Published in final edited form as:

Analyst. 2014 February 21; 139(4): 786–792. doi:10.1039/c3an02154h.

PROBING FOR DNA METHYLATION WITH A VOLTAMMETRIC DNA DETECTOR

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Abstract

A label-free electrochemical detection of DNA hybridization is used for probing synthetic methylated ssDNA 27-mer or 33-mer targets from the GSTP1-gene. The method is based on electrostatic modulation of the anion-exchange kinetics of a polypyrrole bilayer film deposited on platinum-microelectrodes to which a synthetic single-stranded 15-mer GSTP-1 promoter probe DNA has been attached (DNA detector). The effect of the contact of this DNA-detector with non-methylated and methylated complementary DNA sequences in Tris-buffer is compared using cyclic voltammetry (CV). The DNA-hybridization taking place at the electrode surface leads to a significant decrease of the CV area recorded after exposure to complementary target DNA in comparison to the CV change recorded for non-complementary DNA target. The performance of this miniaturized DNA detector was optimized with respect to hybridization time, temperature, and concentration of the target. It was also evaluated with respect to selectivity, sensitivity, and reproducibility. These results are significant for their possible use as a screening test for hypermethylated DNA sequences.

Label-free electrochemical method for the detection of prostate cancer using a DNA biomarker.

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1. Introduction

Prostate cancer (PCa) is the second leading cause of cancer death among American men, following only lung cancer. The American Cancer Society (ACS) estimates that prostate cancer will claim the lives of approximately 28,170 men and 241,740 new cases of PCa will be diagnosed in 2012.¹ Despite the large number of men affected by this disease, the exact causes of prostate cancer have yet to be discovered. However, some common risk factors including age, race, family history, and diet have been identified. Men over the age of 65, African American (AA) men, and men with brothers or fathers who have had prostate cancer are all at higher risk of developing prostate cancer.² African-American men are twice as likely to die of PCa, while it occurs least often among native Asian men.³

Diagnosing prostate cancer in its early stages is a difficult task based on current detection methods. Specifically for patients in Stage 1 prostate cancer, the cancer cannot be seen by sonogram, nor can it be diagnosed during a digital rectal exam (DRE). The treatment of prostate cancer is costly, and because detection of the disease can be difficult, even watchful waiting strategies are expensive.³ The cost of treating prostate cancer patients is about \$10 billion/year in the United States.⁴ As life expectancy continues to increase, a large number of men are being diagnosed with this disease, thus effectively increasing the economic burden of the disease.³ Earlier detection and treatment of clinically relevant prostate cancer has the potential to decrease the morbidity and mortality of this disease, and decrease the cost of screening and diagnosis.²

The serum Prostate-Specific Antigen (PSA) is presently used clinically to screen for prostate carcinoma.² Though it is effective in determining an increased level of PSA, there are serious limitations to this method. Its major limitations include low specificity and high prevalence of detecting benign prostatic hyperplasia, particularly in older men.⁵ Even with the development of methods such as the PSA index to try and increase specificity, PSA levels still do not allow for a differentiation between prostate cancer and other benign prostate conditions. As a result, an urgent need for more specific prostate cancer biomarkers is present so that earlier detection of clinically relevant prostate cancer can be made, decreasing the morbidity and mortality of the disease, as well as the cost of screening and diagnosis.²

Several studies have indicated that hypermethylation of the Glutathione S-transferase P1 (GSTP-1) gene can be used as a possible biomarker of prostate cancer. GSTP-1 encodes a detoxifying enzyme that defends cells against free radical damage to DNA and cancer initiation. Hypermethylation of the GSTP-1 gene leads to the loss of expression of this gene, possibly leading to damaged DNA and a greater likelihood for prostate cancer development. A common sequence rich in C and G is present near the promoter of genes involved in prostate cancer (and in other cancers as well). Hypermethylation in these regions is one of the most common alterations in the carcinoma tissue DNA of the prostate. Because hypermethylation of these CpG islands is not present in normal cells, CpG hypermethylation can be used as a biomarker for the diagnosis and detection of prostate cancer.^{3,6}

Since hypermethylation of the 5' region of the GSTP1 gene appears to be a frequent and early event in prostate cancer development, we approached its use for an early detection using an electrochemical screening method. Fluorescence resonance energy transfer⁷, surface plasmon resonance,⁸ optofluidic ring resonator,⁹ electrochemiluminescence^{10,11} and surface enhanced Raman spectroscopy¹² are just a few of the various methods that have been previously used in the investigations of genomic methylation status. While such methods may hold some advantages, they are often very time consuming, require costly instruments, or are labor intensive.^{13,14} The promising ability to evaluate DNA methylation using electrochemical techniques has been demonstrated, and holds the potential to determine DNA methylation status in a fast, convenient, and precise way.¹⁵ For example, Kato et al. have described a method for detecting both cytosine (C) and methylcytosine (mC) in oligonucleotides by measuring differences between oxidation currents of C and mC using Electron Cyclotron Resonance (ECR) nanocarbon film electrodes.¹⁶ Quantification of DNA methylation information based on the specific interaction between guanine (G) residues and methylene blue indicator has also been used as an electrochemical approach,¹⁴ as well as selective labeling of 5-mC through osmium complexation.¹⁷

Treating DNA with bisulfite can be utilized to determine methylation status, based on the differing chemical reactivity of Cytosine (C) and methylcytosine (mC). Upon treatment with bisulfite, the C residue is readily deaminated and transformed to uracil (U) (Fig. 1), while methylated cytosine resists bisulfite treatment.

Several methods of using bisulfite in genomic methylation profiling are discussed by Palecek et. al.^{18,19} A recent study by Bartosik et al. demonstrated the use of bisulfite for methylation profiling using square wave voltammetry. Uracil residues strongly decreased square wave voltammetric C reduction peaks, while the 5-methylcytosine (mC) residues resist bisulfite treatment and display almost unchanged reduction peak.²⁰

Our label-free electrochemical DNA detector that combines base pair recognition of DNA probes with a miniaturized sensing recognition platform have already gained attention for their potential use in screening for disease detection.²¹ This detector relies on the ability to immobilize probe oligonucleotides to the microelectrode surface modified with polypyrrole bilayers. Using the availability of their phosphonate-functional groups and Mg^{2+} -ions as a linker an activated modified microelectrode is prepared that is ready to bind the probe ssDNA.²² The ability of the target ssDNA to form a duplex with the ss-probe indicates that the nucleotide sequence of the target DNA (tDNA) is complementary to the sequence of the probe.¹⁸

In the present study, we describe the use of a 15mer ssDNA detector to recognize the complementary target of 27- or 33-mer methylated ssDNA from GSTP-1 promoter region, after they have been treated with an epigenetics bisulfite conversion kit. Selected sequences related to hybridization event of methylated complementary ssDNA against non-methylated ssDNA (representative of a healthy GSTP-1 gene) were tested for recognition of hybridization event. The use of the rapid electrochemical detection of the hypermethylation for practical applications was examined by optimizing parameters such as incubation temperature, time of the target and concentration of the tDNA.

2. Experimental

2.1 Chemicals

Pyrrole monomer (Py, 98%) was purchased from Sigma-Aldrich (St. Louis, MO). 2, 5-dithienyl-(N-3-phosphorylpropyl) pyrrole (TPT) is not available commercially, and was synthesized according to Hartung et al.²³ Magnesium chloride hexahydrate, potassium chloride, tetrabutylammonium perchlorate (TBAP), Tris-HCl buffer, acetonitrile (AcCN) and silver nitrate were all purchased from Sigma-Aldrich and were used without further purification. The Tris-HCl buffer (0.1 M, pH 7.2) was used as the supporting electrolyte.

The Epigenetics Bisulflash DNA Modification Kit was purchased from Epigentek (USA)

Hazards—Pyrrole and acetonitrile are flammable liquids, therefore, keep away from sparks, hot plates, and open flame. Acetonitrile is considered a human carcinogen. Avoid inhalation of pyrrole and acetonitrile vapors since they may cause irritation of the digestive and the respiratory tract and irritation of the nervous system. Handle them in adequately ventilated space (fume hood). Pyrrole, acetonitrile, Tris-HCl buffer, tetra-n-butylammonium perchlorate may lead to eye and skin irritation. Silver nitrate is a strong oxidizer, therefore, contact with other reducing agents may cause fire. It causes skin inflammation and discoloration owing to exposure.

2.2 Oligonucleotides

The sequences of the probe oligonucleotides (15-mer), and the non-methylated target DNA (27-mer), and methylated target DNA (27-mer) strands were custom-made by Integrated DNA Technologies, Inc. (Coralville, IA) (Table 1). All chemicals were free of RNase and DNase and were received in 1 μ mole pellet form.

The concentrations of probe DNA, target DNA, and methylated target DNA stock solutions were 2.5×10^{-5} M, and 3×10^{-5} M, and 1.05×10^{-5} M, respectively. All samples were kept in a refrigerator at 2.3°C. The 15-mer DNA Probe sequences are shown in Table 1 and Table 2. The probe DNA used in experiments was prepared by serial dilution with 0.1 M Tris-HCl buffer to give a final concentration of 0.1 μ M probe DNA.

The 27 and 33-mer tDNA sequences were selected from the GSTP-1 promoter region, and a corresponding probe was chosen for attachment in the middle of the target DNA sequences.

2.3 Apparatus

Cyclic Voltammetry (CV) and amperometry were carried out using an electrochemical workstation 600D, CH instruments Electrochemical, Austin, TX. All electrochemical experiments were performed at room temperature (25 °C). A three-electrode cell with a volume of 5 mL, comprising a 25 μ m-diameter platinum disk working electrode encased in glass, a Ag/AgCl in 1M KCl//1M KNO₃ reference electrode, and a platinum wire counter electrode were used for all Tris-HCl experiments. A detailed description of the fabrication of the microelectrodes is given elsewhere.²⁴ Cleaning of the 25 μ M Pt-electrodes was achieved by applying a constant potential of -0.9 V for 10 seconds in 0.5 M H₂SO₄. This followed by

cycling the potential between -0.80 V and $+2.0$ V (2 cycles) and -0.61 to $+1.0$ V (10 cycles) in 0.5 M H_2SO_4 .

ACCU-SCOPE'S 3035 Inverted Metallurgical Microscope was used to inspect the microelectrode tip to ensure no deposited layers remained.

2.4 Sodium bisulfite conversion of target DNA sequences

Target DNA bisulfite treatment was conducted on 27- and 33-mer DNA sequences shown in Table 1 and Table 2 after their serial dilution to 0.1 μM DNA with in 0.1 M Tris-HCl buffer. Following this, 5 μL of the non-methylated ssDNA target and the methylated ssDNA target were each treated with Epigenetics Bisulflash DNA Modification Kit using the protocol outlined in the bisulfite kit manual. 5 μL of the 0.1 μM DNA samples were used for optimal efficiency of conversion, as recommended by Epigentek's protocol.²⁵ The DNA conversion process on target DNA strands was completed in about 1 hour. Approximately 20 μL of DNA was collected from each sample for experimental use after bisulfite conversion. Thereafter, the DNA obtained from the bisulfite kit modification procedure was stored at 2.3°C before use. Non-methylated cytosines were converted to uracil, while in the methylated ssDNA, the methylated cytosine remain as cytosine, Table 3.

DNA protection reagents sustain DNA denaturation status throughout the entire bisulfite DNA conversion process. This process enables 100% of DNA to be modified in single stranded form without chemical and thermophilic degradation. Accelerated conversion of all cytosine to uracil with negligible methylcytosine deamination is accomplished with this method. The non-toxic DNA capture solution enables DNA to tightly bind to the column filter, thus DNA cleaning can be carried out on the column to effectively remove residual bisulfite and salts.²⁵

2.5 Fabrication of the modified Pt-microelectrode

The assembly of the DNA detector has followed the procedure described by Riccardi et al. It starts with a sequentially polymerized polypyrrole (PPy) and then poly [2,5-dithienyl-(N-3-phosphorylpropyl)pyrrole] (pTPT) on the Pt-microelectrode.²² The polymerization of polypyrrole (PPy) was carried out from 0.1 M of Bu_4NBF_4 in AcCN in the presence of 0.1 M pyrrole by applying a constant potential of 0.7 V vs. Ag/ 0.1 M AgNO_3 in AcCN// 0.1 M TBAP in AcCN until the total charge reached 1.0×10^{-6} C. After polymerization the electrode was rinsed with AcCN and the TPTC3- PO_3H_2 was polymerized.²⁶ The pTPTC3- PO_3H_2 polymerization was carried out at constant potential of 0.7 V until the polymerization charge reached 5.5×10^{-7} C. Then, the PPy-pTPTC3- PO_3H_2 modified Pt-electrode was immersed in 5×10^{-3} M MgCl_2 solution and kept there under stirring for at least 15 min and then thoroughly washed with 0.1 M Tris-HCl buffer. The functionality of this microelectrode was tested by recording a series of CVs in the potential range of 0.3 V to -0.3 V in 0.1 M Tris-HCl buffer solution at room temperature.

2.6 Assembling of the DNA detector

The immobilization of the 15-mer DNA probe on the surface of the Pt/PPy-pTPTC3- PO_3H_2 - Mg^{2+} microelectrode was initiated by dipping it in 0.1 M Tris-HCl buffer containing

0.1 μM probe DNA and thermostated for 30 min at 45°C in a heat block. Following the incubation period, the detector was washed for 5 min in 0.1 M Tris-HCl, to remove any of the probe-DNA that were not involved in the bidentate complex formation with the Mg^{2+} linker attached to the PPy-bilayer of the modified electrode. Then five consecutive CV's were recorded within a potential range from +0.3 V to -0.3 V (vs Ag/AgCl at 0.05 V s^{-1}) in 0.1M Tris-HCl buffer (pH 7.2). Only the last recorded CV of the series was used as the standard reference for the detector.

2.7 Hybridization and detection of methylated DNA

The principle of operation of the DNA detector is shown in Fig. 2.

The hybridization event at the DNA detector forms a space charge barrier at the electrode surface. That represents a hindrance for the chloride ion to move from the 0.1 M Tri-HCl buffer to the surface of the Pt electrode where the ionic charge is transferred into then electronic. That the transduction of the biochemical signal into an electrical signal manifest the hybridization event.

The DNA detector (PPy-pTPTC3- $\text{PO}_3\text{H}_2\text{-Mg}^{2+}$ /probeDNA(15-mer)-modified electrode) was placed in a solution of the target DNA (0.1 μM non-methylated DNA) dissolved in buffer after treating with the bisulfite kit, Table 3 for 30 min at 45°C After that it was immediately washed in 0.1 M Tris-HCl for 10 minutes and transferred to the electrochemical cell and five CVs were recorded. These recorded CV's were used to evaluate if any non-specific interactions took place. Subsequently, the same detector was exposed to 0.1 μM solution of the methylated target DNA for 30 min at 45°C and then rinsed in 0.1 M Tris-HCl for 10 min. In follow up again CVs were recorded at room temperature and used for identification of the hybridization event The CV's were always initiated at 0.3 V. The stable fifth CV (last one in series) that was recorded for each step was used for data analysis.

3. Results and Discussion

3.1 Evaluation of the detector performance

In order to determine specificity of the DNA detector, the area under the last in series recorded CV in Tris-HCl buffer was determined and used for the calculation of % changes for each trial. The CV area information was possible to obtain directly from applied evaluation program built into the CHI600D instrument. The calculation of % differences for each trial was conducted using the following equations:

$$(A_{\text{probe}}) - (A_{\text{non-methylated target}}) / (A_{\text{probe}}) \times 100 = \% \text{ difference for non-methyleted target} \quad \text{Eq. 1}$$

$$(A_{\text{probe}}) - (A_{\text{methylated target}}) / (A_{\text{probe}}) \times 100 = \% \text{ difference for methyleted target} \quad \text{Eq. 2}$$

$$\% \text{ of Total change of the DNA detector} = \text{Eq.2} - \text{Eq.1} \quad \text{Eq. 3}$$

where A_{probe} was recorded after exposure of the DNA detector to buffer only, $A_{\text{non-methylated target}}$ was recorded after exposure of the DNA detector to the non-methylated target and $A_{\text{methylated target}}$ was recorded after the same DNA detector in follow up was exposed to the methylated target.

Equation 1 shows the formula for calculating the percent difference in the area for each non-methylated target strand, while Equation 2 shows the percent difference between the probe and methylated target. Once these % differences were calculated for each trial, the value derived from Eq. 3 was used for the comparison of optimal detection parameters.

Replicate experiments were conducted to determine the variability from electrode-to-electrode between experiments. Five trials were conducted, using the same parameters for detection in each. For each set of experiments, the temperature was held constant at 50°C, for an incubation time of 30 minutes, and a DNA concentration of 0.1 μM .

3.2 Effect of bisulfite conversion on target DNA strands

After completing the bisulfite conversion of the 27-mer and 33-mer target DNA, strands of different methylation statuses have been obtained, Table 3. Looking at the 27-mer of non-methylated target DNA strand in Table 1, it can be seen that the five positions of non-methylated cytosine is available for bisulfite modification. The resulting DNA strand leaves the 3 positions on the modified non-methylated target (Table 3) no longer complementary to the previously 15-mer single stranded probe DNA. The sites where the uracil base is present are no longer a base pair match to the guanine bases on the complementary 15-mer probe DNA.

The methylated cytosine bases are not affected by the bisulfite treatment and thus remain able to hybridize with the complementary sites of guanine in the 15-mer immobilized probe (Table 3). Following bisulfite conversion and collection of DNA samples after the desulphonation process, the DNA activated biosensor is then ready to be used for the detection of DNA hybridization. The methylated cytosine bases are not affected by the bisulfite treatment and thus remain complementary to the previously immobilized 15-mer probe, where it can preferentially hybridize at the transducer surface.

3.3 Target detection of methylated cytosine

In order to use the label-free DNA hybridization detector for the detection of GTSP1 hypermethylation, it was important to examine the effect of the non-methylated and methylated target strands (27-mer, 1.0×10^{-6} M) on the electrode response. In Figure 3, it can be observed that no significant difference is seen between the recorded CV in the absence and presence of the 27-mer non-methylated target DNA (curve a and b). However, in the presence of the methylated target oligonucleotide (27-mer, 1.0×10^{-6} M), a significant change of the shape of the CV (curve c) was observed. A 33% decrease in CV area response was determined when compared to the CV area of the detector before exposure to any of the targets. The hybridization events can also be represented by subtraction of the voltammetric currents recorded for methylated or non-methylated target DNA (complementary and non-complementary interactions) from the CV recorded for the DNA probe CV as shown in Figure 3 (curve d and e). As previously explained, the addition of negative charges to the

modified electrode surface due to phosphate groups of the complementary strand decreases the chloride ion exchange in the detector.

3.4 Evaluation of the effect of target length on the hybridization event

After incubation of the 15-mer probe with the 33-mer non-methylated target (Table 2), no significant change of the voltammogram in the presence of the 33-mer non-methylated target DNA (Table 3) was observed as shown in Figure 4. However, in the presence of the methylated target oligonucleotide (33-mer, 1.0×10^{-6} M), a significant change of the shape of the CV was observed corresponding to a 71% decrease of the CV area when compared to the signal derived from the CV of the detector before hybridization. Similarly to the 27-mer target study, a significant decrease in CV signal occurred after the probe was incubated with the methylated target DNA. The hybridization of the 33-mer methylated target to the 15-mer probe immobilized at the transducer surface was again confirmed by the decrease in CV response due to the addition of negative charges at the modified electrode surface.

3.5 Variability, reproducibility, and reusability of the Detector

Steps were replicated for each trial, incubating the biosensor at 50 °C for 30 min in 0.1 μ M probe, non-methylated, and methylated DNA. After percent differences using different electrodes ($n=5$) were calculated (Eq.3), an average percent difference of 25.4% with SD = 4.2 and RSD = 17%. Variations between trials due to the bisulfite conversion may significantly contribute to variations, as the desulphonation process can sometimes lead to unconverted cytosine bases. Although percent hybridizations vary between trials, hybridization is detectable consistently based on the noticeable change between the non-methylated and methylated target CV's. In addition, variations from electrode to electrode are owed to the individual steps in the preparation of the activated biosensor, allowing for human error to occur. The sensitive nature of the DNA itself is also a potential source of variation. However, the strength of this method is in its ability to give a "yes" or "no" answer based on the notable change in CV above a certain threshold. Future studies in this method will work to alleviate variations through the use of a 16-channel potentiostat that will be able to manipulate parameters individually and simultaneously in order to more effectively collect data at each electrode. Repeated usability is a desired feature for biosensors in practical applications. With this electrochemical sensor, we have previously shown that chemical regeneration is possible using 50×10^{-3} M HCl solution.²² The electrode response returned to its original signal after 30 min of treatment, indicating that the double-stranded DNA hybrid was dissociated into single strands and that the signal of the immobilized probe DNA was not destroyed after the regeneration.

3.6 Optimization of the analytical performance of the detector

To identify the conditions for optimal detection of this 27-mer methylated GSTP-1 sequence, the incubation time, temperature, and target concentration were varied one at the time by maintaining the other variables the same.

3.6.1 Effect of incubation temperature—Experiments were conducted at 40 °C, 45 °C, 50 °C, and 55 °C by maintaining the incubation time at 30 minutes, and DNA concentration at 0.1 μ M. After incubation, the detector was removed from the DNA solution and placed in

Tris-HCl buffer and allowed to sit for 10 minutes before recording CV's. The CV's were recorded at room temperature. Following Eq. 3, the effect of incubation temperature on the total change of the CVs is compared in Figure 5. The greatest change in CV area was determined for the incubation temperature set at 45 °C, with a 26.7% of the total difference. Based on these results, optimal incubation temperature in this range was determined to be 45 °C, and was used for all sequential studies.

3.6.2 Effect of incubation time—The incubation temperature was kept constant at 45 °C, and the DNA concentration was 0.1 μM during the study. Figure 6 shows the effect of the incubation time of 10, 15, 30, and 45 minutes on the hybridization. The greatest change in CV area was determined for the incubation time of 30 minutes, with a 31.2% difference as defined by Eq.3.

3.6.3 Effect of target DNA concentration—The incubation temperature was kept at 45 °C, and incubation time was left at 30 minutes. From the current differences obtained in the target concentration range of 0.1 nM to 1.0 μM, it is evident that the varied DNA concentrations affected the change in the CV's areas for each the probe, non-methylated, and methylated targets. This relationship is shown in the bar graph below (Fig. 7), where the DNA concentration corresponding to the greatest change in CV area was shown to be 0.1 μM, with a 29.1% difference. Based on these results, optimal DNA concentration in this range was determined to be 0.1 μM, and was used for all sequential studies.

3.7 Future implications for analysis of human DNA

Methylated GSTP-1 is detectable in urine samples of prostate cancer patients, as well as in other bodily secretions. Studies have suggested that the detection of *GSTP1* methylation in prediagnostic urine may improve the specificity of PSA and help distinguish men with prostate cancer from those with benign prostatic hyperplasia.²⁸ The ability to probe for methylated GSTP-1 in urine provides the possibility of using this ability to detect GSTP-1 differences, as a much less invasive method of detecting abnormal results in PCa patients as compared to PSA testing.²⁹ With further study, we propose that it would be possible to select for methylated sequences in urine since the sensor is specific to complementary single-stranded DNA. Although this paper is intended to demonstrate the proof-of-concept for detecting methylation differences in synthetic strands of DNA, we believe that it has practical future applications to cancer research and diagnostics.

Conclusion

A label-free detection scheme based on conducting-modified polypyrrole films deposited at a microelectrode surface can be applied for identification of short methylated GSTP1 related DNA sequences (27- and 33-mer) from the GSTP-1 promoter region. To study the hybridization event of methylated complementary ssDNA against non-methylated ssDNA a bisulfite conversion was applied to perform methylation profiling. The non-methylated cDNA acted as a control experiment upon bisulfite conversion, becoming a non-complementary ssDNA sequence (representative of a healthy GSTP-1 gene). The proposed method was successfully applied also to the 33-mer non-methylated DNA target. It was

selected from the GSTP-1 promoter region, along with a corresponding methylated target DNA.

The hybridization of methylated target DNA sequences (27-mer, 33-mer) could be determined with average percent difference of 25.4% with SD = 4.2 and RSD = 17%, while the non-methylated targets were shown to be non-complementary to probe DNA after bisulfite conversion.

Based on reproducibility of replicate experiments the optimal parameters for detecting the 27-mer sequence the following optimum parameters have been determined: incubation temperature of 45 °C, incubation time of 30 minutes, and a target concentration of 0.1 μM. The results presented here are promising for future research for early detection of prostate cancer.

Acknowledgments

Research reported in this publication was supported by the National Institute On Minority Health And Health Disparities of the National Institutes of Health under Award Number P20MD001085. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Fig. 1.
Mechanism for bisulfite-mediated conversion of cytosine to uracil.

Fig. 2.
Schematic of the operation of the electrochemical DNA-detector.

Fig. 3.

Cyclic voltammograms of DNA detector recorded in 0.1 M Tris-HCl buffer (pH 7.2) with a scan rate of 20 mV/s; Curve (a) - Detector with 15mer-DNA-Probe (Table 1) before exposure. Curve (b) - after exposure to **27-mer** (Table 3) of 0.1 μ M non-methylated target DNA. Curve (c) - after exposure of the same detector to 27-mer of 0.1 μ M methylated target DNA (Table 3). Curve (d) - subtraction of the responses to methylated target DNA and probe DNA (curves a–c). Curve (e) - subtraction of the responses of non-methylated target DNA and probe DNA (curves a–b).

Fig. 4.

Cyclic voltammograms recorded in 0.1 M Tris-HCl buffer (pH 7.2) for the detection of DNA hybridization of **33-mer** target (Table 3) incubated at 60°C. Curve (a) - Detector with 15mer-DNA-Probe (Table 2) before exposure. Curve (b) - after exposure to 33-mer (Table 3), 0.1 μ M non-methylated target DNA. Curve (c) - after exposure of the same detector to 33-mer of 0.1 μ M methylated target DNA (Table 3). Curve (d) - subtraction of the responses to methylated target DNA and probe DNA (curves a–c). Curve (e) - subtraction of the responses of non-methylated target DNA and probe DNA (curves a–b).

Fig. 5. Changes in total % difference in voltammetric area obtained after interactions of the DNA detector with the non-methylated and then the methylated 27-mer target (Table 3) as a function of the used incubation temperature. The % difference was evaluated using Eq. 3.

Fig. 6.

Changes in total % difference in hybridization area between the non-methylated and methylated CV's (Eq. 3), as a function of incubation time.

Fig. 7.

Changes in the total % difference in hybridization area between the non-methylated and methylated CV's (Eq. 3) as a function of target concentration.

Table 1

DNA Inventory (15-mer Probe, 27-mer Targets)

DNA	Sequence
Probe	5' TCG CCG CGC AAC TAA 3'
Target(non-methylated)	5' TTT CGG TTA GTT GCG CGG CGA TTT CGG 3'
Target (methylated)	5' TTT mCGG TTA GTT GmCG mCGG mCGA TTT mCGG '3

Table 2

DNA Inventory (15-mer Probe, 33-mer Targets)

DNA	Sequence
Probe	5' ACC CCG AAC GTC GAC 3'
Target (non-methylated)	5' CGT TTT TTT GCG GTC GAC GTT CGG GGT GTA GCG 3'
Target (methylated)	5' mCGT TTT TTT GmCG GTmC GAmC GTT mCGG GGT GTA GmCG'3

Table 3

Modification of Target DNA (27-mer & 33-mers) after bisulfite conversion

27-mer Target (non-methylated)	5' TTT UGG TTA GTT GUG UGG UGA TTT UGG 3'
27-mer Target (methylated)	5' TTT mCGG TTA GTT GmCG mCGG mCGA TTT mCGG 3'
33-mer Target (non-methylated)	5' UGT TTT TTT GUG GTU GAU GTT UGG GGT GTA GUG 3'
33-mer Target (methylated)	5' mCGT TTT TTT GmCG GTmC GAmC GTT mCGG GGT GTA GmCG 3'