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Notes & Tips

DNA biosensor based on hybridization refractory mutation system approach for single mismatch detection

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ABSTRACT

We report on a simple approach to enhance solid-phase hybridization-based single base mismatch discrimination at high ionic strength based on the deliberate insertion of a natural DNA base mismatch in the surface-tethered probe. A large drop in hybridization signal of single base mismatched alleles using the designed probe as compared with the conventional probe, from 80% to less than 25% of the signal obtained with the fully complementary, non-mutation-containing sequence, when using colorimetric detection was further improved to 20% when using electrochemical detection, attributable to a difference of spacing of immobilized probes. Finally, the designed probe was used for the electrochemical detection of the DQA1*05:05 allele amplified from real human blood samples.

Single nucleotide polymorphisms (SNPs)[2] are the most common form of genetic variations in the human genome [1,2]. Increasing knowledge gleaned from the HapMap and 1000 Genomes projects have highlighted the importance of SNPs for disease diagnosis and detection of disease predisposition, and it is anticipated that in the future paradigm of medicine SNPs will be used for patient stratification, facilitating personalized medicine and theranostics. Electrochemical and optical biosensors have received considerable attention for the detection of single base mismatches [3–6]. Nevertheless, designing highly specific and selective probes for SNP detection is challenging and not always easy to achieve. To overcome this problem, several strategies have been proposed exploiting high-stringency conditions during the hybridization step, including elevated temperature [4], low ionic strength [7], and the addition of destabilizing agents (e.g., formamide) [8] or, alternatively, the use of probes with functional structures such as hairpin probes [9] and the use of DNA analogues such as peptide nucleic acids [10] and locked nucleic acids [11].

The amplification refractory mutation system (ARMS) was first introduced by Newton and coworkers to increase polymerase chain reaction (PCR) primer specificity [12]. The proposed system consisted of the deliberate insertion of an additional mismatch near the 3′ end of a primer to prevent primer extension with non-completely complementary sequences, hence increasing primer specificity.

Guo and coworkers [13] and Burgner and coworkers [14] reported a method to increase the discrimination of SNPs in DNA hybridization by inserting a universal base analogue of 3-nitropyrrole as an artificial mismatch in the oligonucleotide probes. However, 3-nitropyrrole modification is expensive and not widely available commercially.

In this work, we report on a method based on the deliberate insertion of a natural DNA base mismatch in a surface-tethered probe for enhancement of single base mismatch discrimination via hybridization at high ionic strength. The developed approach was tested for the selective detection of the human leukocyte antigen (HLA) DQA1*05:05 allele because the specific identification of this allele is needed for the high-resolution identification of the haplotype DQ2 trans associated with predisposition to celiac disease.

All reagents used in this work were of analytical grade. Trizma hydrochloride, sulfuric acid, ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), phosphate-buffered saline (pH 7.4) with 0.05% (v/v) Tween 20 (PBS–Tween), and 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system for enzyme-linked immunosorbent assay (ELISA) were purchased from Sigma. Potassium dihydrogen phosphate (KH_2PO_4), sodium dihydrogen phosphate (NaH_2PO_4), sodium hydroxide (NaOH), and sodium chloride (NaCl) were obtained from Scharlau. 6-Mercaptohexanol (MCH) was received from Fluka. Reacti-Bind Maleimide Activated 96-Well Plates were supplied by Thermo Scientific (Madrid, Spain). DNA probes, horseradish peroxidase (HRP)-modified reporting sequence, and

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[2] *Abbreviations used:* SNP, single nucleotide polymorphism; ARMS, amplification refractory mutation system; PCR, polymerase chain reaction; HLA, human leukocyte antigen; ELONA, enzyme-linked oligonucleotide assay; HRMS, hybridization refractory mutation system.

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synthetic analogues of relevant PCR amplicons were supplied by Biomers (Ulm, Germany).

Preparation of enzyme-linked oligonucleotide assay (ELONA), electrochemical experiments, PCR amplification, and single-stranded DNA (ssDNA) generation was performed as outlined previously [15,16] and can be found in the online supplementary material.

A detailed description of the optimized design of the probes for the HLA typing of DQ2 and DQ8 genes, together with the description of relevant interfering alleles, has also been detailed previously [15,16].

Because DQA1*05:01 and DQA1*05:05 alleles are involved in the genetic predisposition to celiac disease detection, clear discrimination between them is required. First, a probe able to detect both alleles, namely DQA05, was designed and investigated [15,16]. Subsequently, discrimination between the DQA1*05:01 and DQA1*05:05 alleles was obtained by the design of a DQA0505 probe (5′-CTTGTCCAGTCCCCAGT-SH-3′) located between codons 170 and 175 of the DQA1 exon 3 region, where a single base difference (A → C at codon 172) is present.

This DQA0505 probe was tested using alleles in the DQA1 locus and represented by DQA1*05:01, DQA1*02:01, DQA1*03:02, and DQA1*06:01 synthetic oligonucleotides having one, two, and three base mismatches (see Fig. S1 in supplementary material).

The ability of the DQA0505 probe to discriminate between the target allele and possible interfering alleles was evaluated using both electrochemical and colorimetric detection. The DQA0505 probe failed to discriminate the DQA1*05:05 allele from the single point mutation interfering allele (DQA1*05:01), with this single mismatched allele generating a response comparable to (~80%) that recorded for the fully complementary sequence (Fig. 1). Lucarelli and coworkers reported that employing shorter oligonucleotide probe improves the probe selectivity [17], so a range of shorter 15-mer probes were designed by either removing one base from each end or removing two bases from the 3′ end. However, these probes also failed to discriminate between the DQA1*05:05 and DQA1*05:01 alleles (data not shown).

As mentioned in the introductory paragraphs, the deliberate introduction of a mismatch into the oligonucleotide sequence (ARMS) has been successfully employed to improve the specificity of PCR-based genotyping [12,18]. Taking inspiration from the ARMS approach, we propose the hybridization refractory mutation system (HRMS) for the design of DNA probes with enhanced specificity for on-surface hybridization. Lee and coworkers [19] published guidelines for incorporating non-perfectly matched oligonucleotides into hybridization probes for a DNA microarray. These can be summarized as follows: (i) find the sequence similarity area with other non-target genes, (ii) exclude the last three end positions, (iii) search for a G mutation, and (iv) avoid mutations from A to G or from T to G.

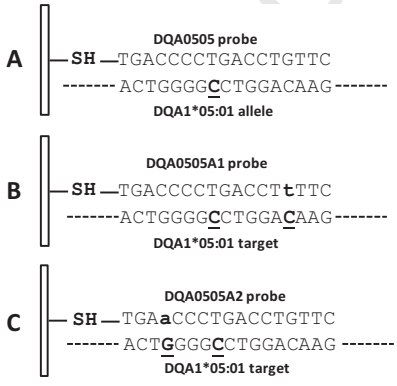

Fig. 1. Schematic highlighting hybridization of different probes with single base mismatched DQA1*05:01 allele: (A) DQA0505 probe; (B) DQA0505A1 probe; (C) DQA0505A2 probe. Base in lowercase letter indicates deliberated mismatch, and base in bold and underlined letter indicates mismatch.

However, their guidelines were based on theoretical T_m calculation for solution-phase hybridization of two oligonucleotides.

In the reported work, two different probes have been designed by inserting different single base mismatches near each end of DNA probe. Because the original probe has a high GC content (58.8%), we replaced either G or C with T or A to decrease the GC content to 52.9%. Specifically, probe DQA0505A1 (5′-CTTTTCCAGTC CCCAGT-SH-3′) was designed by replacing G with T at the fourth nucleotide position from the 5′ end (underlined base), resulting in a (C–T) mismatch for all alleles, whereas probe DQA0505A2 (5′-CTTGTCCAGTCCCAAGT-SH-3′) was designed to have a mismatch at the fourth nucleotide position from the 3′ end, which was achieved by replacing C with A (underlined base), resulting in a (G–A) mismatch for all alleles (Fig. 1).

The selectivity of both probes was tested using the model of interfering alleles described in Fig. 1 with the ELONA colorimetric assay. Fig. 2 summarizes the response as percentage ratios between those of the target (DQA1*05:05) and those of the mismatched alleles for all three probes. Both HRMS probes (light gray and white bars) showed an improvement in the discrimination. The use of the DQA0505A2 probe resulted in a drop of the DQA1*05:01 allele signal to approximately 60% of the fully complementary signal (in comparison with 80% recorded with the original DQA0505 probe). However, a tremendous improvement in the selectivity was achieved using the probe DQA0505A1, where the colorimetric signal recorded for the single base mismatched DQA1*05:01 allele dropped to less than 25% of the signal obtained with the DQA1*05:05 allele.

This result demonstrates that the insertion of the artificial mismatch can significantly enhance the discrimination ability of the immobilized oligonucleotide probe without adjusting the stringency conditions (e.g., salt concentration, use of destabilizing agents) of hybridization.

Following the colorimetric assay, an electrochemical hybridization assay was used to detect and discriminate DQA1*05:01 and DQA1*05:05 alleles using synthetic oligonucleotides and real clinical samples. In the Fig. 2 inset, the percentage of the amperometric signal recorded from the original probe (DQA0505) and the HRMS probe (DQA0505A1), with a significant decrease (from 80 to 20%) of the response for the single base mismatched DQA1*05:01 allele, was recorded and showed an improvement over the results obtained with the colorimetric assay, probably attributable to the surface chemistry on the electrode surface, which results in optimally spaced probes for improved access to the region of the

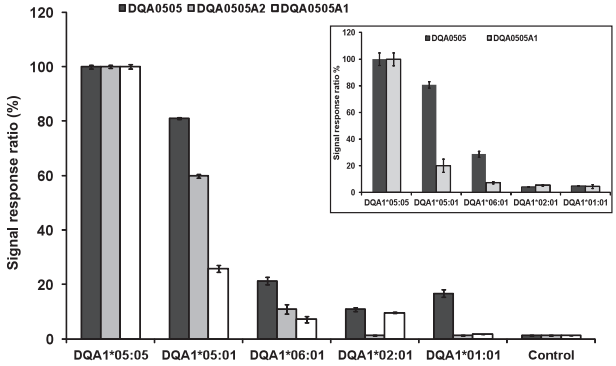

Fig. 2. Colorimetric evaluation of the DQA0505, DQA0505A1, and DQA0505A2 probes in the presence of the target and possible interfering alleles. Hybridization was performed in the presence of 20 nM synthetic DNA for 5 min at 37 °C and the capture of reporting sequence for a 10-nM solution for 20 min at 37 °C. Inset: Normalized electrochemical evaluation of the DQA0505 and DQA0505A1 probes in the presence of the target and possible interfering alleles. Hybridization was performed in the presence of 20 nM synthetic DNA for 10 min at 37 °C and the capture of reporting sequence for a 10-nM solution for 20 min at 37 °C.

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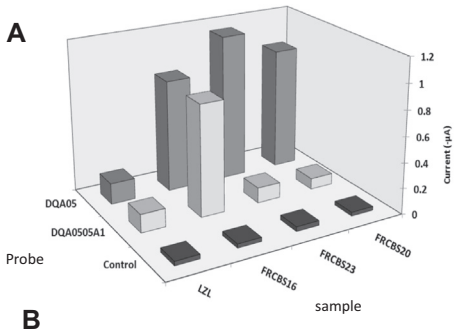

B

Sample	DQA1		DQB1	
FRCBS 20	**05:01**	**05:01**	02:01	02:01
FRCBS 16	01:02	**05:05**	03:01	06:02
FRCBS 23	02:01	**05:01**	02:01	02:02
LZL	05:03	05:03	03:01	03:01

Fig. 3. (A) Electrochemical multiplex detection of DQA1*05:01 and DQA1*05:05 alleles from real human samples. (B) Table of the HLA typing of the samples obtained with the reference approaches (shaded) used in this work: Luminex HLA-DQA1/DQB1 typing and confirmed by Olerup DQA1 or DQB1 SSP typing kits.

immobilized probe containing the introduced mismatch. The higher selectivity of the DQA0505A1 probe in comparison with the DQA0505A2 probe could be explained by the type and position of HRMS mismatch. Allawi and SantaLucia [20,21] reported that an A–G mismatch (as in the case of DQA0505A2) forms a more stable duplex than a C–T mismatch (DQA0505A1). Another factor is the position of the HRMS mismatch of the DQA0505A1 probe far from electrode surface, which has been reported to be more destabilizing than a mismatch closer to the surface [22,23], despite some reports demonstrating that a mismatch near the electrode surface is more destabilizing to the DNA duplex [6].

PCR product of clinical samples, previously typed at the accredited laboratory of the Finnish Red Cross Blood Service (Helsinki, Finland) was then analyzed for the presence of the DQA1*05:01 and DQA1*05:05 alleles using the DQA0505A1 probe. Four clinical samples were used in this study: two samples carrying the DQA1*05:01 allele (FRCBS 20 and FRCBS 23), one sample carrying the DQA1*05:05 allele (FRCBS 16), and one sample negative to both the DQA1*05:01 and DQA1*05:05 alleles carrying the DQA1*05:03 allele (from the LZL cell line).

As can be seen in Fig. 3, a positive electrochemical signal for both the DQA05 and DQA0505A1 probes was obtained for sample FRCBS 16, which indicates the presence of the DQA1*05:05 allele (with or without the DQA1*05:01 allele), whereas a positive electrochemical signal of the DQA05 probe and a very low signal (negative) of the DQA0505A1 probe for samples FRCBS 20 and FRCBS 23 indicates the presence of the DQA1*05:01 allele and the absence of the DQA1*05:05 allele in the samples. Sample LZL did not show any significant hybridization signal with either probe, indicating that LZL does not contain either the DQA1*05:01 allele or the DQA1*05:05 allele.

In summary, we have demonstrated an approach for enhancing single base mismatch discrimination via solid-phase hybridization in the presence of high ionic strength. Insertion of a selectively positioned deliberate mismatch in the surface-tethered DNA probe was demonstrated to improve genosensor selectivity without affecting sensitivity. The developed genosensor was used to analyze amplified clinical samples for selective detection of DA1*05:05 from a single base mismatched DQA1*05:01 allele, and an excellent correlation with laboratory-based genotyping was obtained.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ab.2014.11.021.

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