

An electrochemical detection scheme for identification of single nucleotide polymorphisms using hairpin-forming probes

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ABSTRACT

Single nucleotide polymorphisms are implicated as having a significant role in regulating growth, development and, thereby, human health and disease. We have developed a method for identifying single nucleotide genetic alterations by combining hairpin-forming DNA probes and electrochemical detection of sandwich DNA hybridization. Incorporation of hairpin-forming competitor probes and the catalyzed reporter deposition amplification system further improves assay specificity by 7-fold and sensitivity by 100-fold. We have demonstrated that the system successfully identified the factor V Leiden mutations from human blood specimens.

INTRODUCTION

Averaging about 1 in every 1000 bases, single nucleotide polymorphisms (SNPs) are the most common type of sequence variation among individual genomes and have been found to play a significant role in a wide range of human diseases (1,2). The Human Genome Project has provided a first draft of the human genome sequence (3,4). An important aspect of further analysis is to identify and map SNPs by screening sequences of multiple individuals. Efficient schemes to detect SNPs are critical for applications ranging from disease gene mapping to clinical diagnostics. Throughput, accuracy, speed and cost-effectiveness are among the most important criteria used to evaluate any SNP typing method used for large-scale screening. Currently, the most definitive approach for scoring SNPs is direct sequencing of the region of interest, as indicated by the widespread use of direct sequencing in the NCBI dbSNP database entries (http://www.ncbi.nlm.nih.gov/SNP). However, despite significant advances in DNA sequencing technology, SNP detection methods for large population studies and clinical diagnostics are still far from being optimized. Recently, a wide variety of alternative high-throughput approaches focusing on identification of nucleotides at specific positions of interest have been developed. Several reviews discussing current and emerging technologies have appeared recently (5–9). Among these methods, homogeneous assay systems based on the molecular beacon concept have proven promising in several studies (10–13). Molecular beacons are oligonucleotide probes that emit fluorescence only when bound to complementary nucleic acids (11). Thermodynamic analysis of molecular beacons has shown that the hairpin structures contained within molecular beacons are responsible for the extraordinary specificity required for detecting single base pair mismatches (10,14).

Among the wide variety of approaches for determining DNA sequence information, sandwich DNA hybridization is one of the well-established techniques. In most implementations, it has been used with an enzyme-linked immunosorbent assay system coupled to optical detection schemes. The incorporation of enzymes for generating output signals offers the potential for signal amplification. An alternative to the optical detection methods is an electrochemical system where electrochemical sensors can quantitatively detect electrical currents generated by enzymes using appropriate substrates (15). Recently, our group has developed a highly sensitive electrochemical DNA sensor based on micro-electromechanical system technology that is able to detect very small numbers of bacteria without PCR amplification (16). However, it does not have the capability to identify single nucleotide variations within nucleotide sequences. Currently, to our knowledge, no existing electrochemical method has been shown to inherently contain sufficient specificity for detection of single base pair mismatches.

We present in this report a system that is capable of detecting single nucleotide sequence variations by incorporating the advantages offered by the molecular beacon approach into an established electrochemical detection method. We shall refer to the system as EDEMNA, for electrochemical detection of mismatches in nucleic acids. In EDEMNA, nucleic acid molecules (DNA or RNA) are hybridized with two types of single-stranded DNA probes: capture probes conjugated with biotin for anchoring hybridization complexes onto electrochemical

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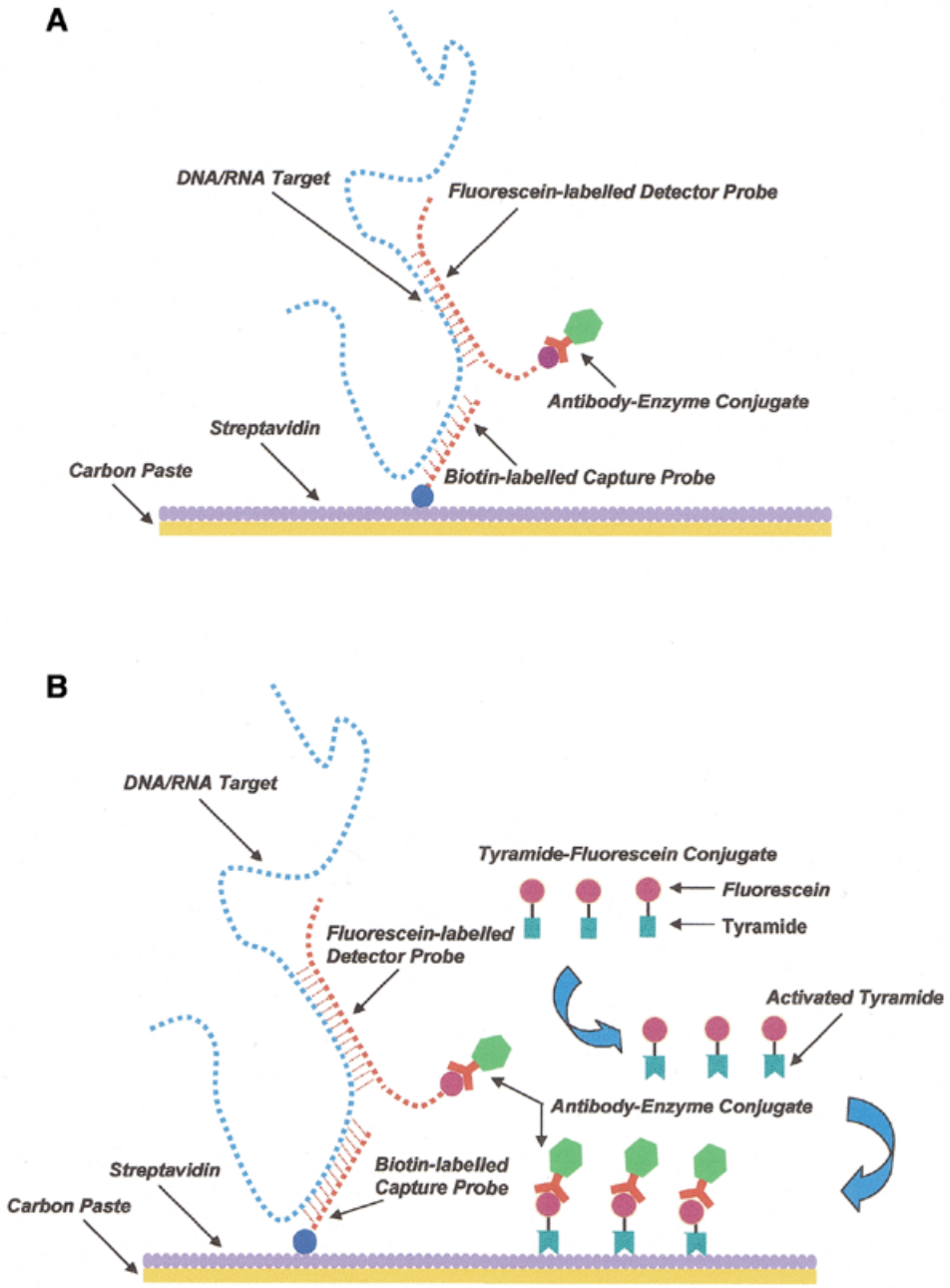

Figure 1. (**A**) Basic EDEMNA scheme. See text for details. (**B**) Adaptation of CARD to EDEMNA. Following the addition of HRP-conjugated anti-fluorescein monoclonal antibodies, instead of adding the HRP substrates, fluorescein-labeled tyramide molecules are added to the mixture. The HRP-mediated reactions deposit the additional fluorescein labels around the enzymes, creating additional targets for the HRP-conjugated antibodies to bind and resulting in higher output signals.

sensor surfaces and fluorescein-labeled detector probes for enzyme binding (Fig. 1A). The hybridized complexes are immobilized through biotin binding with the streptavidin-coated sensor surface while the unbound components are washed away. Horseradish peroxidase (HRP)-linked anti-fluorescein antibody–enzyme conjugates are then loaded onto each hybrid. Following addition of a HRP substrate and mediators such as tetramethylbenzidine, HRP-catalyzed reactions then generate amperometrical signals which can be measured quantitatively and are proportional to the number of immobilized hybrids on the sensor surface.

We have also incorporated the use of competitor probes and the catalyzed reporter deposition (CARD) technique into EDEMNA with improvements in both assay specificity and sensitivity. CARD is a signal amplification scheme based on the deposition of hapten-labeled tyramide molecules mediated by peroxidase enzymes through creation of highly reactive products (17). The deposited haptens can in turn serve as targets for additional antibody–enzyme conjugates to bind. CARD has been used widely in immunohistochemistry and in nucleic acid detection assays (18). This signal amplification technique has so far only been measured optically. In this

Table 1. Oligonucleotide sequences

Control experiments using oligonucleotide targets	
Perfect match target (NDH-PM)	5'-GCTAAAGTAACGCTGGTTGATCGCAATGCCACCCATTTATGGAAAC-3'
Mismatched target	5'-GCTAAAGTAACGCTGGTTGATCGCAATGCCACCGATTTATGGAAAC-3'
Negative control	5'-TTATCTCGGGCAAAATGGCGATACGCAAATTCACGGTTTTGAATTA-3'
Capture probe	Biotin-5'-TCAACCAGCGTTACTTTAGC-3'
Linear detector probe	Fluorescein-5'-CATAAATGGGTGGCA-3'
Hairpin detector probe	Fluorescein-5'-GCGAGCATAAATGGGTGGCACTCGC-3'
Competitor probe	5'-GCGAGCATAAATCGGTGGCACTCGC-3'
Factor V-Leiden mutation detection	
Capture probe	Biotin-5'-CTTCTAATCTGTAAGAGCAG-3'
Detector probes	Fluorescein-5'-GCATGTGGACAGGCNAGGAATACCATGC-3' (N is A, G, C, or T)

report we demonstrate that CARD can also be incorporated into an electrochemical detection scheme. Figure 1B shows the EDEMNA system modified to incorporate the CARD amplification system. To our knowledge, this report is also the first one to improve the match-to-mismatch signal ratio by using hairpin-forming oligonucleotides as competitor probes.

MATERIALS AND METHODS

Oligonucleotides

All of the oligonucleotides used in this report were purchased from Integrated DNA Technologies (Coralville, IA). The oligonucleotide sequences are listed in Table 1.

Basic EDEMNA assay protocol

The protocol is largely based on the AndCare *Escherichia coli* assay (AndCare, NC) with slight modifications. Briefly, the general assay protocol is as follows. Aliquots of 65 µl of nucleic acid targets in 0.17 M NaOH were first incubated at room temperature for 5 min. To this was added 25 µl of probe solution containing 200 nM capture probe and 100 nM detector probe (plus specific concentrations of competitor probes as indicated) in 1.05 M Tris, 0.5 M NaCl, 1 mM $MgCl_2$ and 0.05% BSA, pH 7.8, followed by incubation at 65°C for 10 min. The mixtures were then loaded onto commercial electrochemical sensors (AndCare) and further incubated for 10 min at room temperature. Following a wash step using biotin wash solution (Kirkegaard and Perry Laboratories, MD), 50 µl of HRP-conjugated anti-fluorescein monoclonal antibody (Roche) at a concentration of 0.75 U/ml was added to the sensor surfaces and incubated at room temperature for 10 min. After one more wash step, the sensors were placed in AndCare Pulse Amperometric Monitors (AndCare) and 50 µl of K-blue substrate (Neogen, MI) was dispensed onto the sensors. The amperometric signals were determined 70 s after addition of the substrate solution.

Incorporation of CARD

To incorporate the use of CARD, the basic assay protocol outlined in the previous section was modified in the following way. After the first wash step, 50 µl of HRP-conjugated anti-fluorescein monoclonal antibody, at 0.15 U/ml, which was different from the concentration used in the basic assay protocol, was added to the sensors and incubated for 10 min at room temperature. Following one more round of washing, instead of placing the sensors in the amperometric monitors and adding K-blue substrate, 25 µl of fluorescein-labeled tyramide (NEN Life Science, MA) working solution, prepared according to the manufacturer's instructions, was added, followed by a third round of washing with biotin wash solution. The basic assay protocol was then resumed at this point with the addition of 50 µl of HRP-conjugated anti-fluorescein monoclonal antibody (0.75 U/ml). After another round of washing, the sensors were placed in the amperometric monitors and K-blue substrate was added to the sensors before signals were determined.

Factor V Leiden mutation detection

The factor V Leiden mutation status of each sample was established first by the UCLA Diagnostic Molecular Pathology Laboratory according to standardized protocols (UCLA Department of Pathology and Laboratory Medicine Special Testing–Molecular Pathology; 19,20). The results were not revealed to T.J.Huang and M.Liu, who carried out the EDEMNA assay (including the PCRs), until the EDEMNA results were independently obtained and interpreted. The PCRs were carried out according to a previously described protocol (21) and the products were purified using a QIAquick PCR Purification Kit (Qiagen). The EDEMNA assay was carried out according to the basic protocol as described above without the use of CARD or competitor probes.

RESULTS

Analysis of single base variations in oligonucleotide targets

To facilitate optimization and reduce the effects of variability between DNA samples, we began by determining single base variations in oligonucleotide targets. A 46 bp oligonucleotide (NDH-PM) corresponding to bp 6262–6307 of the *Haemophilus influenzae* NADH dehydrogenase gene *ndh* was chosen as the detection target. As a negative control, we used bp 3962–4007 of the unrelated *Streptococcus pneumoniae* penicillin-binding protein 1b gene *pbp*1b.

Theoretically, in a sandwich DNA hybridization-based mismatch detection system such as EDENMA, the expected mismatch position could be designed into either the capture probe or the detector probe. Using NDH-PM as the target, experiments were conducted in which the mismatched base pair was located either in the middle of a hairpin-forming detector probe or a hairpin-forming capture probe. The results showed that the match-to-mismatch signal ratio was significantly higher when the mismatched base pair was located within the loop of the detector probe (data not shown). Subsequent

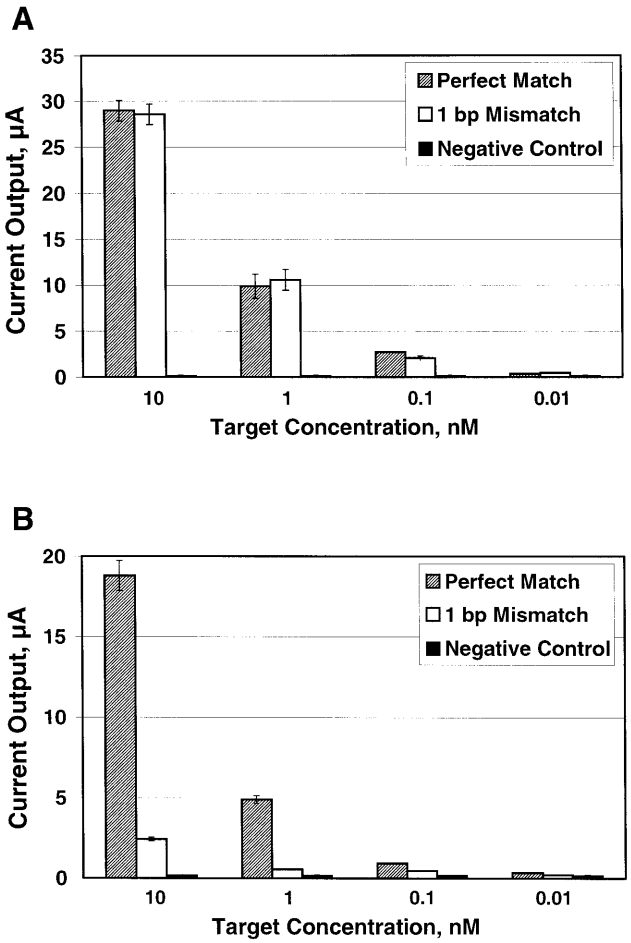

Figure 2. Comparing the ability of linear detector probe and hairpin-forming detector probes to detect the single nucleotide difference between the NDH-PM (Perfect Match) and mismatched (1 bp Mismatch) targets. (**A**) Using linear detector probe. (**B**) Using hairpin-forming detector probe.

experiments described in this study therefore incorporated the combination of a linear capture probe with 100% complementarity and a hairpin-forming detector probe for mismatch detection.

Specificities of hairpin probes and linear probes

Linear oligonucleotide probes are commonly used to identify target nucleic acids containing complementary sequences (22). However, single mismatched base pairs have relatively marginal effects on the stability of a duplex when the linear probes are long enough to distinguish a particular sequence (10). To determine the change in specificity, if any, resulting from addition of hairpin stems to linear probes, we compared the ability to distinguish single mismatched base pairs using a hairpin-forming detector probe with that of a linear detector probe without hairpin-forming sequences. The experiments were carried out using NDH-PM as the perfectly complementary target and a corresponding mismatched target containing a single base alteration. The results suggest that the linear detector probe-based systems would be unable to detect mismatched base pairs (Fig. 2A). Using the hairpin-forming detector probe, the mismatched target–probe hybrids produced significantly lower signals than that generated by the perfectly

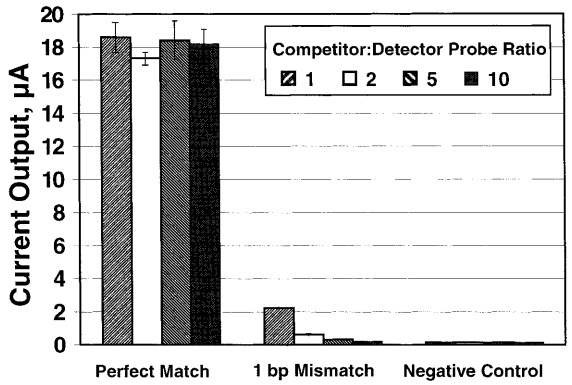

Figure 3. Effect of incorporating competitor probes into EDEMNA. With increasing competitor probe concentrations (100 nM–1 mM), signals generated by the mismatched target (1 bp Mismatch) were progressively reduced to levels comparable with the background with no significant effect on the signals generated by NDH-PM (Perfect Match).

matched target–probe hybrids (Fig. 2B). At the target concentration of 10 nM, the match-to-mismatch signal ratio produced with the hairpin-forming probe is ~10-fold higher than that generated with the linear detector probe.

Addition of unlabeled hairpin competitor probes

We hypothesized that addition of unlabeled, hairpin-forming competitor probes containing sequences complementary to mismatched targets could increase the sensitivity and specificity of the system by decreasing the formation of mismatched target–probe hybrids. The hairpin competitor probes differ from the hairpin detector probes in two ways. First, only the detector probes are labeled with fluorescein. Second, the loop sequences of the competitor probes are complementary to the mismatch targets, which are designed to differ by only a single nucleotide. Using target concentrations of 10 nM, increasing concentrations of the competitor probe progressively reduced the signals produced by the mismatched target–probe hybrids to levels not significantly different from the signals generated by the negative control (Fig. 3). However, as shown in Figure 3, the signal levels from the perfectly complementary target–probe hybrids and the negative controls were not significantly affected. The match-to-mismatch signal ratio was increased by >7-fold when the ratio of the competitor probes to the detector probes was increased to 10. A specificity check using another 'competitor probe' with unrelated sequence showed that the effect was sequence specific, as non-specific competitors had no effect on output signals (data not shown).

Adaptation of CARD

To further improve the detection limit of the EDEMNA implementation described above, we hypothesized that the CARD amplification system could be incorporated into EDEMNA to amplify output signals. We first determined that the CARD system, which in previous reports had been used in conjunction with optical detection, could be detected using the electrochemical detection method used in EDEMNA (data not shown). An experiment using the same oligonucleotides showed that CARD was able to improve the nucleic acid detection limit by ~100-fold, from 1 to 0.01 pM, with only a 7-fold increase in the background signal levels (Fig. 4).

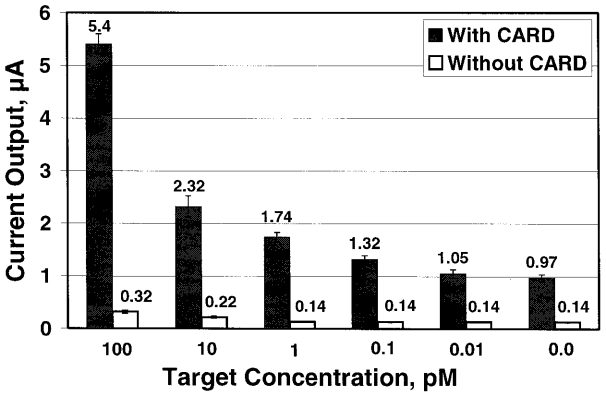

Figure 4. Signal amplification using CARD. Using NDH-PM as the target, without CARD, the output signals became indistinguishable from the background at the target concentration of 1 pM. With CARD, the output signals became indistinguishable from the background at 0.01 pM.

Application of EDEMNA to detect the factor V Leiden mutation

As a functional test, we showed that EDEMNA could be used to screen the factor V Leiden mutation from clinical samples (Fig. 5). The factor V Leiden mutation is a single base pair mutation (guanine to adenine) in the human factor V gene that predisposes carriers and homozygotes to venous thrombosis (21,23). PCR was first used to amplify the relevant region from genomic preparations (21). The PCR products were then subjected to interrogations composed of four reactions each using a hairpin detector probe containing A, C, G or T at the position of the factor V Leiden mutation. Figure 5 shows the results for both homozygous wild-type (Fig. 5A) and heterozygous carriers (Fig. 5B). The results obtained using EDEMNA agreed completely with the results determined independently by the UCLA Diagnostic Molecular Pathology Laboratory.

DISCUSSION

We describe in this report the adaptation of probes containing hairpin-forming stems to a HRP-based sandwich DNA hybridization assay using a electrochemical detection method. The data show that this approach, EDEMNA, is able to identify point mutations accurately. Our results also imply that the hairpin structures can confer sequence specificity independent of other elements present in the molecular beacon design. Hairpin-forming DNA probes should be applicable to a wide variety of assays where single nucleotide specificity is required.

Tyagi *et al.* (11) have suggested that divalent cations, such as magnesium, present in hybridization buffers significantly enhances stem hybrid stability. Within the EDEMNA assay set-up, increasing stem lengths beyond a minimum number would therefore not be likely to dramatically increase the performance of hairpin-forming probes. Based on the results of using different stem lengths in EDEMNA, we also concluded that in the presence of appropriate divalent cations there was no significant increase in specificity, as measured by the match-to-mismatch signal ratio, when the stem length was increased beyond 5 bp to 14 bp (data not shown).

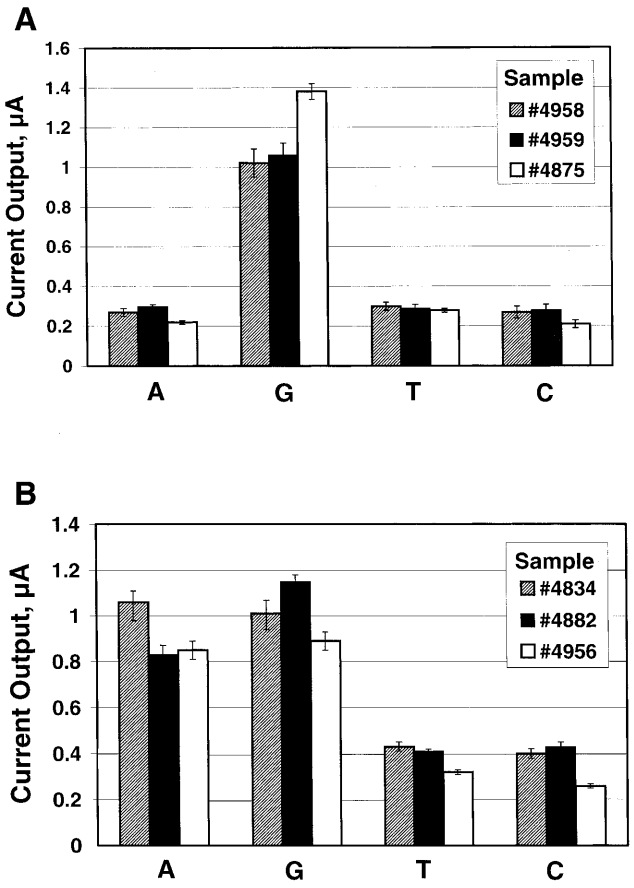

Figure 5. Screening for the factor V Leiden mutation using EDEMNA. (**A**) Homozygous wild-type. (**B**) Heterozygous carriers.

We have also shown that addition of competitor probes, which are essentially unlabeled hairpin-forming probes with loop sequences complementary to expected mismatch targets, can increase the match-to-mismatch signal ratio. In control experiments, the increase was ~7-fold, from 13 to 93. These competitor probes most likely work by binding to the mismatch targets to inhibit binding by the detector probes. They do not appear to interfere with the binding of detector probes to perfectly complementary targets.

In an enzyme-based method for the detection of nucleic acid hybridization such as EDEMNA, enzyme-mediated signal amplification can potentially alleviate the need for nucleic acid amplification. We showed that using off-the-shelf reagents, CARD could be incorporated into EDEMNA and was able to increase the detection limit of EDEMNA in control experiments by approximately two orders of magnitude. Further optimization of the CARD-based amplification scheme could omit the need for the nucleic acid amplification step as part of the EDEMNA assay.

In its current implementation, EDEMNA still has room for continued research and further improvement. Like most enzyme-based assays, EDEMNA is a heterogeneous assay requiring multiple steps involving washing and addition of reagents. Once automated, however, we expect that these steps could be carried out consistently and efficiently. Furthermore, using EDEMNA, only 1 nt can be interrogated in each

reaction, whereas optical detection schemes such as molecular beacons have the ability to multiplex and detect multiple nucleotides in a single reaction. We are currently attempting to alleviate this by microfrabrication technology involving dense electrochemical sensor arrays with significant improvements in detection limit over the single-well commercial sensors used in this study (16).

The ability to detect changes in nucleic acid sequences or identify differences between them are important to predicting, understanding and managing many human diseases. We have presented in this report EDEMNA, an electrochemical method for identifying single nucleotide differences in nucleic acids. On-going work will further explore its potential as a practical method for detecting nucleic acid variations in a variety of applications.

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