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Toehold-Mediated Nonenzymatic DNA Strand Displacement As a Platform for DNA Genotyping

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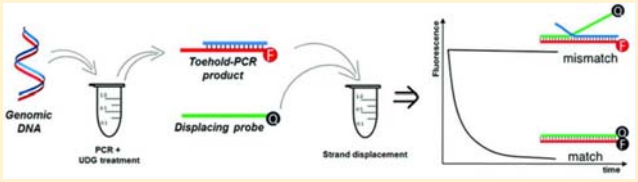
Supporting Information

ABSTRACT: Toehold-mediated DNA strand displacement provides unique advantages in the construction and manipulation of multidimensional DNA nanostructures as well as nucleic acid sequence analysis. We demonstrate a step change in the use of toehold-mediated DNA strand displacement reactions, where a double-stranded DNA duplex, containing a single-stranded toehold domain, enzymatically generated and then treated as a molecular target for analysis. The approach was successfully implemented for human DNA genotyping, such as gender identification where the amelogenin gene was used as a model target system, and detecting single nucleotide polymorphisms of human mitochondrial DNA. Kinetics of the strand displacement was monitored by the quenched Förster resonance energy transfer effect.



INTRODUCTION

DNA displacement reactions between double-stranded (ds)-DNA with strands of unequal length and single-stranded (ss)-oligonucleotides using a toehold structure, as a trigger point, enables DNA rehybridization in a fast "base-by-base" programmable controlled manner.[1,2] The advantage of this is that the route of strand displacement can easily be predicted due to the fact that the displacement is always known at the commencement of the displacement. Moreover, provided the entire sequence of the reacting oligonucleotides is known and taking into account all mismatches, such as point mutations, deletions, or insertions occurring during strand exchange, it is possible to predict the outcome and the kinetic and thermodynamic behavior of the displacement reaction.[3]

Since the discovery of toehold-mediated nonenzymatic DNA strand displacement reactions by Yurke et al.[4] in 2000 there has been a plethora of innovation in this new era of DNA nanotechnology.[5,6] Such innovations include the construction of artificial DNA nanoactuators,[7,8] computation DNA logic elements,[9−12] and 2-D and 3-D DNA nanoconstructions.[13,14] In particular, biosensing techniques based on strand displacement were targeted at synthetic nucleic acids,[3,15−20] proteins[21,22] and low molecular weight organic and inorganic molecules and ions.[15,23] However, to date, little attention has been paid to manipulating DNA strand displacement reactions for the analysis of real-life DNA samples, such as those of human, bacterial, or viral genomes.

Recently, a platform based on toehold-mediated strand displacement and atomic force microscopy (AFM) for label-free single nucleotide polymorphism (SNP) genotyping was described by Zhang et al.[16] The authors constructed a DNA-origami chip anchored with single-stranded capture probes which in turn were hybridized with partially complementary streptavidin labeled reporter probes. Application of fully complementary oligonucleotides (analytes) to the origami chip allowed the authors to perform toehold exchange of strands which were identified by the disappearance of streptavidin "white bulges" on the AFM images. This approach was subsequently applied to SNP typing using synthetic single-stranded target oligonucleotides, unrelated to real DNA diagnostics. A similar approach was demonstrated by Subramanian et al.[19] In another approach Picuri et al.[15] focused on the creation a universal translator for nucleic acid diagnostics. A toehold-mediated DNA strand displacement reaction was applied to the polymerase chain reaction (PCR) independent translation of biologically relevant DNA and RNA sequences into unique unrelated DNA sequences. This in turn could be recognized by another generic technique of choice. The technology was then used to identify synthetic oligonucleotides which mimic sequences of hepatitis C (HCV), influenza, and chicken pox viruses.

Previous work in literature[16−18,24] has predominately focused on short artificial ss-oligonucleotides as targets for identification, where the ds-DNA duplex containing a ss-toehold domain acted as a molecular probe. Moreover, the concentrations of the targets were well beyond those conventionally observed in real-life nucleic acid analyses. This in turn imposes strict limitations on the application of these techniques for the analysis of real DNA samples.

Seminal work by Pourmand et al.[25] described the analysis of human DNA length polymorphisms (short tandem repeats (STRs)). In this work the DNA strand displacement phenomenon was used as an auxiliary technique to remove

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imperfectly hybridized (overhanging) ss-DNA from specific capture probes immobilized on a microarray surface. This approach consisted of three nested PCRs in order to obtain a ss-DNA target. Three consecutive hybridization procedures were then used to hybridize the ss-DNA target with an immobilized probe and remove all imperfectly hybridized ds-DNA, likely restricting this method for clinical or forensic applications.

Herein, we describe a new simpler approach for the analysis of real-life DNA samples using a toehold-mediated DNA strand displacement reaction where a partially complementary duplex, with a ss-toehold sequence, was used as a target for analysis. The approach consisted of two stages (Figure 1): (i) PCR amplification was used to generate a PCR product with a contiguous ss-toehold domain (further named toehold-PCR product). This was achieved using a substitution of deoxythymidine for deoxyuracil (dT→dU) in one of the PCR primers. Using this dU substituted primer then resulted in a PCR product with incorporated uracil bases. By treating the PCR product with uracil-DNA glycosylase (UDG) short ss-oligonucleotides were released from the 5′ of the PCR product, making them suitable for toehold-mediated strand displacement reactions; and (ii) toehold-mediated DNA strand displacement reactions between the target toehold-PCR product and single-stranded chemically synthesized oligonucleotide molecular probes were performed using a quenched Föster resonance energy transfer (FRET) technique to monitor how the reaction proceeded. The analytical sensitivity of the entire approach is determined by the sensitivity of the first PCR stage and could potentially reach a level as little as 5−10 DNA molecules.[26] The amelogenin gene, commonly used both in forensic and medical applications for human gender identification, was used as a model system for human DNA genotyping.[27,28] Using this model system the enzymatic generation of the toehold-PCR products of the male and female human genome, with subsequent strand displacement assisted discrimination, was shown. Moreover, the developed approach was directly adapted for SNP testing. As an example, a C-to-T (C16223T) single nucleotide substitution at the 16223 position of the hyper-variable region 1 (HVR-1) of the mtDNA found in one of the authors DNA samples was discriminated. It is envisaged that this model system could be easily adapted to other DNA genotyping molecular diagnostics.

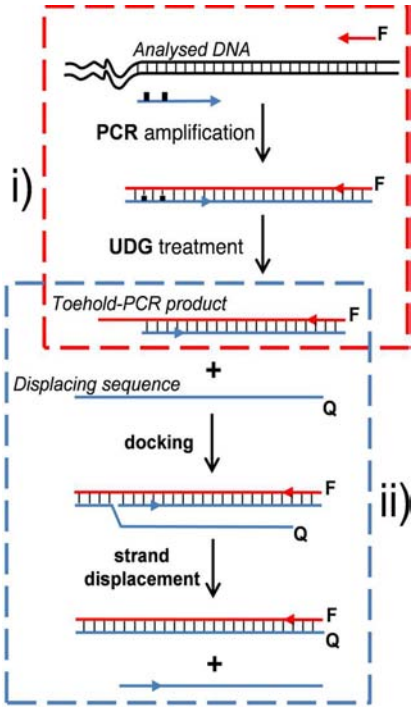

Figure 1. Schematic representation of the approach proposed for the analysis of real-life ds-DNA samples using toehold-mediated strand displacement reaction. (i) Deoxyuracil (■) modified forward and fluorophore (F) labeled reverse primers are used for the amplification of the target DNA. Following treatment of the dU modified amplification product with uracil-DNA glycosylase a ds-PCR product containing a ss-toehold sequence (toehold-PCR product) was generated. (ii) Toehold-mediated strand displacement between the toehold-PCR product and a chemically synthesized displacing sequence labeled with quencher (Q) oligonucleotide molecular probes. Displacement is monitored using a quenched FRET technique.

MATERIALS AND METHODS

Nucleic Acid Isolation and PCR Amplification. Human genomic and mitochondrial DNA were coextracted from the authors' own blood samples using a QIAamp DNA blood mini kit (Qiagen, Germany), according to the recommendations of the manufacturer. PCR primers, including dU modified and fluorescently labeled primers (FAM and HEX) (see Table 1) were purchased from IDT DNA Technology, USA, and used at a concentration of 0.2 μM. Melting temperatures were calculated using Oligo Analyzer 3.1 (IDT DNA Technology, USA) under the following conditions: an oligonucleotide concentration of 0.2 μM, Na^+ concentration of 100 mM, Mg^{2+} concentration of 2.5 mM, and deoxyribonucleotide triphosphates (dNTPs) concentration of 0.8 mM. Both amelogenin and SNP targeted PCRs were performed using a HotStar Taq DNA polymerase (Qiagen, Germany) within 1× HotStar Taq PCR buffer with a final $MgCl_2$ concentration of 2.5 mM. The concentration of the standard deoxynucleotide triphosphates (dATP, dTTP, dGTP, dCTP) was 0.2 mM. A PCR amplification regime of 95 °C for 15 min, 30 cycles of 94 °C for 20 s, 61 °C for 30 s, 72 °C for 30 s, and a final elongation of 72 °C for 3 min was used. A template of 5 ng of the extracted DNA samples was used.

Directly after the PCR, uracil-DNA glycosylase (NEB, USA) (2.5 U) was added to the PCR solution, and the entire mixture was then gently mixed via pipetting. After incubation at room temperature for 5 min the mixture was heated to 95 °C for 5 min and then cooled back to room temperature. The PCR-UDG mixtures were purified using a Qiaquick PCR purification kit (Qiagen, Germany) for the amelogenin system and Amicon Ultra-0.5 30K (Millipore, USA) for SNP testing, according to the recommendations of the manufacturers. The nucleic acids were then eluted from the Qiaquick PCR purification kit, or recovered Amicon Ultra-0.5 30K, with a displacement TEM buffer (pH 8), consisting of Tris·HCl (10 mM), EDTA (1 mM), and $MgCl_2$ (12.5 mM). Quantification of the eluted PCR products was carried out using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). Capillary electrophoresis (CE) analysis was carried out on a ABI-3130XL Genetic Analyzer (Life Technologies, USA), using a GeneScan 500 LIZ size standard (Life Technologies, USA).

Strand Displacement Reaction. Displacing sequences (X*i*, Y*i*, SNP-C*i*, and SNP-T*i*, see Table 1) labeled at the 3′ termini with the fluorescent quencher carboxytetramethylrhodamine (TAMRA), for FRET analysis, were purchased from Eurogentec, Belgium.

All displacement reactions were carried out at 30 °C, if not mentioned otherwise, in a reaction volume (15 μL) using a RotorGene 3000 real-time PCR thermocycler. Prior to the displacement reaction the toehold-PCR product (2 pMole, dissolved in 1× TEM buffer) was placed in a thin-wall PCR tube (0.2 mL) and briefly centrifuged. Then, the displacing sequence (20 pMole, dissolved in 2 μL of 1× TEM buffer), if not mentioned otherwise, was carefully applied into the lid of the same PCR tube. The tube lid was then carefully closed and the tube placed into the thermocycler carefully avoiding mixing of the

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Table 1. Sequences and Melting Temperatures of Primers and Displacing Oligonucleotides

	Sequence	T_m, °C
	PCR Primers	
AMEL-F-dU-4	5′- CCC ***dU***GG GCT CTG TAA AGA ATA GTG-3′	65.3
AMEL-F-dU-9	5′- CCC TGG GC***dU*** CTG TAA AGA ATA GTG-3′	65.3
AMEL-F-dU-4,9	5′- CCC ***dU***GG GC***dU*** CTG TAA AGA ATA GTG-3′	65.3
AMEL-F-HEX -dU-4,9	5′-HEX-CCC ***dU***GG GC***dU*** CTG TAA AGA ATA GTG-3′	65.3
AMEL-FAM-R	5′-FAM-ATC AGA GCT TAA ACT GGG AAG CT-3′	65.1
HVR-F-dU-5,9	5′-CTA G***dU***G GG***dU*** GAG GGG TGG CT-3′	68.0
HVR-FAM-R	5′-FAM-ATGCTTACAAGCAAGTACAGCAAT-3′	64.1
	Displacing Sequences	
X*i* (106)	5′-CCCTGGGCTCTGTAAAGAATAGTGTGTTGATTCTTTATCCCAGAT - - - - - - GTTT CTCAAGTGGTCCTGATTTTACAGTTCCTACCACCAGCTTCCCAGTTTAAGCTCTGAT-TAMRA-3′	
Y*i* (112)	5′-CCCTGGGCTCTGTAAAGAATAGTGGGTGGATTCTTCATCCCAAAT**AAAGTG**GTTT CTCAAGTGGTCCCAATTTTACAGTTCCTACCATCAGCTTCCCAGTTTAAGCTCTGAT-TAMRA-3′	
SNP-C*i*	5′-CTAGTGGGTGAGGGGTGGCTTTGGAGTTGCAGTTGATGTGTGATAGTTGA**G**GGTT GATTGCTGTACTTGCTTGTAAGCAT-TAMRA-3′	
SNP-T*i*	5′-CTAGTGGGTGAGGGGTGGCTTTGGAGTTGCAGTTGATGTGTGATAGTTGAAGGTT GATTGCTGTACTTGCTTGTAAGCAT-TAMRA-3′	

toehold-PCR product and the displacing sequence before the run protocol started (for details see Supporting Information, S2). As controls the UDG untreated PCR products and the single FAM labeled reverse primer (R-FAM) were used.

Acquisition of fluorescent signals was performed within the SYBR Green/FAM channel at a gain of 10 within a time interval of 20 s between fluorescent reads. Normalization of the raw fluorescent (see Figure S5) signals was made by dividing the signal values by the initial signal value, as described previously.[2]

Curve-fitting of the kinetic data was carried out using the nonlinear curve fitting function in OriginPro 8 software (Origin Corporation, USA). The data were fit to the first-order equation as described by Baker et al.[29]

RESULTS AND DISCUSSION

Model System/Target. The amelogenin gene was chosen as a target model system because the Y chromosome has a six base pair insertion compared to the homologue on the X chromosome. Typically, PCR amplification of intron 1 of the amelogenin locus with specific primers produces a homogeneous PCR product for female samples (two X chromosomes, XX), while producing a heterogeneous product for male DNA samples (X and Y chromosomes, XY).[30]

Primer Design. A DNA duplex with a ss-toehold domain sequence was required for the toehold-mediated DNA strand exchange. In order to achieve this, the forward PCR primer had the deoxythymidine substituted for deoxyuracil (dT→dU). This resulted in the generation of a PCR product with incorporated dU bases at the primer location. Consecutive treatment of the PCR mixture with uracil-DNA glycosylase, an enzyme which removes uracil bases from DNA by glycosidic bond hydrolysis[31] and heating of the reaction to 95 °C lead to the hydrolysis of the DNA phosphate backbone producing the PCR products with a ss-toehold domain (Figure 1i).

A primer set with sequences based on those from Power Plex 16 System (Promega, USA),[32] for amplification of the amelogenin gene, was used. These are primers that are typically used in the majority of forensic PCR systems for human STR analysis, except with the dT→dU substitutions in the sequence of the forward primer. Three different dU substituted forward primers (Table 1) were used in order to investigate the influence of toehold sequence length on the efficiency of the displacement. The primers F-dU-4 and F-dU-9 were generated with one dT→dU substitution at the fourth and ninth position (counting from the 5′ termini), respectively. These substitutions therefore resulted in the formation of PCR products with 4 and 9 nucleotide toehold lengths. In the primer AMEL-F-dU-4,9, dT→dU substitutions were made at two positions to provide a 9 nucleotide toehold length as well. This was achieved by removing the uracil bases from both the fourth and ninth positions simultaneously.

The reverse primer (AMEL-R-FAM) for PCR amplification was labeled with FAM fluorescent dye at its 5′ termini, which was then used as a reporter fluorescent dye for monitoring reaction kinetics using the quenched FRET effect (Figure 1ii).

In order to evaluate the efficiency of the PCR amplification with dU modified primers and subsequent UDG assisted generation of a ss-toehold sequence, the AMEL-F-dU-4,9 primer was labeled with HEX fluorescent dye (AMEL-F-HEX-dU-4,9) at its 5′ termini in order to be observed using CE.

Amelogenin Gene PCR Amplification and Formation of a Toehold Sequence. For the PCR amplification with dU modified primers special attention was required in the selection of the DNA polymerase due to the fact that native DNA polymerases isolated from archaea, for example, Pfu DNA polymerase (Promega, USA), Pfx DNA polymerase (Life Technologies, USA) or Phusion DNA polymerase (NEB, USA), bind strongly to the uracil-containing DNA template and stall further polymerization.[33] For this reason only DNA polymerases originating from bacterial host strains or a mutant Pfu DNA polymerase, for example, as described by Norholm,[34] should be used while working with dU modified primers. Therefore, HotStar Taq DNA polymerase (Qiagen, Germany), isolated from *Thermus aquaticus*, was used in this research.

The analysis of the PCR amplification using dU substituted primers and the subsequent UDG treatment was then performed. To achieve this the PCR amplification of human both female and male DNA samples (5 ng, each) using AMEL-F-HEX-dU-4,9 and AMEL-R-FAM primers was conducted. Immediately after the PCR, the mixtures were treated with UDG. The results of the reactions were then confirmed using CE (see Supporting Information, S1). Figures S1A, S1B, S3A, and S3B show the results of the CE analysis of the PCR products before the UDG treatment. The peaks at the positions (approximately) of the 106 and 112 nucleotides belong to X and Y chromosomes, respectively. It can be seen that the peaks corresponding to the HEX fluorescent dye (Figures S2B and

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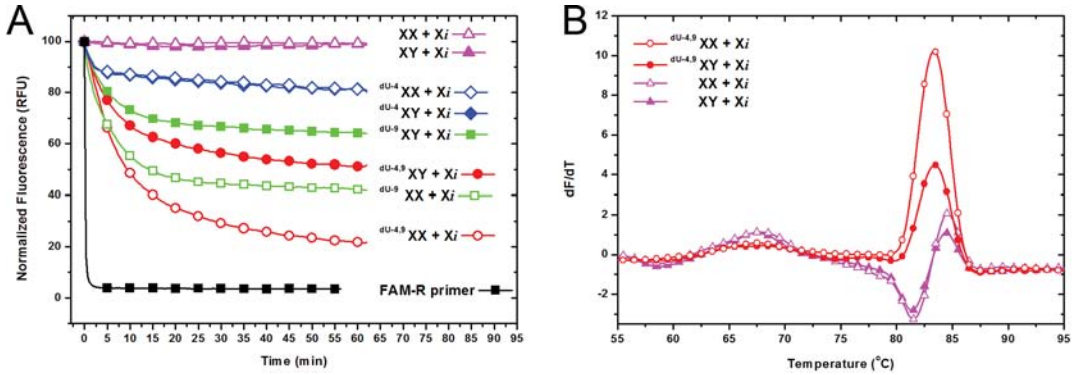

Figure 2. (A) Effect of the toehold length on the efficiency of the strand displacement reaction between the amelogenin PCR products (XX, female = hollow symbols; XY, male = solid symbols) and X*i* displacing sequence. Toehold lengths of 4 ($^{dU-4}$toehold-PCR products, ◆ and ◇) and 9 nucleotides ($^{dU-9}$- and $^{dU-4,9}$toehold-PCR products, □ and ■ and ○ and ●, respectively) were investigated. The female (XX, △) and male (XY, ▲) PCR products with no toehold sequence as well as the fluorescein labeled reverse primer (FAM-R, solid squares) were used as controls. (B) Melting temperature analysis of the corresponding displaced products.

S4B) disappear completely after the UDG treatment, indicating that the phosphate backbone at the site of the uracil base had been destroyed.

Six different fluorescently labeled amelogenin toehold-PCR products were obtained via amplification of male and female DNA with three different primer pairs and treatment with UDG. Every primer pair consisted of the AMEL-R-FAM primer and each of three dU substituted primers (AMEL-F-dU-4, AMEL-F-dU-9, and AMEL-F-dU-4,9) (Table 1).

DNA Strand Displacement Monitoring Using FRET. Two chemically synthesized displacing sequences labeled at their 3′ termini with TAMRA (for the FRET quenching effect) (X*i* and Y*i*, shown in Table 1) were designed for reacting with the FAM fluorescently labeled amelogenin toehold-PCR products. The sequence of the X*i* displacing sequence, having a length of 106 nucleotides, was fully complementary to the longer strand of the toehold-PCR product produced from the X chromosome, while the Y*i* displacing sequence, having a length of 112 nucleotides, was fully complementary to the longer strand of the toehold-PCR product of the Y chromosome.

The difference in length between the X*i* and Y*i* displacing sequences was attributed to a 6 nucleotide insertion (AAAGTG) at the 46th position (counting from the 5′ nucleotide of the forward primer). Moreover, addition of single nucleotide substitutions namely T25G, T28G, T36C, G43A, T68C, G69A, and C88T, corresponding to naturally occurring polymorphisms in the X and Y chromosomes of the human genome,[30] was also present in the X*i* and Y*i* displacing sequences. According to literature[2,3,15,19] the influence of these polymorphisms is controversial, but in this particular case, this may have negligible influence on the differentiation between X and Y chromosomes compared to the insertion of six nucleotides.

A 10-fold excess of the TAMRA labeled X*i* sequence was added to both the fluorescently labeled female and male PCR products and the FRET quenching effect monitored. The results of the experiments are shown in Figure 2A. The female (XX) and male (XY) UDG untreated PCR product control samples (Figure 2A; Δ, ▲) showed the degree of conversion to be approximately 2% (±3%), indicating that no reaction occurred.

Among the three toehold-PCR products obtained after the UDG treatment the highest degree of conversion of the strand displacement was observed for the amelogenin toehold-PCR product, generated using the AMEL-F-dU-4,9 forward primer (^{dU4,9}toehold-PCR product) with two dT→dU substitutions. The displacement levels in this case reached 79% for the female toehold-PCR product ($^{dU-4,9}$XX, Figure 2A, ○) and 49% for the male toehold-PCR product ($^{dU-4,9}$XY, Figure 2A, ●).

Comparison was then performed between the degree of conversion for the amelogenin toehold-PCR products made with AMEL-F-dU-9 and AMEl-F-dU-4,9 primers ($^{dU-9}$XX and $^{dU-4,9}$XX, respectively) reacting with X*i*, (Figure 2A). Although the toehold domains in both cases theoretically have the same length (9 nucleotides), the degree of displacement for the reaction with the toehold-PCR product produced with the F-dU-9 primer was noticeably lower and found to be around 58% (±4) and 37% (±3) for female $^{dU-9}$XX (Figure 2A, □) and male $^{dU-9}$XY samples (Figure 2A, ■), respectively. This could best be explained by incomplete dissociation of the digested 5′ sequence consisting of 9 nucleotides and having a melting temperature of 45.3 °C. Most likely this nondissociated sequence acts as a protector for toehold strand displacement.[35] This indicated that a single dU substitution was insufficient to provide a fully unprotected toehold domain of 9 nucleotides in length. Thus, the AMEL-F-dU-9 primer was excluded from further investigation.

The amelogenin ^{dU4}toehold-PCR product with the shortest toehold length of 4 nucleotides gave the lowest degree of displacement at around 18 ± 3% for both female ($^{dU-4}$XX Figure 2A, ◇) and male ($^{dU-4}$XY Figure 2A, ◆) samples. That in turn indicates that, in this particular case, the toehold consisting of 4 nucleotides (T_m < 10 °C) was not able to initiate an effective strand displacement process.[1] As a result the AMEL-F-dU-4,9 primer proved to be the most efficient for the generation of a ss-toehold domain in a PCR product using the proposed method.

Since the displacement reactions were carried out using a real-time PCR thermocycler, this provided the opportunity to perform melting temperature analysis of the displaced products. The melting analysis (Figure 2B) shows the major melting peak of the displaced product at 83.2 °C (79.7 °C calculated) for female (Figure 2B, ○) and male (Figure 2B, ●) PCR products produced with the AMEL-F-dU-4,9 primer. The area under the curve for these samples differs by approximately a factor of 1.87, which closely corresponds to the ratio between of the amount of the X chromosome in female DNA and in male DNA.

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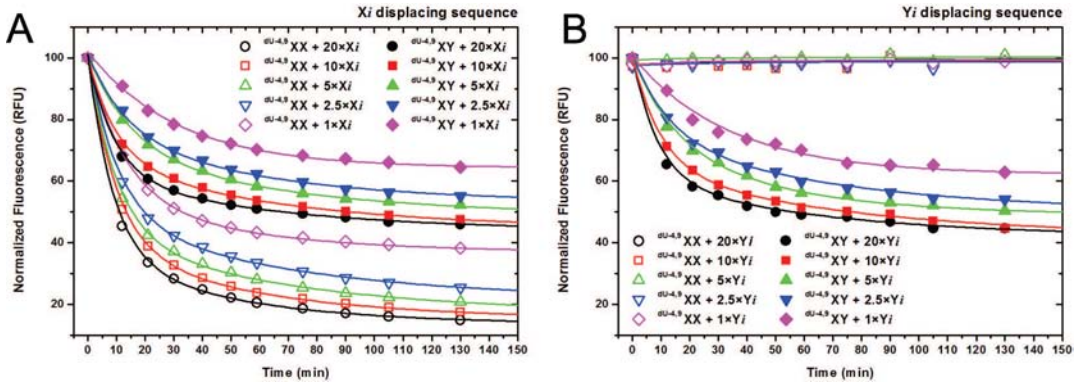

Figure 3. Effect of the displacing sequence excess on the degree of strand displacement conversion. Strand displacement reaction between the amelogenin $^{dU\text{-}4,9}$toehold-PCR products containing a 9 nucleotides long ss-toehold sequence (female $^{dU\text{-}4,9}$XX = hollow symbols, male $^{dU\text{-}4,9}$XY = solid symbols) and 20× (circles), 10× (squares), 5× (up-pointing triangles), 2.5× (down-pointing triangles), and 1× (diamonds) excesses of the X*i* (A) and Y*i* (B) displacing sequences.

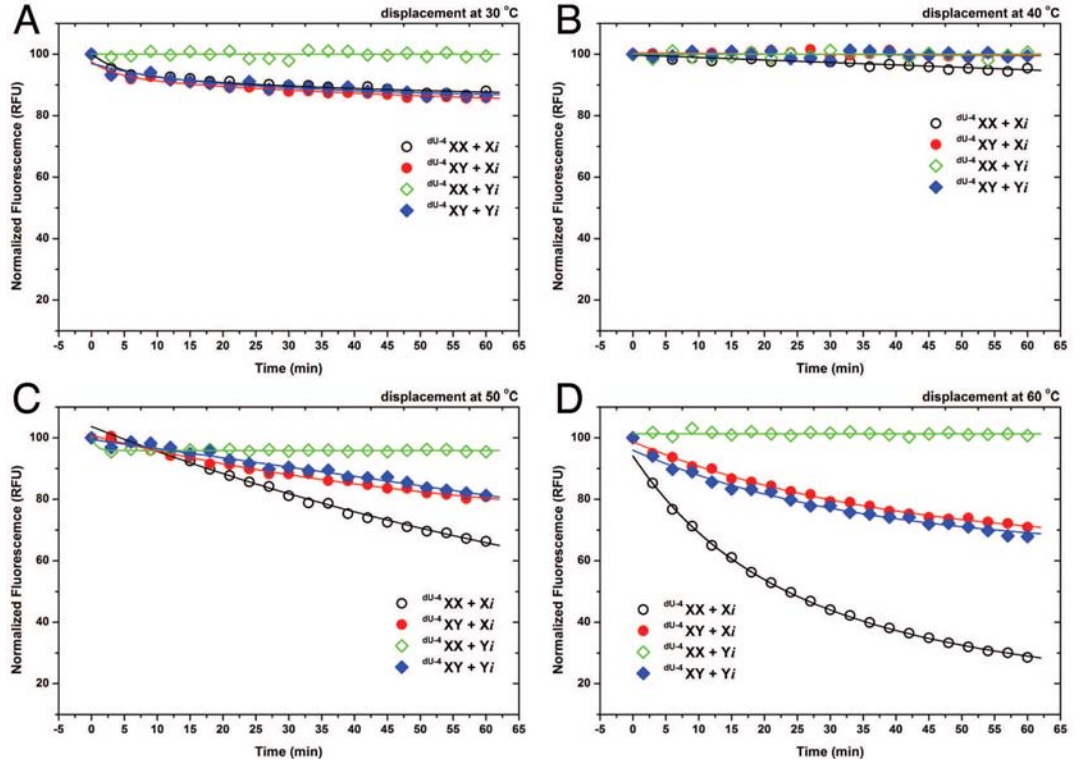

Figure 4. Kinetics of the displacement reactions between the amelogenin $^{dU\text{-}4}$toehold-PCR products and a 10-fold excess of X*i* (circles) and Y*i* (diamonds) displacing sequences performed at the different temperatures: (A) 30 °C, (B) 40 °C, (C) 50 °C, and (D) 60 °C.

The melting curves of the UDG untreated amelogenin samples (no toehold structure was created) showed a different melting behavior (Figure 2B, Δ and ▲). Such behavior represents a dissociative pathway of the DNA strand displacement which occurs at a temperature close to melting.[36] Similar melting trends were observed for the ^{dU4}toehold-PCR products with 4 base length toeholds (data not shown).

To further optimize the efficiency of the strand displacement, the ratio (excess) of the displacing sequence to the amelogenin toehold-PCR products was evaluated. Five different ratios of both X*i* and Y*i* sequences, in the range of a 20-fold excess (20×) to a 1-fold excess (1×), were used. Figure 3A shows the difference between the degree of conversion for both female (^{dU4,9}XX, Figure 3A, hollow symbols) and male (^{dU4,9}XY, Figure 3A, solid symbols) samples reacting with the female displacing sequences (X*i*). In all cases there was a gradual decrease in the degree of conversion. For female samples this degree of conversion was 4.3 ± 1.1% and for male samples this was 3.7 ± 1.1% across the 20×, 10×, 5×, and 2.5× X*i* displacement sequence excess concentrations. However, the samples with equimolar ratio (1×) of the X*i* displacing sequence to the toehold-PCR products (Figure 3A, ◇ female and ◆ male toehold-PCR products) differ from the 2.5× excess by 9.2 ± 1.2% for female samples and 8.3 ± 1.4% for male samples. This difference can be attributed to either incorrect determination of nucleic acid concentration or the side reactions of the displacing sequence with traces of unreacted AMEL-R-FAM primer. However, these processes are rendered negligible when using larger excesses of the displacing sequences.

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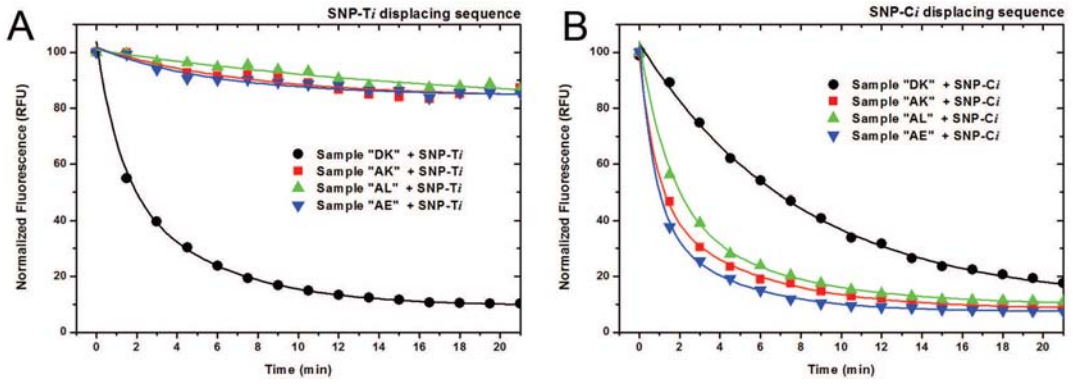

Figure 5. mtDNA C16223T SNP discrimination (A) with the displacing sequence carrying the dA nucleotide at the opposite position to the SNP, (B) with the displacing sequence carrying the dG nucleotide an the opposite position to the SNP. Sample "DK" (circles) has a dT nucleotide at the 16223 position, while samples "AK", "AL", and "AE" (■, ▲, ▼, respectively) have a dC nucleotide at the 16223 position.

Figure 3B shows the male displacing sequence (Y*i*) reacting with both amelogenin female (^{dU4,9}XX, Figure 3B, hollow symbols) and male (^{dU4,9}XY, Figure 3B, solid symbols) toehold-PCR products. Since the female toehold-PCR product (XX) did not contain a sequence that was fully complementary to the Y*i* displacing sequence then the reaction between the Y*i* and the female toehold-PCR product did not occur at any excess of the displacing sequence. On the other hand the kinetics of the strand displacement between the male toehold-PCR product (^{dU4,9}XY) and the Y*i* displacing sequence (Figure 3B, solid symbols) was similar to that observed for the reaction with the X*i* displacing sequence (Figure 3A, solid symbols). All of the evaluated ratios (excesses) were successful at discriminating both female and male toehold-PCR samples within the first 20 min of the displacement reaction. The 10-fold excess (10×) was then chosen for all consecutive experiments.

It was found that by conducting the strand displacement at elevated temperatures, the reaction was noticeably accelerated. The reaction kinetics for the amelogenin $^{dU-4,9}$XX and $^{dU-4,9}$XY toehold-PCR products were studied at four temperatures of namely, 30, 40, 50, and 60 °C (Figure S6). The kinetic data obtained were fitted to the first-order reaction equation according to Baker et al.[29] and Reynaldo et al.[36] using OriginPro 8 software (Origin Corporation, USA). The observed rate constants (k_{obs}) for the displacement reactions are summarized Table S1. Despite the fact that the research presented here uses reasonably long DNA duplexes and displacing sequences, the observed rate constant values ($\sim 10^{-3}$ s^{-1}) were orders of magnitude faster than the values reported for short oligonucleotides immobilized on a surface ($\sim 10^{-4}$ s^{-1}, Baker et al.)[29] or dissolved in solution ($\sim 10^{-6}$ s^{-1}, no toehold-mediated displacement was investigated, Reynaldo et al.).[36] The reaction kinetics for the amelogenin $^{dU-4}$XX and $^{dU-4}$XY toehold-PCR products were also then studied at the same four temperatures as those used for the amelogenin $^{dU-4,9}$toehold-PCR products (Figure 4). Interesting, at 30 °C Figure 4A shows an undistinguishable degree of displacement at 13% for the amelogenin female toehold-PCR product ($^{dU-4}$XX), reacting with X*i*, and the male toehold-PCR product ($^{dU-4}$XY), reacting with both X*i* and Y*i* (Figure 4A ○, ●, ◆, respectively). As expected, the reaction of the female toehold-PCR product $^{dU-4}$XX with Y*i* displacing sequence showed no conversion at all (Figure 4A ◇). Similarly, at 40 °C there was no observed displacement for the male toehold-PCR product ($^{dU-4}$XY) reacting with both the X*i* and Y*i* displacing sequences (Figure 4B ●, ◆, respectively). However, for the female toehold-PCR product ($^{dU-4}$XX) a lower degree of conversion of 3 ± 2% was observed with the X*i* displacing sequence (Figure 4B ○). This fall in conversion (from 13% to 3%) may be explained by the reduced stability of the 4 nucleotide toehold structure at 40 °C. Increasing the reaction temperature to 50 °C and then to 60 °C (Figure 4C,D, respectively) resulted in distinguishable displacements for both amelogenin female and male toehold-PCR products allowing for their discrimination. Since the formation of a toehold structure at these temperatures is unlikely, it is most probable that the strand displacement reactions are activated by means of partial melting of the toehold-PCR products.[36] Moreover, the observed rate constants for the reaction kinetics, shown in Figure 4C,D, were lower compared to those observed for the $^{dU-4,9}$toehold-PCR products (Table S1 and Figure S6).

Activation energies (E_a) (Table S1) were calculated from the slopes of the Arrhenius plots for the reactions of $^{dU-4,9}$XX with X*i*, $^{dU-4,9}$XY with X*i*, $^{dU-4,9}$XY with Y*i*, and $^{dU-4}$XX with X*i* toehold-PCR products (Figure S7 ○, ●, ◆). Table S1 shows that E_a's of the reactions of the amelogenin PCR products containing the 9 nucleotide long toehold domain (Figure S7 ○, ●, ◆) have a similar value (17.1 ± 0.95 kcal/mol). However, the E_a value (36.9 kcal/mol) for the displacement reaction of the $^{dU-4}$XX toehold-PCR product with X*i* was approximately twice as high as the displacement reaction of the $^{dU-4,9}$XX toehold-PCR product with X*i* (E_a = 17.2 kcal/mol). This suggests that in the case of the reaction between the $^{dU-4}$XX toehold-PCR product with X*i*, the dissociative activation of the displacement occurs at elevated temperatures. On the other hand, the displacement reaction between both the female $^{dU-4,9}$XX and the $^{dU-4}$XX toehold-PCR products and Y*i* displacing sequence (Figure S6 ◇, and Figure 4 ◇, respectively) did not occur at any of the temperatures tested.

SNP Testing. SNPs are the predominant variant in the human genome, and their detection plays a pivotal role in medical diagnostics, prediction of treatment, and the outcome of genetically determined diseases. Typically, SNPs are distributed throughout the human genome, including the mitochondrial genome. Two hypervariable regions (HVR-I and HVR-II) within the mitochondrial DNA (mtDNA) contain an abundance of SNP markers which provide highly useful information for determining human maternal ancestry.[37,38] Four DNA samples of the authors' own blood specimens were sequenced within the positions 16106−16339 of the HRV-1

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region (according to the Gene Bank Acc. no. J01415.2; details in Supporting Information, S6). The DNA sample "DK" contained substitution C-to-T at the position 16223. In order to distinguish the "DK" sample from others, a new set of HRV-1 specific dU and FAM modified primers and two displacing sequences (labeled with TAMRA at 3′ termini) was designed (Table 1). The dU modified primer contained two dU nucleotides at the fifth and ninth position providing a toehold length of 9 nucleotides within the total length of the PCR product (80 base pairs). The displacing sequences, SNP-C*i* and SNP-T*i*, contained dG and dA nucleotides at the 51 position, respectively, which are able to distinguish the C16223T substitution in the analyzed DNA samples. After PCR amplification the dU modified PCR products were treated as per the amelogenin system described previously. The displacement reaction was performed at 30 °C with 10-fold displacement sequences excess. Figure 5 shows the results of the displacement. The "DK" toehold-PCR product, with the C16223T substitution, was easily discriminated from the other three authors' samples containing no substitutions at the position 16223 using the SNP-T*i* displacing sequence (Figure 5A). The "DK" sample showed a displacement level of ∼90.0 ± 5.3% after 20 min (Figure 5A ●), while the others − only 13.5 ± 6.7% (Figure 5A ■, ▲, ▼). Figure 5B shows the displacing reaction using the SNP-C*i* displacing sequence. It can be seen that within the first 10 min of the reaction, the "DK" sample can be reliably distinguished from the other three samples. However, the longer incubation time leads to a almost common plateau for all four analyzed samples. These results are in full accordance with those showed by Picuri et al.,[15] where the authors correlated the difference in discrimination ability of the C-to-T substituted displacing sequence with a secondary structure located throughout the toehold domain.[15] However in our case no hindered toehold domain structures, calculated using Mfold web server,[39] were found for both displacing sequences and this phenomenon requires further investigation.

■ CONCLUSION

DNA strand exchange processes have recently been introduced as a major technique for DNA nanostructuring, machinery, computation, and biosensing. A "zipper" mode or a consecutive "base-by-base" rehybridization mechanism allows for the highly efficient discrimination of reacting ds-DNA. However, DNA diagnostic related work in this area, thus far, has almost exclusively focused on the analysis of comparatively high concentrations of synthetic single-stranded nucleic acids. Here, we successfully demonstrate a new approach to the use of the strand displacement reaction for the analysis of ds-DNA using the amelogenin gene as a model system for gender discrimination. PCR products with length of 106 and 112 bp of the amelogenin locus on the X and Y chromosomes, respectively, of this gene were prepared with a ss-toehold sequence which were then subjected to strand displacement using synthetic complementary oligonucleotides. It was found that the ss-toehold domain consisted of 9 nucleotides allowed the discrimination between male and female samples in <10 min of the strand displacement reaction conducted at 30 °C. By increasing the reaction temperature to 60 °C, the displacement level >70% was achieved within 1 min. The use of a toehold domain with a length of 4 nucleotides was only found to be successful at discrimination when using elevated temperatures of 50 and 60 °C.

In addition the approach was demonstrated for the SNP genotyping of real-life ds-DNA samples using mtDNA as a target. The C-to-T substitution in 80 bp PCR products was discriminated with a displacing sequence carrying a dA nucleotide at the opposite position to the substitution. However, the displacing sequence with a dG nucleotide allowed only kinetic discrimination within first several minutes of the reaction. Again, our data demonstrate that the current knowledge of SNP influence on the efficiency of toehold-mediated strand displacement is still controversial and requires additional investigations.

In summary, a novel genotyping approach has been developed which requires only a simple substitution of deoxythymidine for deoxyuracil in one of the existing PCR primers and UDG treatment followed by PCR. This makes this method easily applicable to most DNA genotyping systems. The approach is especially useful for systems which deal with the analysis of nucleic acids with similar hybridization efficiencies and those prone to cross-hybridization.[22] Finally, the strategies described herein are directly adaptable to microarray technologies.

■ ASSOCIATED CONTENT

Supporting Information

CE assessment of the efficiency of the PCR amplification using dU substituted primers with UDG treatment. Strand displacement real-time monitoring workflow. An example of raw fluorescent data. Kinetic data of the strand displacement reactions. DNA sequencing. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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