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Diagnosis and genotyping of *Plasmodium falciparum* by a DNA biosensor based on quartz crystal microbalance (QCM)

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Abstract

Background: Malaria infection with *Plasmodium falciparum* is an important basic health problem in the tropical and sub-tropical countries. The standard diagnostic method is blood film examination to visualize parasite morphology. However, in cases of low parasitemia or mixed infection with very low cryptic species, microscopy is not sensitive enough. Therefore, molecular techniques have been widely employed.
Methods: A label-free DNA biosensor based on quartz crystal microbalance (QCM) to diagnose and genotype *P. falciparum* was developed. Avidin-biotin interaction was used to coat the specific biotinylated probe on the gold surface of QCM. The gene encoding merozoite surface protein 2 (*msp2*) was amplified and the PCR products were then cut with restriction enzyme (MwoI). Enzymatic cutting made the PCR products suitable for QCM development. Hybridization between probe and enzymatic cutting DNA fragments resulted in frequency changes of the QCM.
Results: The newly developed QCM was tested for its diagnosis ability using both malaria laboratory strains and clinical isolates. The biosensor was sensitive at the sub-nanogram level, specific for only *P. falciparum* detection, no cross-reaction with *P. vivax*, and stable at room temperature for up to 6 months. Selection of *msp2* as a target gene and a genotyping marker made the QCM potentially useful for falciparum diagnosis simultaneously with genotyping. Potency was tested by genotyping two allelic families of *P. falciparum*, FC27 and IC1, using malaria laboratory strains, K1 and 3D7, respectively.
Conclusions: The dual function QCM was successfully developed with high sensitivity and specificity, and was cost-effective, stable and field adaptable.

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Introduction

Direct microscopic visualization of the parasite on a well-prepared and well-stained blood film is the gold standard diagnostic method of malaria infection. However, the efficacy of diagnosis is subject to many factors. Manual microscopic examination requires special training and considerable expertise. So it is not a reliable method when performed by non-experts, and it can miss a substantial proportion of malaria infections. Therefore, an automated computer vision of malaria without human intervention has been introduced (1). However, sometime no parasites can be seen in blood smears from patients with severe infections. This might possibly be due to partial antimalarial treatment or to sequestration of parasitized erythrocytes in deep vascular beds (2). In these cases, diagnosis by conventional microscopy is unlikely. Sub-microscopic infections need more sensitive methods based on identification of the malarial DNA by a molecular technique. Polymerase chain reaction (PCR) and other molecular methods have enabled measurement of sub-microscopic infections (3). The objective of this report was to create a new DNA biosensor based on quartz crystal microbalance (QCM) as a post-PCR method to diagnose *Plasmodium falciparum*. This new DNA biosensor can also be used in falciparum genotyping since the target gene is *msp2*, the most suitable single genotyping marker (4). We optimized and validated this new falciparum *msp2* biosensor on the genomic DNA of the laboratory strains and then applied this new method to a small set of clinical isolates. It is worth noting that this is the first study to develop a dual function QCM biosensor for malaria diagnosis and genotyping.

Materials and methods

Specimen preparation

This study was reviewed and approved by the Mahidol University Institutional Review Board (COA. No. MU-IRB 2008/170.1111).

An in vitro culture of *P. falciparum* was synchronized at the ring stage as described previously (5, 6). The parasite density was determined by microscopic examination. Parasite DNA was extracted using the PureGene DNA blood isolation kit (Qiagen, USA) according to the manufacturer's protocol. The total DNA concentration was measured using a spectrophotometric assay. The purified DNA was stored at –20°C until use. Two *P. falciparum* laboratory strains, K1

and 3D7, were used as representatives of the two *msp2* allelic families, FC27 and IC1, respectively.

Infected clinical blood samples (n = 11) were obtained from an endemic area and diagnosed by Giemsa-stained thick blood films. Parasite density was determined by Giemsa-stained thin blood films. Collection of blood samples on filter paper and DNA extraction from filter papers was performed by the TE (Tris-EDTA) buffer-based DNA isolation method (7). The DNA extract was stored at −20°C until use.

Preparation of amplified *msp2* targeted products and enzymatic cutting

The oligonucleotide sequences of primers to amplify the *msp2* gene were designed as described previously (8). The forward primer, ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA, and reverse primer, CTT TGT TAC CAT CGG TAC ATT CTT, were synthesized (1st BASE Pte Ltd., Singapore). The polymorphic repetitive regions, block 3 of *msp2*, was amplified by polymerase chain reaction (PCR) using Master Mix Solution (iNtRON Biotechnology, Seongnam, Korea) for 25 cycles. The PCR products were kept at −20°C until use. An aliquot of PCR products was verified by electrophoresis using 1.2% agarose gel (Invitrogen, USA) in Tris-borate-ethylenediamine tetraacetic acid buffer, pH 8.3. The gel was stained with ethidium bromide and visualized under UV transillumination.

The PCR products were further cut with the restriction endonuclease, HpyF10VI (MwoI) (Fermentas, USA), according to the manufacturer's protocol. After cutting, the expected DNA fragments were verified using 1.2% agarose gel electrophoresis and kept at −20°C until use.

Preparation of the falciparum *msp2* biosensor

Biotinylated probe complementary to the polymorphic block 3 of falciparum *msp 2* gene (5′-CTAGAACCATGCATATGTCC-3′) was synthesized (1st BASE Pte Ltd., Singapore) based on the sequence described previously (9).

The 12 MHz AT-cut quartz crystals with 4 mm gold electrode (0.1257 cm^2 area) fabricated on both sides were purchased from Kyocera-Kinseki Company, Thailand. During each measurement, only one side of the crystal was exposed to aqueous solution. The crystal frequencies were monitored using an in-house frequency counter (10). Major components of this counter are a PIC-microcontroller, oscillator circuit and the read out display. All experiments were performed at 22 ± 1°C.

The gold QCM surfaces were cleaned by exposure to a freshly prepared hot piranha solution consisting of H_2O_2 (30%), H_2SO_4 (70%) in a 1:3 ratio for 20 s. Next, the crystal was thoroughly washed with distilled water and dried in a vacuum chamber. The baseline resonance frequency of the cleaned QCM surface was recorded. Then the QCM surface was exposed to 10 mM of 3-mercaptopropionic acid (MPA) for 1 h, followed by water rinsing. Five microliters of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, 20 mM) was then placed on the surface, followed immediately by 5 μL of N-hydroxysuccinimide (NHS, 50 mM). These solutions were allowed to interact for 30 min in a 100% humidity environment to prevent evaporation of solution. The QCM surfaces were rinsed with water and immersed in an aqueous solution containing avidin at various concentrations (0–0.2 mg/mL) for 2 h, and then rinsed again. The optimal concentration of avidin was assessed.

To measure the number of avidin molecules immobilized on the QCM surfaces, the avidin layer at optimal concentration (0.1 mg/mL) was dried after adsorption and the frequency change measured. These dried QCM were not used for subsequent biotin interaction, as avidin was denatured upon drying. The deposited mass of avidin dried on the QCM surface was estimated by a Sauerbrey equation (11). The number of adsorbed avidin molecules were calculated using the molecular weight of avidin.

In order to be subsequently used, freshly prepared avidin adsorbed onto the QCM surface was exposed to 1 mM ethanolamine hydrochloride in a moisture chamber for 30 min and rinsed with distilled water. The synthesized biotinylated probe at various concentrations (0–2 μM) was placed on the surface and allowed to interact for another 30 min. The gold surface was then washed, dried and the change in frequency measured. The optimal concentration of biotinylated probe was assessed. The mass and number of immobilized probes on the QCM surfaces at optimal concentration (1.0 μM) was calculated (11). This probe immobilized QCM was then ready to be used as a falciparum *msp2* biosensor to diagnose and genotype *P. falciparum*.

Validation of the falciparum *msp2* biosensor

The diagnostic ability of the falciparum *msp2* biosensor was first tested using a laboratory strain of *P. falciparum*, K1. The malaria was grown and synchronized. The DNA was extracted and purified. Next, the block 3 of the *msp 2* gene was amplified and cut with MwoI. After cutting, the single stranded (ss) DNA was prepared by heating at 95°C for 10 min and placed on ice immediately for another 1–2 min. Ten microliters of the serial dilutions (0–250 ng/mL) of melting ss-DNA were applied on the surface of the *msp2* biosensor soaking with hybridization buffer. The hybridization reaction was performed in a moisture chamber for 30 min. Then the sensor was thoroughly washed, dried and the QCM frequency changes were measured. Hybridization was evidenced by in situ frequency changes compared to a control without DNA. Each DNA dilution was tested independently three times. Means and standard deviations were obtained.

Clinical application of the falciparum *msp2* biosensor was verified using malaria-infected blood samples. Eleven samples diagnosed by microscopic examination to have infection with malaria were collected using a filter paper technique. The DNA was extracted, amplified and cut as described. After enzymatic cutting, the DNA was denatured and hybridized with the falciparum *msp2* biosensor. The resonance frequency shift was recorded. The hybridization of each sample was performed in triplicate.

The genotyping ability of the falciparum *msp2* biosensor was investigated using two known allelic families of *P. falciparum*. Malaria laboratory strains K1 (a representative of FC27) and 3D7 (a representative of IC1) were cultivated and synchronized. The block 3 of *msp 2* gene of both clones was amplified, cut with MwoI and denatured by heating. The ss-DNA was hybridized with the *msp2* biosensor in a moisture chamber for 30 min. The sensor was washed and dried. Hybridization was validated by the frequency changes.

Sensor stability following storage in a sealed cap at room temperature for various time intervals was tested by hybridization with the known parasite DNA of K1 and 3D7. The stability of the biosensor was tested every 15 days up to 180 days. Each stability test was performed in triplicate.

Statistic analysis

All data shown in this study were means of the separate triplicate experiments. All comparison data were analyzed by a two-tailed Student's t-test. p-Values < 0.05 were considered to be statistically significant.

Results

Preparation of amplified *msp2* targeted products and enzymatic cutting

The polymorphic block 3 region of falciparum *msp 2* was amplified using the designed primers. The product of FC27 allelic family (K1 strain) was 727 bp, while that of IC1 (3D7 strain) was 703 bp (Figure 1). These PCR products could not be discriminated based on the number of base pairs. Therefore, cutting with a specific restriction enzyme was assessed. HpyF10VI (MwoI) was selected since it could cut the amplified products of both FC27 and IC1 into fragments with different suitable size for QCM development. After cutting, the product of FC27 showed two fragments at 555 bp and 172 bp, while that of IC1 was at 556 bp and 147 bp (Figure 2). The probe binding site of FC27 was on the 555 bp fragment while that of IC1 was on the 147 bp fragment.

Preparation of the falciparum *msp2* biosensor

The biotinylated probe was immobilized on the gold electrode of QCM surface based on the specific interaction between avidin and biotin. Prior to this immobilization, the QCM surface was pre-treated with MPA, EDC and NHS to add the ester group on the surface in order to bind with the amine group of avidin. These pretreatment steps could not be assessed since the QCM frequency changes were not measurable.

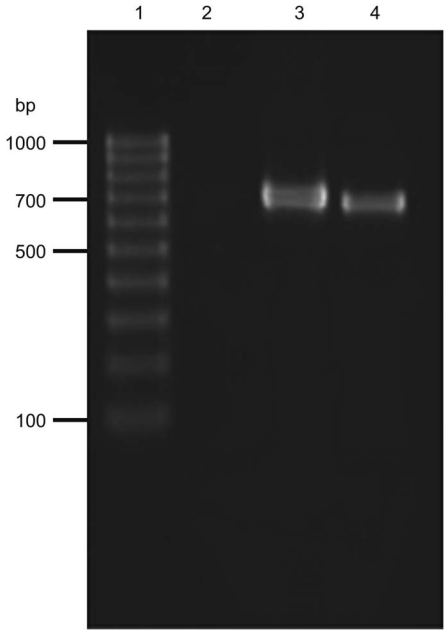

Figure 1 The amplified products of *msp2* from malaria laboratory strains (K1 and 3D7).
The malaria DNA was extracted and amplified by PCR using primers as described. The size of the target DNA was verified by 1.2% agarose gel electrophoresis. Lane 1 is DNA molecular weight marker. Lane 2 is control without DNA. Lane 3 and 4 are amplified *msp2* of K1 (FC27 allelic family) and 3D7 (IC1 allelic family).

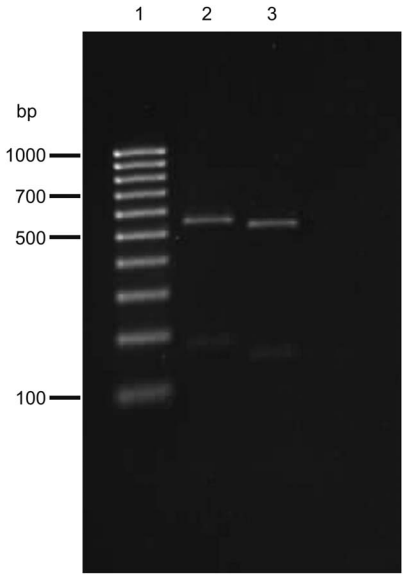

Figure 2 The amplified *msp2* of K1 and 3D7 after cutting with MwoI.
The PCR products of *msp2* of K1 and 3D7 were digested with a single cutter, MwoI, restriction enzyme. Lane 1 is DNA molecular weight marker. Lane 2 and 3 are amplified *msp2* gene of K1 and 3D7 after cutting.

Avidin adsorption at various concentrations (0–0.2 mg/mL) was monitored by the QCM frequency changes, and we found that the optimal concentration was 0.1 mg/mL (Figure 3). The frequency change at this concentration was 139.8 ± 4.60 Hz ($n = 3$). By converting the frequency change to deposited mass based on Sauerbrey equation, the number of adsorbed avidin molecules could be predicted. There were $4.94 \pm 0.16 \times 10^{11}$ molecules of avidin adsorbed on the QCM surface. The surface was treated with ethanolamide hydrochloride to block the non-specific binding sites before interacting with the biotinylated probe at various concentrations (0–2 μM). The avidin-biotin interaction was monitored in situ and found that the optimal probe concentration was 1 μM. The frequency change at this concentration was 79.0 ± 12.12 Hz, ($n = 3$) (Figure 3). Using a similar approach, the predicted number of biotinylated probes bound to avidin on the QCM surface was $1.97 \pm 0.30 \times 10^{12}$ molecules. The avidin: biotin binding ratio on the QCM biosensor surface was 1:3.9.

Validation of the falciparum *msp2* biosensor

The behavior of malaria DNA hybridization on the QCM surface is shown in Figure 4. The hybridization of target DNA to the biotinylated probe was saturated and was concentration dependent. The linear relationship between target DNA concentrations and QCM frequency changes were seen from 0–25 ng/mL. The binding of target DNA to probe showed saturation at concentrations from 25–250 ng/mL. As also shown in Figure 4, the falciparum *msp2* biosensor could detect target DNA as low as 0.025 ng/mL since the frequen-

cy change at this concentration was significantly higher than that of control without DNA ($p < 0.05$).

The diagnostic potential of the falciparum *msp2* biosensor was also evaluated using 11 clinical isolates. The results of frequency changes of all samples are summarized in Table 1. Samples infected with *P. vivax* (number 3 and 11) showed frequency changes < 10 Hz, which was not statistically different from that of the control ($p > 0.05$). However, all blood samples diagnosed either with single *P. falciparum* infection (number 1, 2–9) or mixed infection of *P. falciparum* and *P. vivax* (number 10) showed significant frequency changes when compared to the control ($p < 0.05$).

The genotypic ability of the *msp2* biosensor is shown in Figure 5. The malaria laboratory strains, K1 and 3D7, were used to represent the FC27 and the IC1 allelic family of *P. falciparum*, respectively. The DNA was extracted, amplified, cut, denatured and hybridized with the biotinylated probe of falciparum *msp2* biosensor. The QCM frequency changes after binding with target DNA of K1 strain were 89 ± 7 Hz, which was statistically different from that of 3D7 (36.67 ± 5.51 Hz, $p < 0.05$).

The stability of the *msp2* biosensor after storage at room temperature was evaluated using malaria laboratory strains, K1 and 3D7. The sensor could be kept for up to 180 days with no change in diagnosis and genotyping ability (Figure 6). The frequency changes at all time intervals were not significantly different from that of the freshly prepared sensor ($p > 0.05$).

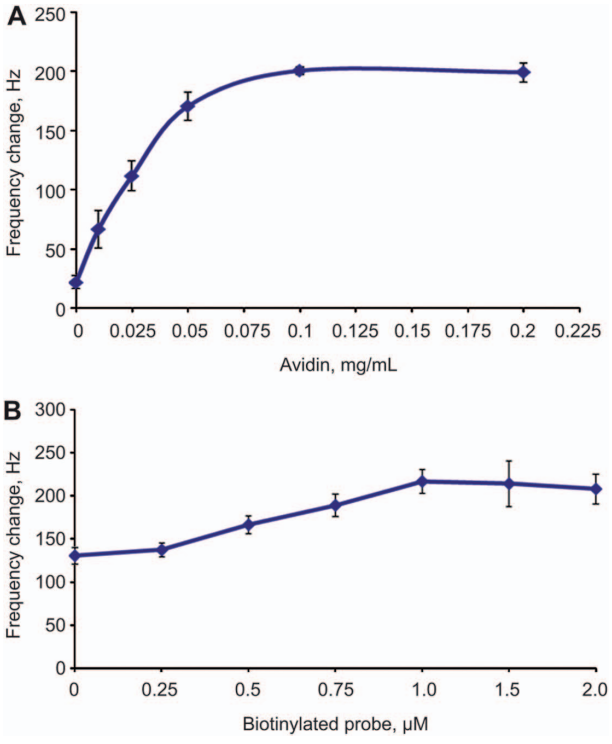

Figure 3 The optimal concentration of avidin (A) and biotinylated probe (B) immobilized on the gold surface of the quartz crystal.
The crystal surface was modified by MPA EDC and NHS, respectively. Various concentrations of avidin (0.0–0.2 mg/mL) were coated and frequency changes were measured. Then another quartz crystal surface was coated with optimal avidin concentration (0.1 mg/mL) followed by various concentrations (0.00–2.0 μM) of bioninylated probe solution. The frequency changes were also measured. The experiments were done in triplicate and the mean ± SD was shown.

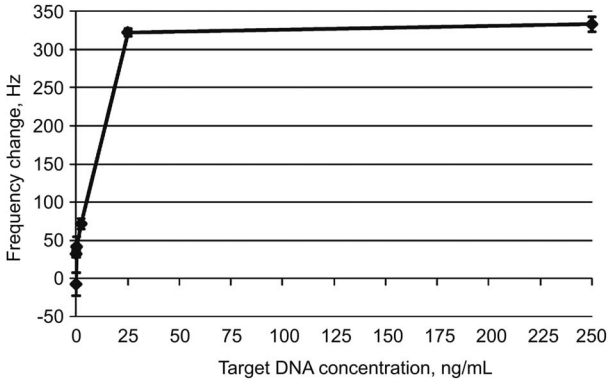

Figure 4 Relationship between falciparum target DNA concentrations and frequency changes after binding of the DNA to the falciparum *msp2* biosensor.
The DNA of parasitized blood of malaria laboratory strain, K1, was extracted, amplified, cut with MwoI and denatured. The DNA concentration was measured, diluted and hybridized with the sensor. The experiments were done in triplicate and the mean ± SD was shown.

Discussion

We created a novel technique to diagnose and genotype malaria infection due to *P. falciparum*. This technique was based on DNA piezoelectric biosensor using gold electrode of quartz crystal microbalance (QCM). The deposited mass due to DNA hybridization on the QCM surface resulted in a shift of the quartz resonance frequency (11). In this QCM development, we focused on the hybridization between the immobilized bionylated DNA probe and the specific DNA fragments of polymorphic block 3 of falciparum *msp2* on the

Table 1 Clinical application of the falciparum *msp2* biosensor to diagnose specimens infected with malaria as diagnosed by microscopic examination.

	Frequency change, Hz	Microscopic diagnosis
Target control	-35.33 ± 5.03	–
Sample 1	48.67 ± 4.43	*P. falciparum*
Sample 2	53.00 ± 9.44	*P. falciparum*
Sample 3	8.67 ± 7.24	*P. vivax*
Sample 4	124.00 ± 27.78	*P. falciparum*
Sample 5	148.00 ± 13.23	*P. falciparum*
Sample 6	174.00 ± 64.58	*P. falciparum*
Sample 7	125.67 ± 36.90	*P. falciparum*
Sample 8	139.67 ± 22.19	*P. falciparum*
Sample 9	192.67 ± 36.12	*P. falciparum*
Sample 10	193.33 ± 20.98	Mixed infection
Sample 11	4.33 ± 5.50	*P. vivax*

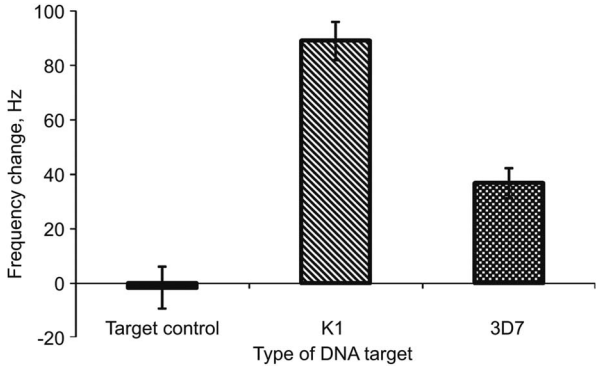

Figure 5 Genotypic ability of the falciparum *msp2* biosensor.
The DNA of blood of malaria laboratory strains (K1 and 3D7) was extracted, amplified, cut with MwoI and denatured. The changes in resonance frequency after hybridization with the *msp2* biosensor were measured and compared to control without DNA. The experiments were done in triplicate and the mean ± SD was shown.

QCM surface. The *msp2* is a suitable single molecular marker for falciparum genotyping (12). Therefore, one QCM sensor could be used simultaneously for both diagnosis and genotyping of *P. falciparum*.

The QCM was adopted in the present study because of its great sensitivity. It is capable of measuring sub-nanogram mass changes and does not require post-treatment of target samples (13). The 12-MHz AT-cut quartz crystal was used because crystals with high-frequency (>10 MHz) can increase the QCM sensitivity (14). The QCM surface was sequentially treated with the immobilization reagents, MPA, EDC and NHS in order to prepare the surface for avidin adsorption. However, these immobilization steps could not be assessed separately because the frequency changes of each step were not measurable. The frequency change could be measured after avidin adsorption.

Various concentration of aqueous avidin were coated and the frequency changes were measured (Figure 3). The optimal avidin concentration (0.1 mg/mL) was consistent with previous studies (15). Since the quartz oscillation was directly proportional to changes in deposited mass on the QCM surface (16), the experimental deposited avidin mass could be obtained using the Sauerbrey equation (11). From this mass and molecular weight, the number of adsorbed avidin could then be calculated as $4.94 \pm 0.16 \times 10^{11}$ molecules. Theoretical calculations based on avidin dimensions ($6.0 \times 5.5 \times 4.0$ nm) (17) and the occupying area on the QCM surface (0.1257 cm^2) indicated that the expected number of avidin molecules adsorbed as a monomolecular layer in a flat orientation should not exceed 5.18×10^{11} molecule. Therefore, the experimental number of $4.94 \pm 0.16 \times 10^{11}$ molecules of avidin in this study could be assumed to be a monolayer.

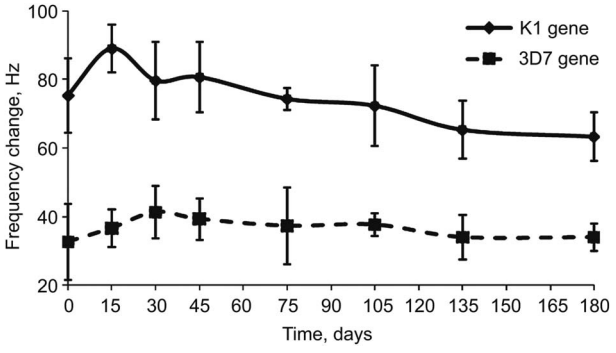

Figure 6 Stability of the QCM-*msp2* biosensor after storage in sealed cap at room temperature for 180 days.
Every 15 days the sensor was tested by hybridization reaction with the amplified and enzymatic cut *msp2* of K1 (above line) and 3D7 (lower line). The frequency changes at each time interval were statistically compared to the freshly prepared sensor. The experiments were done in triplicate and the mean ± SD was shown.

Since avidin was denatured by drying (17), the avidin layers were not dried when subsequently coated with the biotinylated probe. The optimal probe concentration was assessed to be 1 μM which was consistent with the previous reports (15). By similar calculations, it could also be estimated that there were $1.97 \pm 0.30 \times 10^{12}$ molecules of the biotinylated probe bound to avidin on the QCM surface. Therefore, the binding ratio of avidin: biotin was 1:3.9. Although the ratio was close to the theoretical ratio (1:4) but for immobilized avidin the ratio might be too high. The experimental ratio of 1:3.9 indicated some non-specific binding, which could not be completely blocked by ethanolamine hydrochloride as described previously (16). However, this newly developed falciparum *msp2* biosensor was tested for its diagnostic and genotypic ability.

The diagnostic ability was verified using a laboratory-maintained strain of *P. falciparum*, K1. The DNA of K1 was extracted and purified. The target DNA encoding *msp2* gene was amplified, cut and hybridized with the falciparum *msp2* biosensor. Serial dilutions of target DNA from 0–250 ng/mL were assessed by the QCM biosensor. Results shown in Figure 4 indicated a saturation binding of target DNA to the biotinylated probe adsorbed on the QCM surface. As also shown in Figure 4, the detection sensitivity of the new falciparum *msp2* biosensor was ≤ 0.025 ng/mL of target DNA. This sub-nanogram sensitivity is consistent with the characteristic of DNA biosensor (18). However, the target DNA solution contained not only complementary ss-DNA which could bind the probe, but also the non-complementary ss-DNA. Therefore, the true sensitivity might be ≤ 0.0125 ng/mL. It was reported previously that 10 pg of purified malaria DNA was approximately equal to 100 parasites (19). Presumably, the sensitivity of the new falciparum *msp2* biosensor developed in this study was 25 parasites in 10 μL of DNA solution applied on the QCM surface. This finding indicated that the sensitivity of this biosensor was comparable to other molecular techniques, since tests based on PCR can detect as few as 10 parasites/μL blood (20).

The clinical application of the new falciparum *msp2* biosensor was tested using 11 blood samples that were diagnosed by standard microscopic examination. As shown in Table 1, all samples infected with *P. falciparum*, both single and mixed infections, showed significant QCM frequency

changes compared to control. However, blood samples infected with *P. vivax* showed no cross-reaction with the falciparum *msp2* biosensor because the QCM frequency change was not different from that of the control. The results showed high specificity of the new *msp2* biosensor. The high sensitivity (Figure 4) as well as the high specificity (Table 1) of the falciparum *msp2* biosensor indicated the potential of the sensor to identify *P. falciparum* infected as a cryptic species of mixed infection. The cryptic *P. falciparum* infections were reported in 21 of 160 samples diagnosed with vivax malaria (21). It is essential to diagnose falciparum cryptic infection since it is the most severe and fatal malaria infection (22).

In terms of genotyping potency, we tested this falciparum *msp2* biosensor using laboratory-maintained strains. Since the *msp2* gene can be grouped into FC27 and IC1 allelic family (23), K1 and 3D7 were used to represent these two allelic families, respectively. Both K1 and 3D7 were grown and synchronized to the ring stage because this is the stage that is found in most clinical specimens (22). The block 3 region of the *msp2* gene was amplified using the designed primers in order to obtain DNA fragments as short as possible. This is because the suitable DNA used in DNA biosensor technology should not be longer than 500 bp. Long fragments will cause the effect of steric hindrance during DNA binding (24). With the primers used in this study, the obtained PCR products of K1 and 3D7 were 727 bp and 703 bp, respectively (Figure 1). Certainly, these products could not be distinguished based on mass difference. Therefore, digestion with the optimal restriction enzymes was required. The selected enzyme was HpyF10VI (MwoI), since after cutting, the 727 bp and 703 bp produced fragments containing probe binding site at 555 bp and 147 bp, respectively (Figure 2). The QCM frequency changes of enzymatic cut DNA fragments of K1 and 3D7 binding on the QCM surfaces were statistically different ($p < 0.05$) (Figure 5). The results showed clear discriminative ability of the new sensor to genotype two allelic families of *P. falciparum*.

All of the results indicated dual potency of the newly developed falciparum *msp2* biosensor. It could be used to diagnose and genotype falciparum malaria simultaneously. In term of diagnosis, it is sensitive for detecting parasite DNA at the sub-nanogram level. It is specific for detecting only *P. falciparum* with no cross-reaction with *P. vivax*. These two properties widen the sensor potency to detect falciparum cryptic infection. Moreover, the new sensor is cost-effective since both sides of the quartz gold surface can be used separately. Therefore, one quartz crystal can be used twice which will reduce the sensor price to 50%.

In term of genotyping, the addition of enzymatic cutting after PCR is an important step to use one sensor to distinguish two allelic families of *P. falciparum*. It should be noted that this is the first report to develop QCM to genotype malaria infection. Genotyping potency makes the sensor useful for malaria drug resistance surveillance sites. The sensor can be easily transported to be used in the remote area since it is stable at room temperature for up to 6 months. As shown in Figure 6, its diagnostic and genotyping potency after storage were not different from that of freshly prepared sensor. This implied the field utility of the new biosensor.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
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Employment or leadership: None declared.
Honorarium: None declared.

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