 10% FBS. The virus concentrate was stored at -80°C before use.

2.4. Plaque forming unit assay of DENV

Viral PFU assay was performed in BHK-21 cells (ATCC number CCL-10TM). BHK-21 cells (3×10^5 ml⁻¹) suspended in MEM were seeded into each 6-well culture plate (Nalge Nenu, Rochester, NY, USA) and incubated at 37°C in a humidified incubator with 5% CO₂. The cells were cultured overnight before virus adsorption. A series of viral dilutions made in the culture medium at 0.4 ml were adsorbed onto the monolayer of BHK-21 cells for 1 h at 37°C , and every 30 min they were shaken slightly to make virus adsorption on BHK-21 uniform. Each virus-infected culture well was overlaid with 4 ml of 1.1% carboxymethyl cellulose containing 5% FBS for an additional 6 days. The plaque forming wells were fixed with 3.7% formalin for 30 min followed by staining with 0.5% crystal violet in 3.7% formalin. The results were calculated by counting plaques on the four replicate wells.

2.5. DENV RNA extraction

DENV RNA was extracted by the ALS Viral Nucleic Acid Extraction System (ALS, Kaohsiung, Taiwan). For each RNA preparation, 150 μl supernatant of DENV infected cells were mixed with 570 μl VRE buffer and incubated at room temperature for 10 min to lyse the virus. Thirty microliters of 400 U μl^{-1} DNase solution was added to the lysate and treated for 30 min, and then the viral RNA extraction was carried out according to the manufacturer's protocol for the virus (ALS). For each preparation, a total of 50 μl of viral RNA was eluted from the RNA purified column and quantified by determining OD₂₆₀ with a SpectraMax 190 spectrophotometer (Molecular

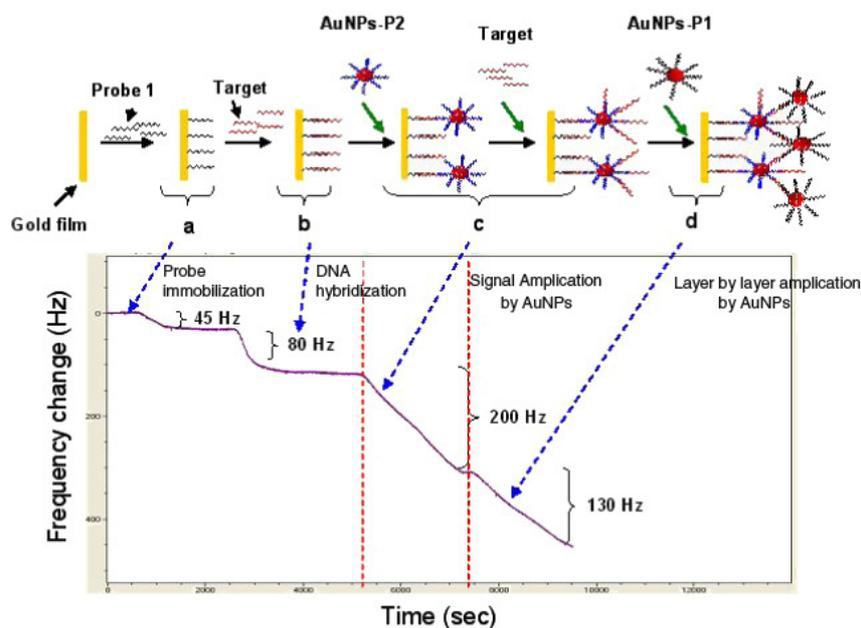
Devices Corp., Sunnyvale, CA, USA). The viral RNA was stored at -80°C until use.

2.6. DENV cDNA preparation

The DENV cDNA was prepared by RT using ReverTra Ace (TOYOBO, Osaka, Japan). Ten micrograms of DENV RNA was used as a template to prepare cDNA and synthesized oligonucleotide, DENV-URT, as the primer. The RT reaction was carried out in PTC-100TM thermal cycle (MJ Research 171 Inc., Watertown, MA, USA) with the following program: extension at 52°C for 30 min and followed by inactivating RT enzyme at 99°C for 5 min.

2.7. Asymmetric PCR amplification

For detecting the DNA sequences of DENV E gene, asymmetric PCR was used to amplify the target DNA (130 bp) for direct hybridization detection [26]. The ratio of forward primer (DENV-UF) and reverse primer (DENV-UR) concentration was 5:1, and the lower concentrated reverse primer acted as a 'limiting primer'. Double-stranded target DNA fragments were produced when both primers were present in the PCR mixture at the beginning of the reaction. However, after the limiting primer was consumed, the remaining primer (forward primer) continued to amplify the target DNA, which resulted in the PCR products predominantly being single-stranded E gene fragments. The PCR reaction was carried out in PTC-100TM thermal cycle (MJ Research 171 Inc.) with the following program: DENV cDNA was initially denatured at 95°C for 3 min, followed by 40 cycles of 30 s denaturation at 95°C , 20 s annealing at 53°C , 30 s extension at 72°C , and 5 min final extension 72°C . One microliter of DENV cDNA (1 μg), 1 μl forward (25 μM), 1 μl reverse primers (5 μM) and other components were added to the 50 μl PCR reaction mixture following the instructions for the PCR reagent (BerTaq DNA Polymerase, Bertec, Taipei, Taiwan). Following amplification, the PCR products were purified by WizardTM PCR Preps DNA Purification System (Promega,



Scheme 1. Schematic illustration of the steps involved in probe immobilization, probes hybridized with target sequences, and layer-by-layer AuNP-probe amplification. One example of the step-by-step procedures corresponded to the QCM flowchart and the recorded oscillatory frequency change (ΔF) of the detection process was shown. (a) Specific probe (DENV2-P1) immobilized onto the QCM chip. (b) DENV2-P1 complement hybridized with the target sequences (partial gene sequences encoding DENV E protein) at 5' terminus. (c) AuNPs-DENV2-P2 complement hybridized with the target sequences at 3' terminus to enhance detection signal, i.e. increasing ΔF . (d) AuNPs-DENV2-P2 linked to AuNPs-DENV2-P1 by the surplus target sequences that play as a bridge for the layer-by-layer AuNP probes hybridized for enhancing ΔF . Specific probes were immobilized onto the chip surface of the QCM sensor or AuNPs by thiol (-SH) groups.

Madison, WI, USA) according to the manufacturer's protocol and then we performed electrophoresis followed by visualizing through a UV transilluminator (Apices Scientific Co., Ltd, MA, USA). The DNA concentration of PCR-amplified products was determined spectrophotometrically (Agilent Technologies, Palo Alto, CA, USA) at 260 nm.

2.8. The circulating-flow QCM system

The circulating-flow QCM system was applied as in our previous reports [17, 19]. The piezoelectric quartz crystals, which consisted of a 9 MHz AT cut quartz crystal slab with a layer of a gold electrode on each side (0.091 cm^2 in area on each side; the detection limit of the QCM instrument in liquid = 1 Hz), were obtained from ANT Technology (Taipei, Taiwan). The flow injection and continuous frequency variation recording were operated using an Affinity Detection System (ADS; ANT Technology). The experimental data were analyzed by P-Sensor software in real-time. The sensor unit was composed of resolution, 0.1 Hz, and sampling period, 1 s. The oscillatory frequency change (ΔF) of 1 Hz corresponds to a mass change of 0.391 ng.

Before the experiments, the gold electrode surface of the QCM chip was pre-treated with hot piranha solution (concentrated H_2SO_4 and 35% of H_2O_2 , 3:1 v/v). After an incubation period of 1 min with piranha solution, the QCM chip was thoroughly rinsed with distilled water and air-dried subsequently [27]. This procedure was repeated twice and then the chip was immersed in distilled water [13, 28].

2.9. AuNP preparation and oligonucleotide modification

The colloidal gold was prepared by reduction of 0.01% HAuCl_4 with 38.8 mM sodium citrate aqueous solution, while the monodispersed AuNPs of different sizes (5, 13, 20 and 50 nm in diameter) were obtained by the same procedure with different amounts of sodium citrate [29, 30]. The sizes of AuNPs were checked by scanning electron microscopy (SEM) images with a JEM 100 CX electron microscope (JEOL Co. Ltd, Tokyo, Japan) at 25 kV and the AuNPs average diameter was estimated by dynamic laser scattering (DLS) (TPCL, Chupei, Taiwan). The AuNPs were functionalized with DENV2-P1 or DENV2-P2 at 37°C for 60 min [31]. To steady the AuNP probes and increase the probe oligonucleotide loading onto the AuNPs, the concentration of NaCl was increased to 0.3 M using phosphate buffer (1 M NaCl and 10 mM Na_2HPO_4 , pH 7.4). The process was: add 20 μl of 0.05, 0.1, 0.2 and 0.3 M NaCl diluted step by step from PBS buffer (1 M NaCl, 1 mM Na_2HPO_4 , pH 7.4) for 6 h, respectively. Then, the AuNP-probe solution was dispersed for 10 s by vortex in every 6 h incubation period at 4°C . To remove excess oligonucleotides, the AuNP probes were centrifuged and the supernatant was removed. The precipitated AuNP probes were then resuspended in 0.3 M phosphate buffer.

2.10. Immobilization of probes, hybridization and signal amplification by AuNP probes

Scheme 1 (upper panel) shows a schematic illustration of the procedures of probe oligonucleotide immobilization, DNA

hybridization of probes and targets, and signal amplification of detection in the DNA–QCM system by layer-by-layer AuNP probes via target sequences. In the procedure, the phosphate buffer was first flushed through the QCM chip at a flow speed of $50 \mu\text{l min}^{-1}$ and we began the study while the frequency of the chip was steady within ΔF within $\pm 1 \text{ Hz}/300 \text{ s}$. The solution containing specific probe sequences (3'-thiolated DENV2-P1; table 1) for detecting the sequences of the DENV E gene was added to the sample tube (total volume: $500 \mu\text{l}$). The probe oligonucleotides were self-assembly-immobilized onto the gold surface of the QCM chip through flow circulation for 10 min. After probe immobilization, the probe solution was removed and then the solution containing the target sequences was added. The hybridization of probe and target sequences on the QCM chip was at a flow speed of $50 \mu\text{l min}^{-1}$ for 10 min. The temperature of the QCM system was maintained at 30°C . The ΔF during the hybridization was recorded in real-time observation. After target–probe hybridization, the first layer of AuNPs–DENV2-P2 (AuNPs conjugated with DENV2-P2; the sequences of DENV2-P2 are complementary with the 3' terminus of the target sequences) was added to enhance the mass on the QCM chip. Following this, the AuNPs–DENV2-P1 (AuNPs conjugated with DENV2-P1; the sequences of DENV2-P1 are complementary with the 5' terminus of the target sequences) was added to specifically link the surplus (not hybridized) target sequences in the reaction. The second layer of AuNPs was formed as the AuNPs–DENV2-P2/target/AuNPs–DENV2-P1 conjugant to amplify the detection signal.

3. Results and discussion

3.1. QCM system and detection

Piezoelectric QCM was applied as a mass biosensor for the detection of specific DNA sequences reverse-transcribed from the DENV RNA genome. In the circulating-flow QCM system for DENV detection, the oscillatory frequency of the QCM chip decreased when the mass on the surface of the QCM chip increased, for which the ΔF could be recorded in real-time. The frequency decreased gradually with the self-assembly immobilization of the probe oligonucleotides and DNA hybridization of probe and target sequences on the chip surface of the QCM via layer-by-layer AuNP probes conjugant. Scheme 1 (lower panel) shows an example of the real-time detection of the QCM system performed in the present study. An oscillatory frequency decrease of approximately -45 Hz was observed after the probe DENV2-P1 ($1.0 \mu\text{M}$) was self-assembly-immobilized onto the QCM chip, which suggested that the gold surface of the QCM chip was successfully functionalized. Subsequently, the target oligonucleotides, DENV-T ($0.5 \mu\text{M}$), were introduced for the DNA hybridization and the result showed an additional ΔF of approximately -80 Hz . Then, for enhancing the detection signal, the DENV2-P2 functionalized-AuNPs (i.e. AuNPs–DENV2-P2) were applied to the system to be the first enhancer and confirmer to increase ΔF when the AuNPs–DENV2-P2 hybridized with the target sequences and ΔF of approximately

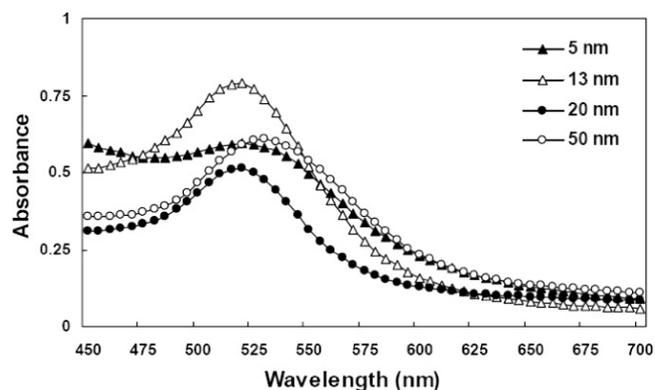


Figure 1. UV–vis absorption spectra of the AuNPs with different diameters. Four sizes of AuNPs with diameters of 5, 13, 20 and 50 nm were measured by a UV–vis absorption spectrophotometer, and they exhibited an absorption maximum at 520–540 nm arising when the AuNPs exhibit noticeable absorption and scattering from the colloid gold localized surface plasmon resonance.

-200 Hz was detected. However, a lot of surplus target oligonucleotides still have not been used in the reaction. In order to achieve full detection of the target sequences, the DENV2-P1 functionalized-AuNPs (i.e. AuNPs–DENV2-P1) were added to the QCM system. The result shows ΔF of -130 Hz after AuNPs–DENV2-P1 was applied in the system. The AuNPs–DENV2-P1 played a role as a catcher to capture the surplus target sequences in the reaction and to act as the second enhancer to amplify the detection signal via the layer-by-layer AuNP-probes' hybridization until the surplus targets were exhausted in the circulating-flow QCM system.

3.2. Identification of AuNP size

AuNPs of 5, 13, 20 and 50 nm in diameter were produced by sodium citrate reduction. Wavelength shifts, ranging from 450 nm to 700 nm, of the AuNPs with different sizes were scanned by a spectrophotometer (figure 1). The maximum absorbance (λ_{max}) of the prepared 5, 13, 20 and 50 nm AuNPs were at about 515, 520, 524 and 530 nm, respectively. The λ_{max} of 13 nm AuNPs was confirmed by a previous report [32]. Therefore, the optical spectra can preliminarily estimate the AuNP sizes produced. Further, the SEM and DLS were used to confirm and measure the AuNP shape and average diameter. The diameters of 5, 13, 20 and 50 nm AuNPs were 5 ± 1 , 13 ± 2 , 20 ± 4 and $50 \pm 6 \text{ nm}$ by DLS assay, respectively. When the probe oligonucleotides were immobilized onto AuNPs, the λ_{max} of the oligonucleotide-functionalized AuNPs shifted slightly; this may be due to the radius of the AuNP probes increasing a little. Besides, the high density of the probe on the AuNPs caused a steric barrier and interfered with the hybridization between the target and the probe. The optimum AuNP-probe concentration was calculated to provide better AuNP-probe stability and DNA hybridization efficiency [33, 34]. Therefore, the concentration of the AuNP probe was controlled at 2.6 nM in the circulating-flow QCM system. At this concentration, the hybridization between AuNP probes and targets would have a better efficiency.

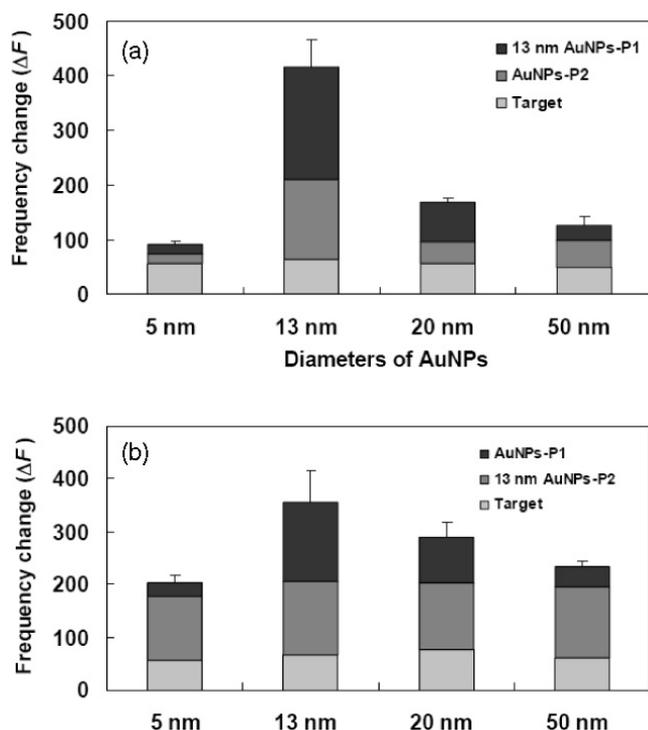


Figure 2. The enhancement of detection signal by different sizes of AuNP probes. (a) In the experiments, 5, 13, 20 and 50 nm AuNPs–DENV2-P2 were used as the first layer and 13 nm AuNPs–DENV2-P1 as the second layer enhancers. (b) In the experiments, 13 nm AuNPs–DENV2-P2 were used as the first layer and 5, 13, 20 and 50 nm AuNPs–DENV2-P1 as the second layer enhancers. Each value was derived from three independent detections and the error bars mean SD.

3.3. Effect of AuNP-probe size on ΔF enhancement

The effects of AuNP-probe size on ΔF were determined (figure 2). In the experiments with 5, 13, 20 and 50 nm AuNPs–DENV2-P2 as the first layer and 13 nm AuNPs–DENV2-P1 as the second layer enhancers, the ΔF for 5, 13, 20 and 50 nm AuNPs–DENV2-P2 applied were -17 ± 5 , -148 ± 48 , -103 ± 6 and -50 ± 16 Hz, respectively (figure 2(a)). In the reverse experiments, 13 nm AuNPs–DENV2-P2 as the first layer and 5, 13, 20 and 50 nm AuNPs–DENV2-P1 as the second layer enhancers, the ΔF for 5, 13, 20 and 50 nm AuNPs–DENV2-P1 applied were -25 ± 5 , -151 ± 30 , -86 ± 13 and -37 ± 5 Hz, respectively (figure 2(b)). The results showed that the largest efficiency for enhancing ΔF was when the 13 nm AuNP probes were applied as the enhancer either for the first or second layer enhancer in the detection.

AuNP probes deposited onto the QCM chip are dependent on the DNA hybridization efficiency between the specific probe and target sequences [22, 35]. Previous reports showed that the density of DNA on a nanogold-modified surface is directly influenced by the hybridization result [35] and the ΔF of the QCM sensor is decreased accompanied by an increase of AuNP size applied in the static solution [36, 37]. This could be attributed to the steric hindrance effect of the nanoparticles. The larger nanoparticles cannot move as freely as the smaller nanoparticles and the larger nanoparticles connected to the

target DNA hinder the approach of further nanoparticles [37]. However, in a circulating-flow condition, our results show that the hybridization rate reaches its maximum value when the average diameter of AuNP probes is 13 nm and then decreases with increasing AuNP-probe size (figure 2). Some speculations could be used to explain the controversial results observed. First, the larger AuNPs could be immobilized with more probe oligonucleotides than the smaller ones, but according to the report by Hurst *et al* [38], the larger AuNPs have fewer probes on the same superficial area than smaller ones. Therefore, the larger AuNP probes that hybridize with the target sequences are harder than the smaller ones, resulting in a decrease of DNA hybridization efficiency when larger AuNP probes were used in the DNA–QCM biosensing system. Second, the flow rate could interfere with the DNA hybridization efficiency in a flow system [39]. In the circulating-flow QCM system, the AuNP probes in a fluid should approach the chip surface and hybridize with their complementary sequences before they flow out of the reaction chamber of the QCM chip. We proposed that the AuNP probes with a size of 13 nm may be more suitably applied in the flow condition used in the present system. Third, the AuNPs carry negative charge which could repel other AuNP probes [40] and the larger AuNP probes would have a stronger repulsion effect resulting in DNA hybridization efficiency decreasing.

Besides, we supposed that 5 nm AuNP probes could easily stay on the QCM chip to hybridize with the target sequences in the circulating-flow system. The amount of 5 nm AuNP probes deposited in the first layer may be more than for other sizes of AuNP probes (the order of deposited efficiency was $5 \text{ nm} > 13 \text{ nm} > 20 \text{ nm} \gg 50 \text{ nm}$) in the QCM chip. But considering the mass effect, the mass of 5 nm AuNPs was about 1/27 times the 13 nm AuNPs, 1/64 times the 20 nm AuNPs and 1/1000 times the 50 nm AuNPs. Therefore, we conjectured that the lower efficiency of ΔF enhancement for the 5 nm AuNP probes is because of a mass effect.

3.4. Observation of AuNP-probes hybridization on a QCM chip

The chip surface of QCM sensors was observed with SEM (figure 3), where the assembled AuNP probes were displayed through DNA hybridization. Figure 3(a) shows the chip surface of immobilized DENV2-P1 probes hybridized with the target sequences on the QCM chip without AuNP probes applied. The SEM image of the first layer of AuNPs–DENV2-P2 (13 nm) deposited onto the QCM chip (figure 3(b)) and of the second layer of AuNPs–DENV2-P1 (13 nm) added onto the QCM chip via the hybridization with target sequences (figure 3(c)) were shown. In figure 3(b), the dispersed AuNP probes were observed because the surface of the AuNP probes was full of negative charge and the AuNP probes would repel each other [41]. Figure 3(c) shows the SEM images of layer-by-layer AuNP probes deposited on the QCM chip, i.e. the AuNP probes linked by the surplus target sequences and at most two to four AuNPs linked together (enlarged view in figure 3(c)).

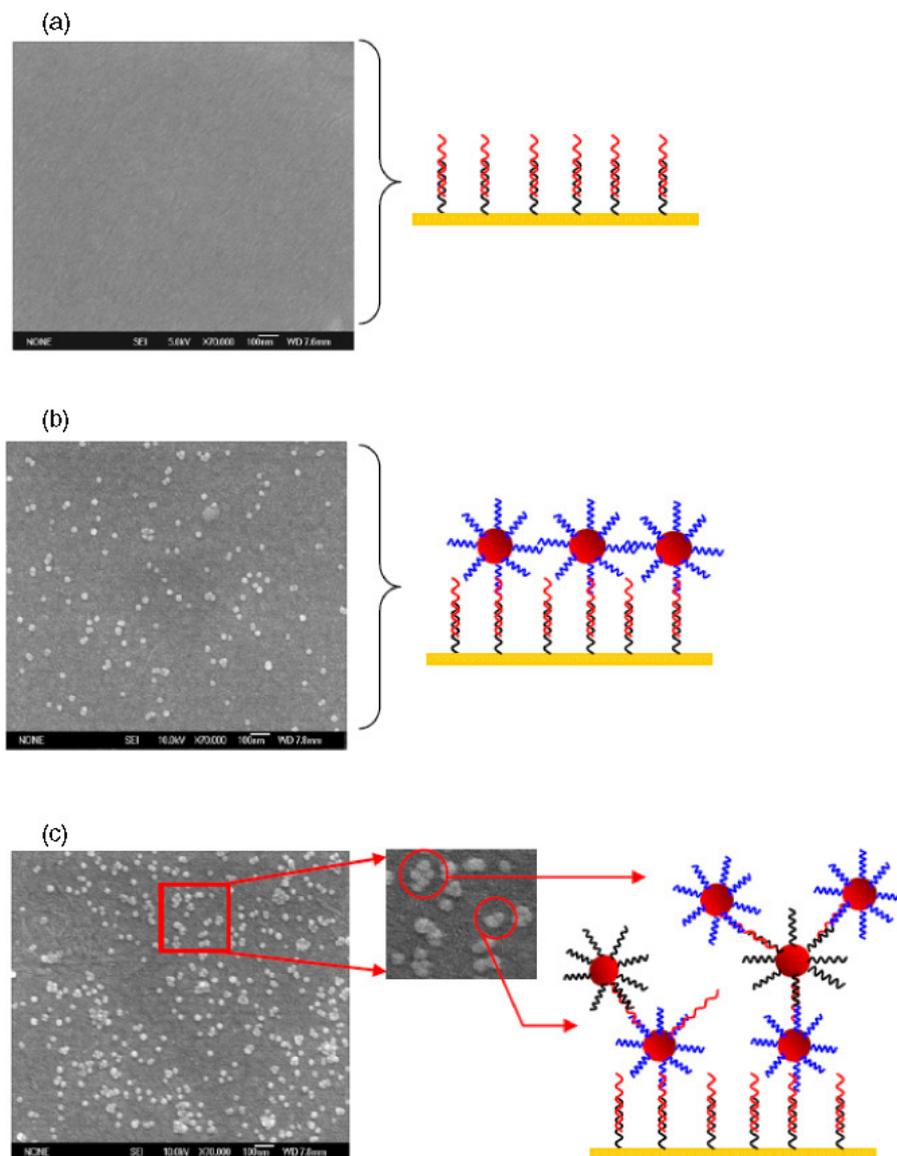


Figure 3. SEM images of the chip surface of a QCM sensor. (a) The chip surface of QCM-immobilized DENV2-P1 probes and hybridized target sequences without AuNP probes applied. (b) The chip surface with the first layer of AuNPs–DENV2-P2 (13 nm) adapted onto the QCM chip via hybridization with target sequences. (c) The chip surface with the second layer of AuNPs–DENV2-P1 (13 nm) added onto the QCM chip via hybridization with target sequences. The enlarged view in panel (c) shows the AuNP probes linked by the surplus target sequences and at most two to four AuNP probes linked together.

3.5. Detection of RT-PCR-amplified DNA from DENV2

DENV cDNA was obtained by RT used DENV-URT as primer and then the DNA fragment of the DENV E gene was obtained by PCR using the universal primer pair, DENV-UF and DENV-UR (figure 4). The PCR-amplified DNA fragment (130 bp) could be obtained from DENV2 and DENV3 which were mixed in human whole blood before the RT-PCR was performed (lanes 2 and 3 shown in figure 4(a), respectively). However, only the DNA fragment from DENV2 could be detected in the QCM system and the detected ΔF in the DENV2 sample reached -115 Hz; this value was 3.4, 4.3 and 4.3 times that in the DENV3 (38 Hz), C6/36 cells (29 Hz) and whole blood (29 Hz) detected (figure 4(b)). The sequence alignment demonstrated that the target sequences between

DENV2 and DENV3 are extremely different (figure 4(c)). This is the reason why the DENV2-specific probes can recognize the DENV2 from the other DENV serotypes.

The PCR-amplified DNA from DENV2 cDNA was able to be detected by the DENV2-P1-immobilized QCM chip and the detected signal could be amplified by AuNPs–DENV2-P2 and AuNPs–DENV2-P1 hybridization. In addition to the signal amplification, the role of AuNPs–DENV2-P2 and AuNPs–DENV2-P1 is also for sequence verification. In figure 4(b), the result demonstrates that the layer-by-layer AuNPs–DENV2-specific probes not only can catch the surplus target sequences but also play a verifier role to provide detection accuracy.

In the present study, only one pair of universal primers was needed to PCR amplify the specific target DNA fragment from four DENV serotypes. The primer pair was selected

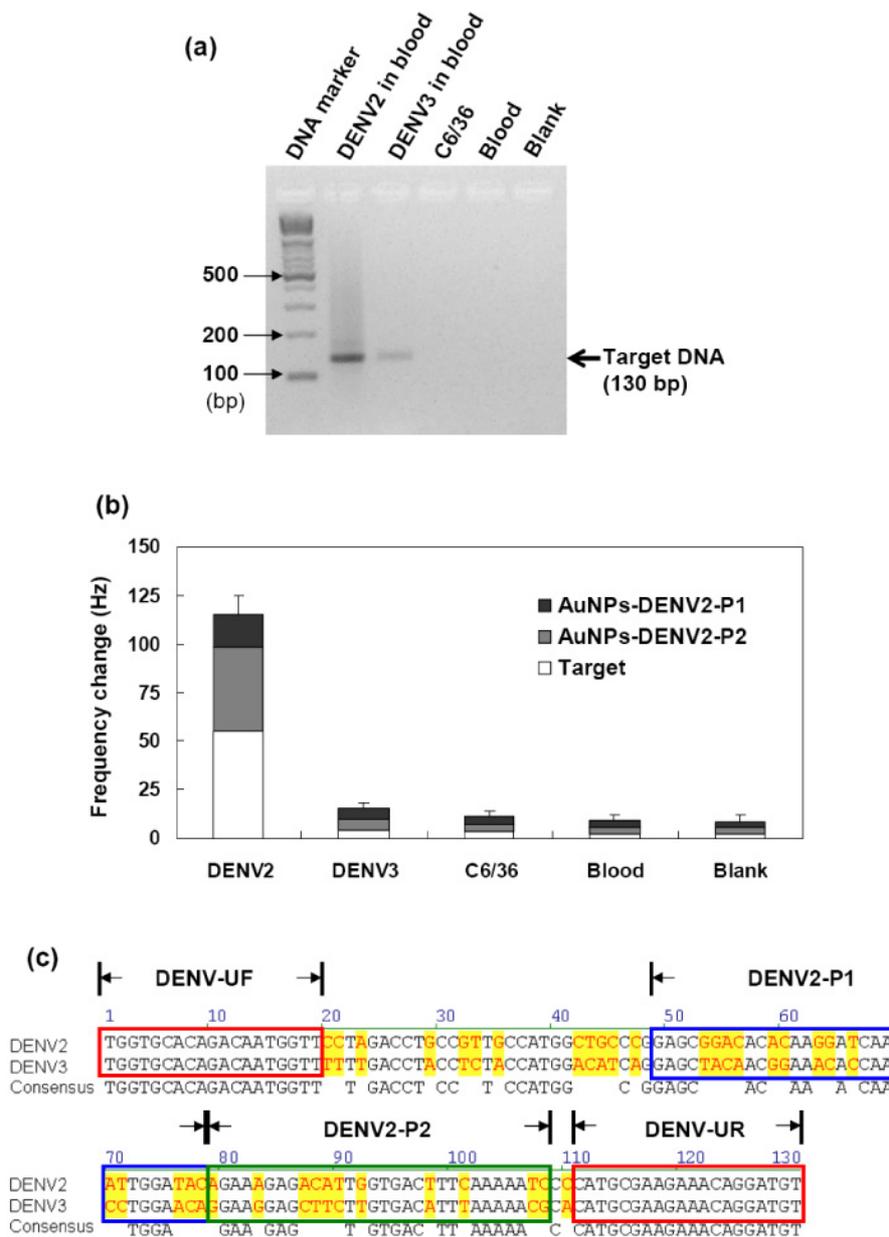


Figure 4. Specificity of the layer-by-layer AuNP probes (13 nm) for DENV sequence detection. The DENV cDNA was obtained by RT using DENV-URT as primer and then the DNA fragment of the DENV E gene was obtained by asymmetric PCR using the universal primer pair, DENV-UF and DENV-UR. (a) A representative result of agarose electrophoresis shows that a 130 bp DNA fragment was obtained in the samples of DENV2 and DENV3 which were mixed into human blood before the RNA was extracted. There was no PCR product observed in the samples of C6/36 cells and whole blood. (b) The results of QCM detection showed that a significant ΔF (-115 Hz) was detected only in the sample of DENV2 in blood. Each value was derived from three independent detections and the error bars mean SD. (c) The sequence alignment show the difference between DENV2 and DENV3 within the target sequences; the sequence similarity of the probe 1 and probe 2 recognized region is 53% and 63%, respectively.

based on highly conserved regions within the DENV genome. The amplified DNA fragment of DENV can be recognized by specific AuNP probes in the DNA-QCM biosensing system. Therefore, the developed method could be used to differentiate DENV serotypes via DNA hybridization.

For the immuno-QCM biosensor developed by Wu *et al* [11], the data showed a high background (10–20 Hz) because of serum interference, even though they used a cibacron blue 3GA gel-heat denature (CB-HD) method to pretreat the clinical samples. As the authors mentioned, however, the pretreatment

procedures would cause a dramatic decrease in analytical sensitivity. In the nucleic acid QCM biosensing method developed in this study, the detection targets of nucleic acid would be purified through the procedures of RNA extraction, reverse transcription and PCR amplification. Therefore, the background signal of QCM detection could be lower than 10 Hz. The extraction process also caused the loss of cellular RNA, but the number of molecules of detection targets could be greatly increased via the procedure of PCR amplification.

Table 2. Comparisons of the present study with the related detection technologies for dengue virus.

Method	Detected entity	Pretreatment procedure	Virus typing	Label-free	Detection time	Detection limit	Timing of infectious virus can be detected	Reference
Plaque assay	Viable virus	None	No	Yes	Days–weeks	1–10 infectious virus	After disease onset	[42, 44]
ELISA	Antigen	Protein extraction	Yes	No	1–3 h	15–50 ng ml ⁻¹	Viremia phase	[45, 46]
Immuno-QCM	Antigen	Protein extraction	Yes	Yes	2 h	7–17 ng ml ⁻¹	Viremia phase	[11]
Fluorescent real-time PCR	Nucleic acid (i.e. viral RNA)	RNA extraction, reverse transcription and PCR amplification	Yes	No	Hours	1–50 PFU ml ⁻¹	Initial stage of infection	[42, 43]
DNA-QCM	Nucleic acid (i.e. viral RNA)	RNA extraction, reverse transcription and PCR amplification	Yes	Yes	1.5 h	2 PFU ml ⁻¹	Initial stage of infection	This study

3.6. Quantitative detection of DENV2 detection in real blood sample

The DNA-QCM sensor was used to detect different titers of DENV2 in real blood samples with/without amplification by AuNP probes (13 nm) (figure 5). The blank control showed the background of $\Delta F = 2 \pm 1$ Hz in the samples without AuNP probes and $\Delta F = 7 \pm 1$ Hz in samples with AuNP-probe application. The ΔF of the QCM sensor was increased by the increase of virus concentrations in the detection either with or without AuNP-probe amplification. The measurements were highly reproducible for all concentrations of DENV2 applied ($n = 3$ for each detection, $RSD < 13.5\%$ without AuNP probes and $RSD < 8.7\%$ with AuNP-probe amplification). In the detection with the layer-by-layer AuNP-probe amplification, a linear relationship ($y_1 = -17.646x + 129.3$, $R^2 = 0.9874$) was found between ΔF versus log (PFU ml⁻¹ of DENV2) from 2×10^0 to 2.0×10^6 PFU ml⁻¹. In the detection without the AuNP-probe amplification, a linear relationship ($y_2 = -10.41x + 49.15$, $R^2 = 0.9301$) was also found, but the relationship was measured from 2×10^2 to 2×10^5 PFU ml⁻¹.

Compared with the blank, the results show that as low as 2 PFU ml⁻¹ of DENV2 could be detected by the QCM system when the AuNP probes were applied in the circulating-flow QCM system. However, the detection limit was around 100 PFU ml⁻¹ of DENV2 without the AuNP probes being applied. It indicates that the layer-by-layer AuNP-probes hybridization can markedly enhance the detection sensitivity by more than a hundred-fold. Beyond the detection limit, the AuNP probe can double as verifying the target sequences; therefore, a decrease in false-positives in the detection could be expected.

In the clinical diagnosis of infectious DENV by the detection of viral nucleic acid, the virus could be immediately detected after virus infection if the detection method was highly sensitive. The detection limit of the present method can reach to as low as 2 PFU ml⁻¹, which means that using the DNA-QCM biosensor combined with the technique of layer-by-layer AuNP-probes hybridization, DENV in clinical samples can be detected at the very early stage of virus infection. Compared with the SyBR green or TaqMan real-time PCR methods, whose detection limits are around 1–50 PFU ml⁻¹ of DENV [42, 43], the sensitivity and

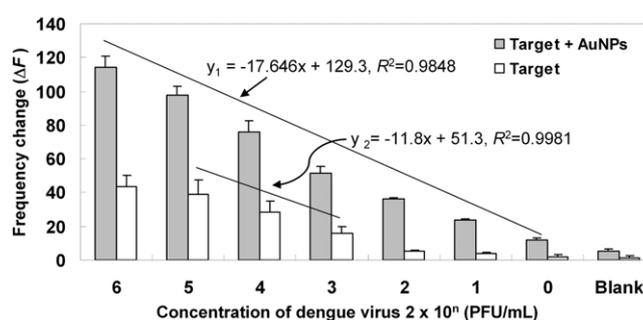


Figure 5. Sensitivity of the circulating-flow DNA-QCM sensor combined with layer-by-layer AuNP-probe (13 nm) amplification. Different titers of DENV2 (2×10^0 – 2×10^6 PFU ml⁻¹) were detected by the QCM system with and without AuNP-probe amplification. The blank control showed a background of $\Delta F = 2 \pm 1$ Hz in the samples without AuNP probe and $\Delta F = 7 \pm 1$ Hz in samples with AuNP-probes application. In the detection with layer-by-layer AuNP-probe amplification, a linear relationship was found between the ΔF versus log (PFU ml⁻¹ of DENV2) from 2×10^0 to 2.0×10^6 PFU ml⁻¹. In the detection without the AuNP-probe amplification, a linear relationship was also found, but the relationship was measured from 2×10^2 to 2×10^5 PFU ml⁻¹. Each value was derived from three independent detections and the error bars mean SD.

specificity of the present DNA-QCM biosensing method with nanoparticle technology showed it to be comparable to the fluorescent real-time PCR methods. However, the DNA-QCM biosensing method described herein was shown to not require expensive equipment, was label-free and had higher sensitivity because the specific AuNP probes were used as both detection amplifiers and verifiers (table 2).

4. Conclusions

DENV is nowadays the most important arthropod-spread virus affecting humans existing in more than 100 countries worldwide [1, 2]. In Taiwan, dengue has been a very important infectious disease since 1981 [47]. Effective surveillance and efficient control of the disease is dependent on rapid and sensitive diagnosis. In this study, we have shown a highly sensitive DNA-QCM sensor combined with the method of layer-by-layer AuNP-probe amplification. The DNA-QCM sensor was fabricated as a circulating-flow system which could

record in real-time the ΔF of the QCM chip. In this method, the specific oligonucleotide-functionalized AuNPs, AuNP probes, are used as both 'mass enhancer' and also 'sequence verifier' to increase the detection specificity and limitation. Using the DNA-QCM system, as low as 2 PFU ml^{-1} of DENV in clinical blood samples could be detected and a linear correlation between detection signal and virus titer was found from 2×10^0 to $2 \times 10^6 \text{ PFU ml}^{-1}$ of DENV. The DNA-QCM biosensing method developed would be useful for the early diagnosis of infectious DENV and very helpful in clarifying the epidemiology patterns of dengue infection. In the future, we will continue to integrate and optimize the detection system and examine clinical specimens or mosquitoes to define a quantitative detection for the infectious virus.

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