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# Allele-Specific Genotype Detection of Factor V Leiden Mutation from Polymerase Chain Reaction Amplicons Based on Label-Free Electrochemical Genosensor

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**An electrochemical genosensor for the genotype detection of allele-specific factor V Leiden mutation from PCR amplicons using the intrinsic guanine signal is described. The biosensor relies on the immobilization of the 21-mer inosine-substituted oligonucleotide capture probes related to the wild-type or mutant-type amplicons, and these probes are hybridized with their complementary DNA sequences at a carbon paste electrode (CPE). The extent of hybridization between the probe and target sequences was determined by using the oxidation signal of guanine in connection with differential pulse voltammetry (DPV). The guanine signal was monitored as a result of the specific hybridization between the probe and amplicon at the CPE surface. No label-binding step was necessary, and the appearance of the guanine signal shortened the assay time and simplified the detection of the factor V Leiden mutation from polymerase chain reaction (PCR)-amplified amplicons. The discrimination between the homozygous and heterozygous mutations was also established by comparing the peak currents of the guanine signals. Numerous factors affecting the hybridization and nonspecific binding events were optimized to detect down to 51.14 fmol/mL target DNA. With the help of the appearance of the guanine signal, the yes/no system is established for the electrochemical detection of allele-specific mutation on factor V for the first time. Features of this protocol are discussed and optimized.**

The detection of specific DNA sequences provides the basis for detecting a wide variety of infectious and inherited diseases. Traditional methods for DNA sequencing based on the coupling of electrophoretic separations and radioisotopic detection are labor-intensive and time-consuming and are thus not well-suited for routine and rapid medical analysis, particularly for point-of-care tasks. Electrochemical hybridization biosensors for the detection of DNA sequences may greatly reduce the assay time and simplify its protocol. Such fast on-site monitoring schemes are required for quick preventive action and early diagnosis. Therefore, electrochemical monitoring of DNA hybridization has recently been an attractive research area.[1−6]

Oligonucleotides labeled with enzymes such as horseradish peroxidase[7] and alkaline phosphatase[8] have been employed in hybridization detection protocols. The basis of the hybridization detection scheme of Umek et al.[9] depended on the ferrocene-modified adenine-containing signaling oligonucleotides. After hybridization with the target DNA, the self-assembled monolayer (SAM) allowed electron transfer between ferrocene and the gold electrode surface. Umek et al.[9] detected several inherited and infectious diseases, including hemochromatosis and HIV, from the polymerase chain reaction (PCR)-amplified samples by using alternating current voltammetry.

The use of inosine-substituted probes and the appearance of a guanine signal upon hybridization with the target opened a new field in electrochemical research. This procedure eliminated the external labels and shortened the assay time. The chemical mechanism of the oxidation of guanine was reported in detail by Steenken et al.[10] Guanine was reported to be the most redox-active nitrogenous base in DNA. Since then, many reports have used the oxidation signal of guanine for the detection of hybridization. For example, Wang et al.[11,12] attached biotinylated inosine-

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substituted oligonucleotides onto strepavidin-coated magnetic beads. Magnetic separation greatly eliminated the nonspecific adsorption effects. Wang et al.[13] described a colloidal gold tag for eletrochemical detection and amplification of DNA hybridization. Biotinylated target strands were used in the hybridization process. These target strands bound the streptavidin-coated gold nanoparticles. The acid dissolution of gold tags was monitored by using disposable carbon strip electrodes. Yang et al.[14] reported a catalytic guanine oxidation protocol for the detection of trinucleotide repeat expansions in connection with cyclic voltammetry at an indium tin oxide electrode with the ruthenium complexes as the oxidation catalysts. Tomschik et al.[15] observed the oxidation signals of guanine and adenine at low concentrations of DNA and PNA by applying chronopotentiometry and differential pulse voltammetry (DPV) and square wave voltammetry (SWV) with a suitable baseline correction system at a pyrolytic graphite electrode (PGE). Wang et al.[16] described an indicator-free electrochemical DNA biosensor protocol that involves the immobilization of inosine-substituted (guanine-free) probe onto a carbon paste electrode (CPE), and the detection of hybrid formation was performed by using the appearance of the guanine oxidation signal of the target in connection with chronopotentiometric stripping analysis (PSA). A pencil-based renewable biosensor for label-free electrochemical detection of hybridization was also reported by Wang et al.[17]

The factor V Leiden mutation, also designated as 1691 G > A or R506Q, is the major heritable risk factor for venous thromboembolism.[18] This mutation in the coagulation factor V gene results in the resistance of factor V to inactivation by activated protein C (APC).[19] Approximately 5% of Caucasians are either heterozygous carriers or homozygous for the factor V Leiden mutation; the prevalence is reported to be lower for other ethnic groups.[20] Potter et al.[21] have developed a simplified and robust assay using oligonucleotide probes for normal and mutant factor V Leiden mutation sequences, labeled with europium and samarium, respectively, and measured by time-resolved fluorescence. The electronic array analysis of amplicons for factor V mutation by using strand displacement amplification was described.[22−24] After electronic addressing of the amplicons to specific array locations, the reporter oligonucleotides modified with either Cy3 or Cy5 were added, and fluorescence at each location was quantified.

Here for the first time, we describe an electrochemical DNA biosensor for the detection of factor V Leiden mutation and the discrimination of the mutation type using the oxidation signal of guanine in connection with DPV. There have not yet been any literature reports about the detection of heterozygous or homozygous mutations from PCR-amplified amplicons by using the guanine signal without any modifications in the native bases or any external labels. The features of the protocol are discussed, and results are compared with the other protocols previously reported.

## EXPERIMENTAL SECTION

**Apparatus.** The hybridization between capture probes and PCR-amplified amplicons was investigated by using differential pulse voltammetry with an AUTOLAB PGSTAT 30 electrochemical analysis system (Eco Chemie, The Netherlands). The UV−visible spectrophotometer (Schimadzu, Japan) was used with quartz cuvettes of 1 mL and 10 mm path length (Starna, U.K.). The three-electrode system consisted of the carbon paste electrode as the working electrode, a reference electrode (Ag/AgCl), and a platinum wire as the auxiliary electrode. The body of the CPE was a glass tube (3 mm i.d.) tightly packed with the carbon paste. The electrical contact was provided by a copper wire inserted into the carbon paste. Carbon paste was prepared in the usual way by hand-mixing graphite powder (Fisher) and mineral oil (Acheson 38) in a 70:30 mass ratio. The surface was polished on a weighing paper to a smoothed finish before use. The convective transport was provided by a magnetic stirrer.

**Chemicals.** The PCR-amplified amplicons were kindly donated by the Department of Medicinal Biology from the Faculty of Medicine in Ege University.

The 23-mer synthetic oligonucleotides of the WT and MT capture probes and their complementary targets were purchased (as lyophilized powder) from Thermo Hybaid (Erlangen, Germany); their base sequences are as follows:

Wild-type (WT) capture probe:
5′-AAT ACC TIT ATT CCT CIC CTI TC-3′
Wild-type target:
5′-GAC AGG CGA GGA ATA CAG GTA TT-3′
Mutant (MT) capture probe:
5′-AAT ACC TIT ATT CCT **T**IC CTI TC-3′
Mutant target:
5′-GAC AGG CAA GGA ATA CAG GTA TT-3′

The capture probes immobilized on the CPE had the same sequence as a region of the gene for factor V. This sequence was included in the DNA fragment amplified by PCR. Factor V Leiden mutation is caused by a point mutation from G to A at the 1691st position of the human genome.[25] A 23-mer inosine-substituted factor V oligonucleotide served as a wild-type capture probe. A second capture probe contained a single G-to-A transition at position 16 from the 5′ end of the oligonucleotide. Thus, capture probes that were perfect complements to the wild-type (WT) or mutant (MT) factor V alleles were used for our investigations. Amplicons containing the SNP were prepared using restriction fragment length polymorphism (RFLP). This PCR technique was


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used for patient DNAs previously characterized as homozygous WT, homozygous MT, or heterozygous. The amplicons were 224 bp DNA fragments. In total, 30 patient samples of each genotype were studied. The amplicons were analyzed independently using both CPEs modified with WT and MT capture probes.

All oligonucleotide stock solutions (100 mg/L) were prepared with TE solution (10 mM Tris−HCl, 1 mM EDTA, pH 8.00) and kept frozen. More dilute solutions of capture probes were prepared using 0.5 M acetate buffer (pH 4.80) containing 20 mM NaCl. More dilute solutions of targets were prepared using 20 mM Tris−HCl buffer solution (pH 7.0) containing 20 mM NaCl. The concentrations of all oligonucleotides and PCR products were determined by following the spectrophotometric method in which one $A_{260}$ unit of double-stranded DNA represented 50 $\mu$g/mL and one $A_{260}$ unit of single-stranded DNA represented 33 $\mu$g/mL.[26]

Other chemicals were of analytical reagent grade. In-house sterilized and deionized water was used in all solutions. All buffer solutions contained 20 mM NaCl to provide desired ionic strength.

**Methods.** The hybridization detection of amplicons was transduced by means of DPV. The guanine oxidation peak height after baseline fitting was used as the analytical signal. The procedure of hybridization detection of the PCR-amplified amplicons consisted of the following steps:

*Capture Probe Immobilization.* The CPE was pretreated by applying +1.70 V for 1 min in 0.05 M phosphate buffer solution (PBS) (pH 7.40). The WT or MT capture probe was subsequently immobilized onto the pretreated CPE by applying a potential of +0.50 V for 5 min in the 0.50 M acetate buffer solution (ABS) (pH 4.8) containing 10 $\mu$g/mL WT or MT probe with 200 rpm stirring. After immobilization, the WT or MT capture-probe-modified CPE was washed with blank ABS.

*Hybridization with the Synthetic Target.* The capture-probe-modified CPE was inverted, and ~10 $\mu$L of 20 mM Tris−HCl buffer solution (TBS) (pH 7.00) containing 15 $\mu$g/mL DNA target was pipetted onto the surface. The hybridization was allowed to proceed for 6 min. Nonspecific adsorption effect was eliminated with the following washing step. The hybrid-modified CPE was dipped into the 1% sodium dodecylsulfate dissolved in TBS (TBS−SDS) for 3 s and then immediately dipped into blank TBS for 3 s. Hybridization with only one-base-mismatch-containing DNA target was monitored following the same method as described above.

*Hybridization with DNA Fragments Obtained from PCR Amplification.* The amplicon obtained from the PCR amplification was diluted with TBS; the diluted sample was then placed in a vial and denatured by heating in a water bath at 95 °C for 6 min and subsequent freezing in an ice bath for 2 min. After the immobilization of the probe, the CPE was inverted, and ~10 $\mu$L of the denatured sample was pipetted directly onto the capture-probe-modified electrode surface. The hybridization was allowed to proceed for 6 min. The hybrid-modified CPE was then dipped into TBS−SDS for 3 s and then immediately dipped into blank TBS for 3 s.

*Voltammetric Transduction.* The oxidation signal of guanine was measured by using differential pulse voltammetry in blank ABS by scanning from +0.80 to +1.40 V. The raw data were treated using the Savitzky and Golay filter (level 2) of the General Purpose Electrochemical Software (GPES) of Eco Chemie (The Netherlands) with moving average baseline correction, using a "peak width" of 0.01.

Control experiments were performed with an amplicon that contained a PCR product that has a sequence complementary to a completely different probe, which was used for the detection of hepatitis B virus (noncomplementary as a negative amplicon). Both of the probes were tested with this negative amplicon. Hepatitis B virus amplicon was obtained from the Department of Gastroenterology in the Faculty of Medicine, Ege University. The WT probe was also challenged with the one-base mismatched amplicon that contained a sequence complementary to the MT probe, and the MT probe was challenged with the one-base-mismatched amplicon that contained a sequence complementary to the WT probe. The MT and WT probes were also challenged with the PCR blank solution, which contained the primers and polymerase without the target amplified DNA.

The amplicons of factor V that were used for the voltammetric measurements had been characterized by the method performed previously[27] with 1.5% agarose gel electrophoresis in the Department of Medicinal Biology of the Faculty of Medicine, Ege University. Thus, the results of the electrochemical biosensor were confirmed with the ones obtained from the conventional method.

## RESULTS AND DISCUSSION

The detection of PCR-amplified amplicons by using an electrochemical DNA biosensor relies on the differential pulse voltammetric transduction of the hybridization reaction between the factor V Leiden wild-type or mutant probes and target DNA sequences, which are present in the PCR-amplified amplicons. An aliquot of the amplified amplicon is simply diluted in the hybridization buffer solution and then introduced onto the probe-immobilized electrode. The detection of hybridization is accomplished with the appearance of the guanine oxidation signal,[10] whereas no guanine signal is obtained from inosine-substituted probes. DPV provides a novel way to monitor hybridization with lower detection limits than the ones obtained by using potentiometric stripping analysis and square wave voltammetry.[28] The decrease in the magnitude of the guanine oxidation peak thus reflects the heterozygous genomic DNA at the hybrid-modified CPE.

Figure 1A represents the guanine signals obtained when the 10 $\mu$g/mL WT capture probe was immobilized on the CPE surface. The best discrimination of hybridization was to be able to see a guanine oxidation signal or not. The appearance of the guanine signal after hybridization with the synthetic target was used as the analytical signal (Figure 1A-a). No guanine signal was observed with the inosine-substituted WT probe-modified CPE (Figure 1A-b). Hybridization of the WT probe with PCR blank solution did not give rise to any guanine peaks (Figure 1A-c).

Figure 1B represents the guanine signals obtained when the 10 $\mu$g/mL MT capture probe for factor V Leiden point mutation was immobilized on the CPE surface. The MT capture probe was also able to detect the 15 $\mu$g/mL factor V MT-DNA target sequence. The appearance of the guanine signal indicated the formation of a hybrid (Figure 1B-a). No guanine signal was observed at the inosine-substituted MT probe-modified CPE

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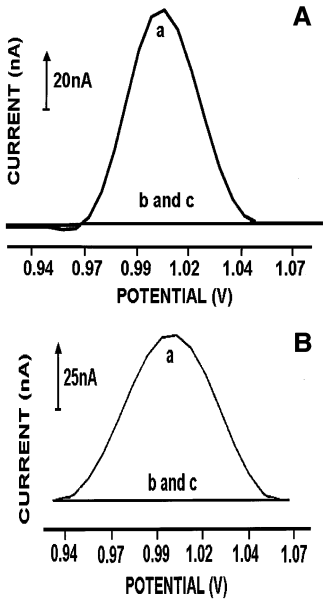


Figure 1. Differential pulse voltammograms for the oxidation signals of guanine in ABS at (A) 10 $\mu$g/mL wild-type (WT) capture-probe-modified CPE after hybridization with (a) 15 $\mu$g/mL synthetic target, (b) no target, and (c) 1/40 diluted PCR blank solution containing TBS; and (B) 10 $\mu$g/mL mutant (MT) capture-probe-modified CPE after hybridization with (a) 15 $\mu$g/mL synthetic target, (b) no target, and (c) 1/40 diluted PCR blank solution containing TBS.

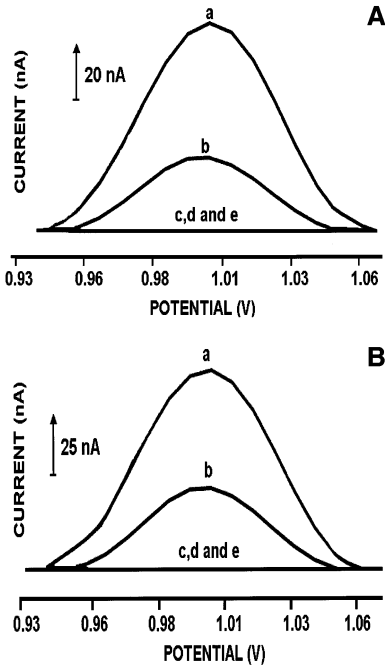


Figure 2. Differential pulse voltammograms for the oxidation signals of guanine in ABS at (A) 10 $\mu$g/mL wild-type (WT) capture-probe-modified CPE after hybridization with (a) 18.25 $\mu$g/mL homozygous WT PCR amplicon, (b) 18.25 $\mu$g/mL heterozygous mutant (MT) PCR amplicon, (c) no PCR amplicon, (d) 18.25 $\mu$g/mL homozygous MT PCR amplicon, and (e) 18.25 $\mu$g/mL negative PCR amplicon containing TBS; and (B) 10 $\mu$g/mL MT capture-probe-modified CPE after hybridization with (a) 15 $\mu$g/mL homozygous MT PCR amplicon, (b) 18.25 $\mu$g/mL heterozygous MT PCR amplicon, (c) no PCR amplicon, (d) 18.25 $\mu$g/mL homozygous WT PCR amplicon, and (e) 18.25 $\mu$g/mL negative PCR amplicon containing TBS.

(Figure 1B-b). Hybridization of the MT probe with PCR blank solution did not give rise to any guanine peaks, either (Figure 1B-c).

Figure 2 represents the hybridization detection studies with PCR amplicons. The appearance of the guanine signal obtained with a positive amplicon containing the target DNA sequence confirmed hybridization. When the WT capture probe was immobilized on the CPE surface, the high guanine oxidation signal (Figure 2A-a) showed that the patient had homozygous WT factor V DNA. A decrease in the voltammetric signal (Figure 2A-b) to approximately one-half its intensity showed that the patient had heterozygous mutation. No guanine signal was observed from the inosine-substituted WT probe-modified CPE (Figure 2A-c). With the help of a TBS−SDS washing step for 3 s, all of the nonspecifically adsorbed PCR product of homozygous mutant patient (Figure 2A-d) was removed. A longer TBS−SDS washing step from 5 s up to 1 min removed all material not firmly attached onto the surface.

Both of the alleles of the homozygous mutant patient contained the point mutation. Thus, none of the strands of the PCR product was complementary to the WT probe, and no hybridization occurred. A negative amplicon (Figure 2A-e), that belonging to the hepatitis B virus gene, did not give rise to any signals, either.

In an inverse setup, when the MT capture probe was immobilized on the CPE surface, the high guanine oxidation signal (Figure 2B-a) showed that the patient had homozygous mutant DNA. An almost one-half decrease in the voltammetric signal (Figure 2B-b) showed that the patient had heterozygous mutation. No guanine signal was observed from the inosine-substituted MT probe-modified CPE (Figure 2B-c). Both of the alleles of the homozygous patient contained no point mutation. Thus, none of the strands of the PCR product was complementary to the MT probe, and no guanine signals appeared (Figure 2B-d). A negative amplicon (Figure 2A-e), which was fully noncomplementary, did not yield any signals, either.

The six subsequent experiments for the detection of hybridization between the WT and MT capture probes and the target DNA from thirty amplicons gave reproducible results. The guanine signal obtained from the WT probe-modified CPE after hybridization with homozygous amplicons gave a relative standard deviation (RSD) value of 8.12%. The guanine signal obtained from the hybridization of the WT probe with the heterozygous amplicons a RSD value of 7.84%. The guanine signal obtained from the MT probe-modified CPE after hybridization with homozygous amplicons gave a RSD value of 9.56%. The guanine signal obtained from the hybridization of the WT probe with the heterozygous amplicons gave a RSD value of 9.43%. The results obtained with the amplicons tested with WT and MT capture probes are shown in Figure 3. The electrochemical microarray assembly that is planned to be microfabricated in our laboratory is illustrated in the inset of Figure 3. No signal was obtained from the MT probe-modified working electrode B when the denatured WT homozygous amplicon was applied on the surface. In contrast, the WT capture-probe-modified CPE (A) gave a very high guanine signal. Nearly one-half of the homozygous guanine signal was obtained from electrodes C and D when heterozygous amplicon was applied. Each one of the strands of the heterozygous amplicon contained a sequence complementary to one of the probes, thus giving rise to guanine signals, as shown by C and D. The washing step with

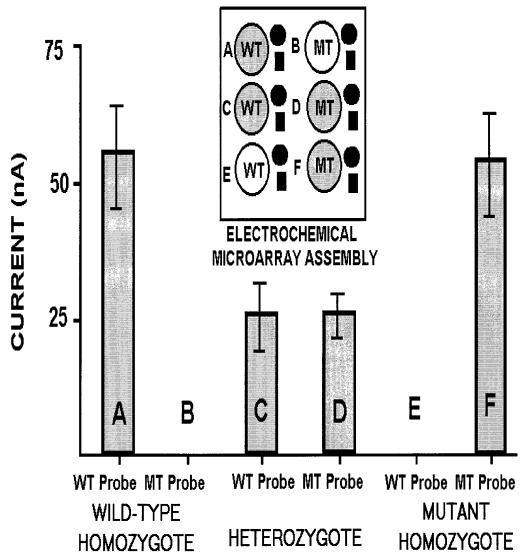


Figure 3. Histograms with error bars of six measurements of the electrochemical signals obtained from the microarray assembly. Working electrodes A, C, and E were modified with the wild-type (WT) capture probe; working electrodes B, D, and F were modified with the mutant (MT) capture probe. WT homozygous amplicon was applied to A and B. Heterozygous amplicon was applied to C and D. MT homozygous amplicon was applied to E and F. Inset: electrochemical microarray assembly design for microfabrication where the gray circle represents the working electrode, the black circle represents the reference electrode, and the black rectangle represents the auxiliary electrode.

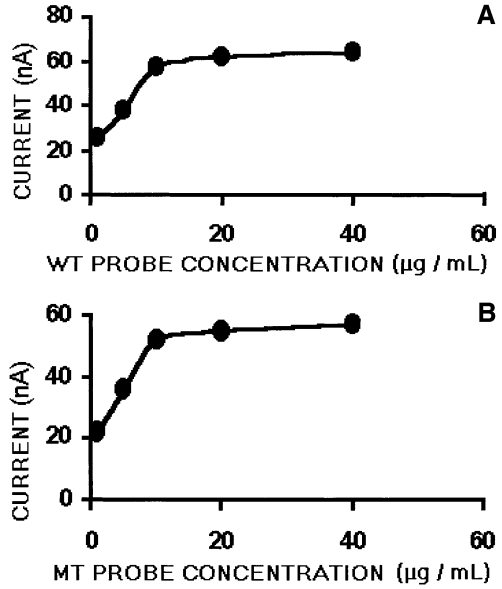


Figure 4. Calibration plot for the dependence of the guanine oxidation signal upon increasing the concentration of the (A) WT and (B) MT capture probe immobilized on the CPE surface.

TBS−SDS removed all of the nonspecifically adsorbed one-base-mismatched MT homozygous amplicon, and thus, no signal was observed at electrode E. MT homozygous amplicon gave a signal only on the MT capture-probe-modified CPE (F).

The effects of probe concentration on the guanine (Figure 4) signals were observed. The target concentration was kept constant at 15 $\mu$g/mL, and the WT (Figure 4A) and MT capture probe (Figure 4B) concentrations were increased from 1 $\mu$g/mL to 40 $\mu$g/mL. When the probe concentration for the hybridization with 15 $\mu$g/mL synthetic DNA target was increased from 1 $\mu$g/mL to 10 $\mu$g/mL, the guanine signal increased. Both of the probes even as low as 1 $\mu$g/mL responded to the target. The highest hybrid signal was obtained from a 10 $\mu$g/mL probe with 15 $\mu$g/mL target, which practically indicated full surface coverage of the probe-modified CPE. When the probe concentration was increased to 20 $\mu$g/mL and more, the guanine signal remained almost constant. Thus, an optimum capture probe concentration of 10 $\mu$g/mL was employed. The detection limits, estimated from S/N = 3, correspond to a 61.64 fmol/mL WT capture probe and a 52.63 fmol/mL MT capture probe at the CPE.

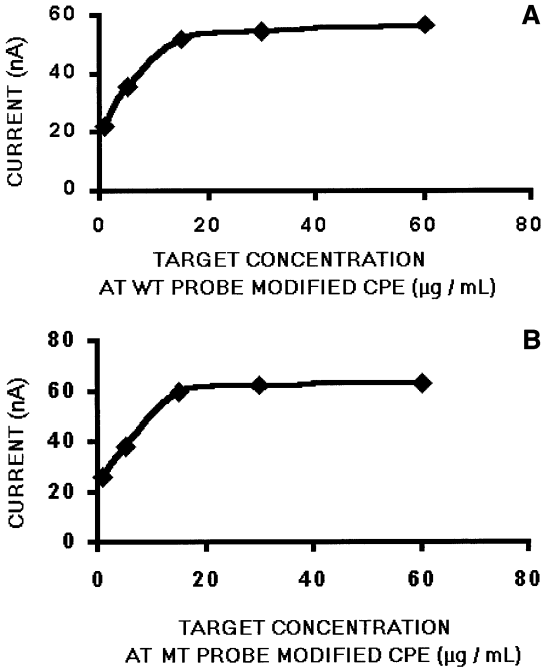


Figure 5. Calibration plot for the dependence of the guanine oxidation signal upon increasing the concentration of the synthetic target at (A) WT and (B) MT capture-probe-modified CPEs.

The effect of target concentration on the guanine signals was also observed in Figure 5. When the target concentration for WT (Figure 5A) or MT (Figure 5B) probe-modified CPEs was increased from 1 to 15 $\mu$g/mL target, the guanine signal linearly increased. The DNA probe slightly responded to target concentrations even as low as 1 $\mu$g/mL. When the capture probes were exposed to target concentrations of 20 $\mu$g/mL and more, the guanine signal remained almost constant until 60 $\mu$g/mL target. It was concluded that as the target DNA concentration increased, the flanking nonspecifically adsorbed oligonucleotides were moved away at the washing step with TBS−SDS. The best discrimination between probe and hybrid signals was observed when 10 $\mu$g/mL capture probe and 15 $\mu$g/mL target DNA were employed. The detection limits, estimated from S/N = 3, correspond to a 60.31 fmol/mL target at the WT capture-probe-modified CPE and a 51.14 fmol/mL target at the MT capture-probe-modified CPE.

The effect of sample dilution upon the hybridization signal was also investigated (Figure 6). The voltammetric signal obtained from the hybridization reaction between the probe-modified CPE and the complementary sequence in the PCR-amplified amplicon increased linearly with sample dilution up to 18.25 $\mu$g/mL and

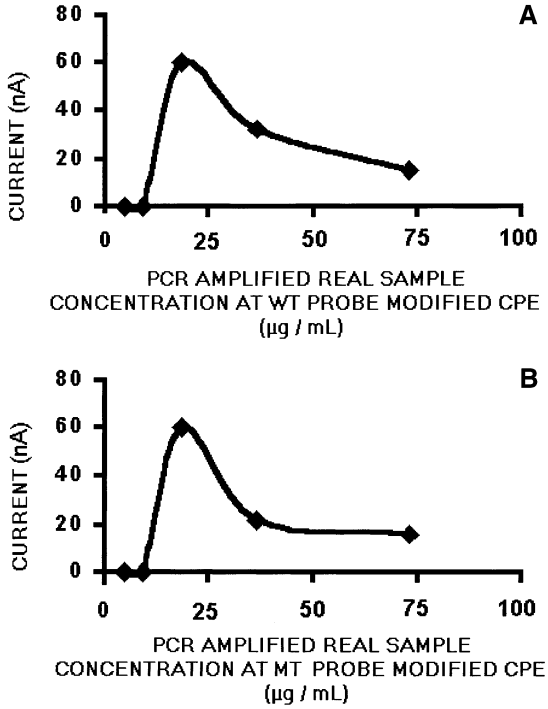


Figure 6. Calibration plot for the dependence of the guanine oxidation signal upon increasing the concentration of the PCR amplicon at (A) WT and (B) MT capture probe modified CPEs.

then dramatically decreased at both the WT (Figure 6A) and MT (Figure 6B) modified CPEs. All subsequent hybridization experiments thus employed a 18.25 $\mu$g/mL PCR-amplified amplicon.

During the optimization studies, it was observed that the constituents of the PCR (primers and polymerase) did not show any voltammetric signals in the potential range at the probe-modified CPEs. Even after hybridization, the constituents of PCR did not increase the nonspecific adsorption effect of guanine at the CPE surface.

In the meantime, an electrophoresis of the amplified samples was also performed in the Department of Medicinal Biology to check the compatibility of the electrochemical test results for the amplified samples (not shown). The results obtained from the gel electrophoresis were in good agreement with the ones obtained from the electrochemical DNA biosensor: 93% of the electrochemical results confirmed the test results obtained from the conventional detection method.

## CONCLUSIONS

The appearance of the guanine signal enables the monitoring of hybridization as a yes/no system in a short time. The main advantage of this new protocol is its simplicity and fast response time. The need for external indicators, such as carcinogenic antitumor drugs, metal complexes, and organic dyes, has been eliminated. The biosensor is able to detect the complementary sequence in the PCR-amplified amplicons by using the oxidation signal of guanine. This procedure also eliminates the use of toxic chemicals, such as ethidium bromide, which was used in the gel electrophoresis step of the reference method[26] in factor V Leiden mutation analyses. Although the feasibility of a factor V microarray has been demonstrated in this study in connection with PCR amplicons, further improvements may be achieved by using several probes for multiplex detection of different genomic targets in connection with DPV. Progress in this laboratory is directed to optimizing the experimental conditions by using renewable pencil graphite electrodes in order to improve the reproducibility. Future work in this laboratory will focus on the design of electrochemical microarrays for detection of target sequences directly from plasma samples without any prior PCR amplification step. The elimination of the PCR amplification step from clinical diagnosis will greatly speed up tests, for example, for point-of-care use.

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