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ARTICLE

Dual-allele dipstick assay for genotyping single nucleotide polymorphisms by primer extension reaction

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We have developed a dry-reagent dipstick test for simultaneous visual detection of two alleles in single nucleotide polymorphisms (SNPs). The strip comprises two test zones and a control zone. Oligonucleotide-functionalized gold nanoparticles are used as reporters. PCR-amplified DNA that spans the interrogated sequence is subjected to primer extension (PEXT) reactions using allele-specific primers. Digoxigenin-dUTP and biotin-dUTP are incorporated in the extended fragments. The primers contain an oligo(dA) segment at the 5′ end. The PEXT products are applied to the sample area of the strip, which is then immersed in the appropriate buffer. As the buffer migrates along the strip by capillary action, the extension products of the two alleles are captured at the test zones from immobilized anti-digoxigenin and streptavidin, whereas the oligo(dA) segment of the primers hybridizes with oligo(dT) strands attached to gold nanoparticles, thus generating characteristic red lines. The excess nanoparticles are captured from immobilized oligo(dA) strands at the control zone of the strip. The test was applied to the genotyping of two SNPs of the Toll-like receptor 4 gene (Asp299Gly and Thr399Ile), one SNP of CYP2C19 gene (CYP2C19*3) and one SNP of the TPMT gene (TPMT*2). Contrary to most genotyping methods, the dipstick test does not require costly specialized equipment for detection of PEXT products. The PCR product is pipetted directly into the PEXT reaction mixture without prior purification. The high sensitivity of the strip allows completion of PEXT reaction in three cycles only (7 min). The visual detection of both alleles is complete in 15 min.
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Keywords: dipstick test; dual-allele detection; SNP genotyping

Introduction

Single nucleotide polymorphisms (SNPs) are single-base changes that occur at a frequency of about 1 in 1000 bases in the human genome and may affect protein structure and level of expression. SNPs constitute a new generation of molecular markers for diagnosis of disease, genetic predisposition and response to various drugs. For this reason, over the past several years, a great deal of effort has been devoted in developing accurate, rapid and cost-effective methods for SNP genotyping.[1–3]

Genotyping methods comprise three main steps, namely, PCR amplification, allele discrimination and detection. Allele-discrimination approaches include restriction fragment polymorphism analysis, hybridization with allele-specific oligonucleotide (ASO) probes, melting curve analysis, oligonucleotide ligation and primer extension (PEXT) reaction. In general, enzymic methods for allele

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discrimination are more robust and easily applicable than ASO hybridization. Detection may be accomplished by electrophoresis, fluorescence polarization, fluorescence resonance energy transfer, flow cytometry,[4] MALDI-TOF mass spectrometry,[5] microtiter well-based chemi(bio)luminometric assays[6–8] and microarray technology.[9] However, all the above detection techniques require highly expensive specialized equipment and reagents. Moreover, most of these methods involve purification of the PCR products prior to allele-discrimination reaction or prior to detection, whereas others include multiple pipetting, incubation and washing steps for detection. As a consequence, the assay cost increases due to the need for highly qualified personnel.

In recent years, there is a strong interest toward the development of simple and low-cost genotyping methods that are suitable for the routine clinical laboratory and/or point-of-care testing. In this context, dipstick-type dry-reagent assays constitute an exceptionally promising alternative because they offer low cost, disposability, simplicity and visual detection with only a few assay steps and a minimum requirement for qualified personnel.

The competitive allele-specific short oligonucleotide hybridization assay (CASSOH) with immunochromatographic strip[10,11] embraces the principle of the ASO hybridization method for allele discrimination in a dipstick-type assay format. Recently, we reported a dry-reagent dipstick assay for SNP genotyping based on the PEXT reaction.[12,13] However, both the CASSOH and the PEXT dipstick assays require two strips per assay, one strip for each allele.

In the present study, we report a novel dry-reagent dipstick assay that enables simultaneous visual detection of both alleles, within minutes, using a single strip. Allele discrimination is based on PEXT reaction. The PEXT reaction products are applied directly to the strip without prior purification. Oligonucleotide-conjugated gold nanoparticles deposited in dry form on the strip are used as reporters. As a model, the assay was applied to the genotyping of two SNPs of the toll-like receptor 4 (TLR4) gene (Asp299Gly and Thr399Ile), one SNP of the cytochrome P450 gene CYP2C19 (CYP2C19*3) and one SNP of the TPMT (TPMT*2) gene. TLR4 gene plays a critical role in the innate immune response to both gram-negative pathogens and respiratory syncytial virus. SNPs within TLR4 result in increased susceptibility to infectious or inflammatory disease.[14–17] CYP2C19 and TPMT are genes encoding enzymes involved in the metabolism of many commonly prescribed drugs.[18,19]

Materials and methods

Instrumentation

PCR and PEXT were performed in the MJ Research PTC-150 thermal cycler (Watertown, MA, USA) and Hybaid Omni-E thermal cycler (Midlesex, UK). A digital camera, Kodak DC 120 and the Gel Analyzer software for DNA documentation were purchased from Kodak (New York, NY, USA). Microcentrifuge Mikro-20 was from Hettich (Tuttlingen, Germany). The TLC applicator, Linomat 5 and the software WinCats were from Camag (Muttenz, Switzerland).

Materials

Antidigoxigenin antibody (anti-Dig, Fab fragments), digoxigenin-11-dUTP (Dig-dUTP) and streptavidin (SA) from *Streptomyces avidinii* were purchased from Roche (Mannheim, Germany). Sephadex G-25 Spin Pure purification columns were from CPG (Linkoln Park, NJ, USA). Agarose was from Bioline and ultra-pure 2′-deoxyribonucleotide 5′-triphosphates (dNTPs) were from HT Biotechnology (Cambridge, UK). Ethidium bromide was purchased from Research Organics (Cleveland, OH, USA). Taq DNA polymerase was from Biotools (Madrid, Spain). Vent (exo-) DNA polymerase was from New England Biolabs (Beverly, MA, USA), $\Phi \times 174$ DNA/*Bsu*RI marker and terminal deoxynucleotidyl transferase (TdT) were from MBI Fermentas (Vilnius, Lithuania), and biotin-11-dUTP (B-dUTP) was obtained from AppliChem (Darmstadt, Germany). Gold nanoparticles (40 nm, 9×10^{10} particles per ml) were obtained from British Biocell (BB International, Cardiff, UK). The wicking pad, glass-fiber conjugate pad and absorbent pad were from Schleicher and Schuell (Dassel, Germany), and nitrocellulose membrane immunopore FP was purchased from Whatman (Germany). Oligonucleotides used as primers and probes in the course of this study were synthesized by the Research and Technology Institute (Irakleion, Crete, Greece) and Thermo Electron (Ulm, Germany). Oligonucleotide sequences are shown in Table 1. A 5′-thiol-modified $(dT)_{30}$ oligo was used for conjugation with gold nanoparticles. A $(dA)_{30}$ oligo was used for the construction of the control zone of the strip. All common reagents were purchased from Sigma (St Louis, MO, USA) or Fluka (Buchs, Switzerland).

Methods

Tailing of oligonucleotide probes with dTTP or dATP

The 5′-thiol-modified $(dT)_{30}$ and $(dA)_{30}$ oligos were tailed at the 3′ end with dTTP and dATP, respectively, using TdT. Tailing reactions were carried out in a total volume of 20 μl, containing 0.2 M potassium cacodylate (pH 7.2), 0.1 mM dithiothreitol, 0.1 ml/l Triton X-100, 1 mM $CoCl_2$, 3.5 mM dTTP (or dATP), 30 U of TdT and 700 pmol of probe. The reaction was held for 1 h at 37°C and stopped by adding 2 μl of 0.5 M EDTA (pH 8.0). The tailed 5′-thiol-modified poly(dT) probe was purified prior to use by size exclusion chromatography on Sephadex G-25 spin-pure columns. The poly(dA)-tailed probe was mixed with 1.5 μl of 40 mM *N*-methylmaleimide solution in DMSO (2.7 mM final concentration) and used without purification. The tailed probes were stored at −20°C.

Table 1 Oligonucleotides used in present study as primers for PCR and PEXT

Oligonucleotide name	*Sequence (5′→3′)*	*Size*
TLR4-M13 Ex3 PCR primers		
5′ primer (634 bp product)	(GTAAAACGACGGCCAGT)AGTCCATCGTTTGGTTCTGGGAGA	41 mer
3′ primer (634 bp product)	(CAGGAAACAGCTATGAC)GCCATTGAAAGCAACTCTGGTGTG	41 mer
CYP2C19 PCR primers		
5′ primer (271 bp product)	AAATTGTTTCCAATCATTTAGCT	23 mer
3′ primer (271 bp product)	ACTTCAGGGCTTGGTCAATA	20 mer
TPMT PCR primers		
5′ primer (197 bp product)	GCATGTTCTTTGAAACCC	18 mer
3′ primer (197 bp product)	CTTGTATCCCAAGTTCACTG	20 mer
TLR4-299 genotyping primers		
5′ TLR4-299 (normal)	$(A)_{24}$AATAAGTCAATAATAT	40 mer
5′ TLR4-299 (mutant)	$(A)_{24}$AATAAGTCAATAATAC	40 mer
TLR4-399 genotyping primers		
3′ TLR4-399 (normal)	$(A)_{24}$GTGATTTTGGGACAAC	40 mer
3′ TLR4-399 (mutant)	$(A)_{24}$GTGATTTTGGGACAAT	40 mer
*CYP2C19*3 genotyping primers*		
3′ CYP2C19*3 (normal)	$(A)_{30}$TTGGCCTTACCTGGATC	47 mer
3′ CYP2C19*3 (mutant)	$(A)_{30}$TTGGCCTTACCTGGATT	47 mer
*TPMT*2 genotyping primers*		
3′ TPMT*2 (normal)	$(A)_{28}$CTACACTGTGTCCCCGGTCTGC	50 mer
3′ TPMT*2 (mutant)	$(A)_{28}$CTACACTGTGTCCCCGGTCTGG	50 mer

Abbreviation: TLR, Toll-like receptor.

Preparation of dry-reagent strip

The dry-reagent strip (4×70 mm) consisted of a wicking pad, a glass-fiber conjugate pad, a nitrocellulose diagnostic membrane and an absorbent pad assembled on a plastic adhesive backing as described previously.[20] Streptavidin, anti-Dig antibody and poly(dA) oligonucleotide were immobilized at the test zone 2, the test zone 1 and the control zone of the nitrocellulose membrane, respectively. Streptavidin diluted in water containing 2% sucrose was loaded at a density of 1.6 μg per 4 mm (width) strip. Anti-Dig was diluted in a 100 mM $NaHCO_3$ solution (pH 8.5), containing 5% methanol and 2% sucrose solution and loaded at a density of 1 μg per 4 mm. The poly(dA) probe diluted in phosphate-buffered saline (10 mM sodium phosphate 1.7 mM potassium phosphate solution 0.14 M NaCl and 2.7 mM KCl, pH 7.4), containing 5% methanol and 2% sucrose was loaded at a density of 2.4 pmol per strip (4 mm). The membrane was then dried in an oven for 1 h at 80°C, and the strips were assembled. The strips were stored dry at room temperature. Poly(dT)-functionalized gold nanoparticles were prepared as described previously,[20] and loaded on the conjugate pad at a density of 6.25 fmol per 4 mm width.

Genomic DNA isolation from whole blood

Whole-blood specimens were obtained from the Medical School of the University of Crete and from St Sophia Children Hospital, Athens, after informed consent. Genomic DNA was isolated using the QIAmp DNA blood mini kit.

Polymerase chain reaction

Following extraction of genomic DNA (50–100 ng/μl^{-1}), a segment in the CYP2C19 gene flanking the CYP2C19*3 polymorphic site, a segment in the TPMT gene flanking the TPMT*2 site and a segment in the TLR4 gene flanking the two SNPs (Asp299Gly and Thr399Ile) were amplified by PCR.

PCRs for CYP2C19 and TPMT genes were carried out in a final volume of 50 μl containing 75 mM Tris-HCl (pH 9.0), 20 mM $(NH_4)_2SO_4$, 50 mM KCl, 2.5 mM $MgCl_2$ for CYP2C19 and 2 mM for TPMT, 0.5 pmol μl^{-1} of each forward and reverse primers (Table 1) for CYP2C19 and 0.2 pmol μl^{-1} for TPMT, 1 U of Taq DNA polymerase for CYP2C19 and 2 U for TPMT 0.2 mM of each of the dNTPs and 100 ng genomic DNA. The cycling conditions were as follows:

- *CYP2C19*: initial denaturation at 95°C for 2 min, 35 cycles at 95°C (15 s), 55°C (15 s), 72°C (30 s) and a final extension step at 72°C for 10 min.
- *TPMT*: initial denaturation at 95°C for 3 min, 35 cycles at 95°C (30 s), 52°C (30 s), 72°C (45 s) and a final extension step at 72°C for 10 min.

PCR amplification of the TLR4 gene was performed using 25 μl of the HotStar Taq Master Mix kit from Qiagen in a total reaction volume of 50 μl consisting of 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.4 μM of each forward and reverse primers (Table 1), 2.5 U HotStar Taq DNA polymerase, 0.2 mM each of the dNTPs and 100 ng of genomic DNA. The PCR conditions were as follows: initial denaturation at 95°C for 15 min, 35 cycles at 95°C (1 min), 65°C (1 min), 72°C

(1 min) and a final extension step at 72°C for 8 min. The amplification products were confirmed and quantified by agarose gel (1.7%) electrophoresis and ethidium bromide staining.

Primer extension reaction

All PEXT reactions were performed in a total volume of 20 μl containing 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Triton X-100, 0.1 pmol of amplified DNA, 1 pmol of either normal (N) or mutant (M) primer, 0.25 U Vent (exo-) DNA polymerase, 2.5 μM of each dATP, dCTP, dGTP and either 1.75 μM dTTP/0.75 μM Dig-dUTP (PEXT reaction with N primer) or 1.25 μM dTTP/ 1.25 μM B-dUTP (PEXT reaction with M primer) and 1 mM $MgSO_4$ for TPMT*2, 1.5 mM $MgSO_4$ for CYP2C19*3 and TLR4-299 and 2 mM $MgSO_4$ for TLR4-399. The thermal cycling conditions for PEXT reactions were: initial denaturation at 95°C for 5 min, followed by three cycles of: denaturation at 95°C for 15 s; primer annealing for 10 s at 40°C (TLR4-299), 55°C (TPMT*2 and TLR4-399) and 60°C (CYP2C19*3); PEXT at 72°C for 15 s (total run time 7 min). All PEXT products were subjected to an additional denaturation step at 95°C for 5 min and then placed immediately on ice for 2 min.

Dual-allele dipstick assay

A 3-μl aliquot of each of the two denatured PEXT reaction products was applied onto the conjugate pad above the gold nanoparticles. The strip was then immersed into 300 μl of developing solution containing 50 mM Tris-HCl (pH 7.5), 75 mM NaCl, 30 g/l glycerol, 2.5 ml/l Tween-20 (polyoxyethylene (20) sorbitan monolaurate) and 0.5 g/l SDS. The visual detection of PEXT products was completed within 15 min.

Results and discussion

Assay principle

A schematic presentation of the proposed dual-allele dry-reagent dipstick test for SNP genotyping by PEXT reaction is shown in Figure 1. Genomic DNA, isolated from whole blood, is first subjected to PCR using primers flanking the polymorphic site. Amplified fragments of 271-bp (CYP2C19), 197-bp (TPMT) and 634-bp (TLR4) were generated. Each PCR product served as template for two separate PEXT reactions, one with a primer carrying at its 3′ end a nucleotide complementary to the normal allele (N primer) and a second reaction using a primer that carries, at its 3′ end, a nucleotide complementary to the mutant allele (M primer). Both N and M primers carry at their 5′ end a $d(A)_{30}$ tail, for hybridization with the poly(dT)-conjugated gold nanoparticles. The PEXT reaction for the normal allele (N-PEXT) is carried out in the presence of a dNTP mixture containing Dig-dUTP, whereas the PEXT reaction for the mutant allele (M-PEXT) takes place in the presence of dNTPs containing B-dUTP. When a perfect match occurs between primer and target sequence, the primer is extended by DNA polymerase and either digoxigenin (N reaction) or biotin (M reaction) is incorporated into the extended products. Aliquots of both PEXT products are applied on to the conjugate pad of the strip near the gold nanoparticles. The immersion pad is then brought into contact with the developing buffer, which migrates upward by capillary action and allows the (dA)/(dT) hybridization between the PEXT products and the gold nanoparticles to occur. The hybrids that correspond to the normal (N reaction) and mutant (M reaction) alleles are captured on the test zones of the strip through Dig/anti-Dig (zone T1) and biotin–streptavidin interaction (zone T2), respectively. The electromagnetic spectrum of gold nanoparticles has maximum absorbance at 520 nm, which depends on the resonance of photons with the conductible electrons of particles (plasmons). This is called localized surface plasmon resonance (LSPR). The range of the LSPR zone depends on the size of the particles. Spherical particles with diameters of 10–40 nm are red. In

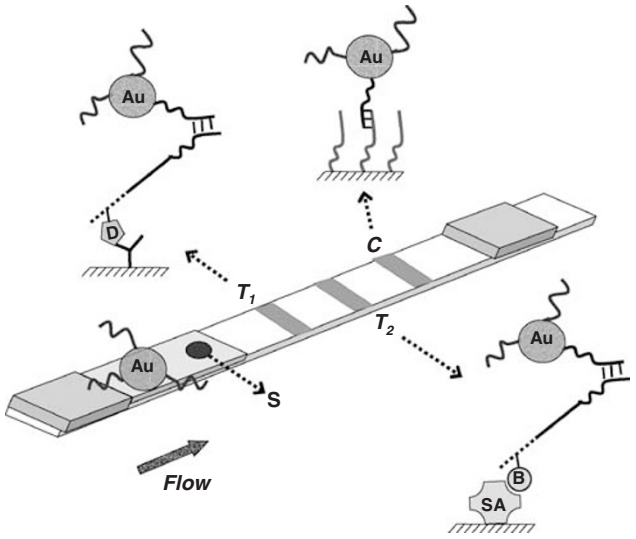

Figure 1 Schematic illustration of the principle of dual-allele dry-reagent dipstick assay for single nucleotide polymorphism (SNP) genotyping by primer extension (PEXT) reaction. PCR-amplified DNA that spans the interrogated sequence is subjected to PEXT reactions using 'normal' and 'mutant' primers. Digoxigenin-dUTP and biotin-dUTP are incorporated in the extended fragments, respectively. The primers contain an oligo(dA) segment at the 5′ end. The PEXT reaction products are applied to the sample area of the strip (S), which is then immersed in the appropriate buffer. As the buffer migrates along the strip by capillary action, the 'normal' and 'mutant' extension products are captured at test zones T1 and T2 from immobilized anti-digoxigenin and streptavidin, respectively, and the oligo(dA) segment of the primers hybridizes with the oligo(dT) strands attached to gold nanoparticles, thus generating characteristic red lines. The excess nanoparticles are captured from immobilized oligo(dA) strands at the control zone of the strip. Au: gold nanoparticles; S: sample application area; T1: test zone for 'normal' allele; T2: test zone for 'mutant' allele; C: control zone; D: digoxigenin; B: biotin; SA: streptavidin.

the present study, gold nanoparticles with 40 nm diameter are used as reporters and the color development in the dry-reagent strip is due to the accumulation of a large number of nanoparticles in the two test zones and the control zone.

Not extended primers (either N or M) carrying a $d(A)_{30}$ tail are also hybridized with gold nanoparticles but not captured on the test zones. The excess of gold nanoparticles form hybrids with immobilized oligo(dA) strands at the control zone of the strip, giving a third red band, which confirms the proper performance of the strip. A sample is characterized as normal or mutant when a red line appears in T1 or T2 zone, respectively. A heterozygote gives red lines in both T1 and T2 zones.

The addition of a buffer is necessary to initiate the migration of reagents along the strip by capillary action. The buffer could be provided either by immersing the strip into the buffer or by applying the buffer directly onto the appropriate part of the strip. Instead of a buffer, a sample could be added of adequate volume (approximately 200 μl).

Optimization studies

The performance of the dual-allele dry-reagent dipstick test was optimized by analyzing mixture of N- and M-PEXT products of a heterozygote for the mutation TLR4-399 or TLR4-299.

The zone intensity increases with the amount of deposited anti-Dig antibody up to about 1000 ng per strip (Figure 2a). Also the intensity increases with the volume of PEXT reaction product applied to the strip and the maximum signal is obtained at 3 μl of each PEXT product (Figure 2b).

We optimized the PEXT reaction conditions to achieve the highest yield and best allele discrimination. The intensity of the test zones (T1 and T2) is influenced by the ratio of B-dUTP/dTTP or Dig-dUTP/dTTP in the dNTP mixture (Figure 2c). The highest intensities are obtained at ratios exceeding 50% modified with biotin and 25% modified with Dig oligonucleotides.

Mg^{2+} concentration is an important parameter because it affects the annealing of the primer to template DNA by stabilizing the primer–template interaction; it also stabilizes the replication complex of polymerase with template-primer. Optimization studies were performed for each SNP with Mg^{2+} concentration in the range of 0.5–2 mM, by a series of PEXT reactions using a normal (N/N) and a mutant (M/M) sample containing 100 fmol of amplified genomic DNA, and 1 pmol of the allele-specific primer. Representative results from TPMT*2 are shown in Figure 2d. We observe that at Mg^{2+} concentrations higher than 1 mM a nonspecific zone appears on the strip, due to nonspecific extension of the primer. Similarly, the Mg^{2+} concentrations that provided the highest specific signal and the lowest nonspecific extension for TLR4-299, TLR4-399 and CYP2C19*3 were 1.5, 2 and 1.5 mM, respectively.

Data pertaining to the effect of annealing temperature for CYP2C19*3 are presented in Figure 2e. With all SNPs

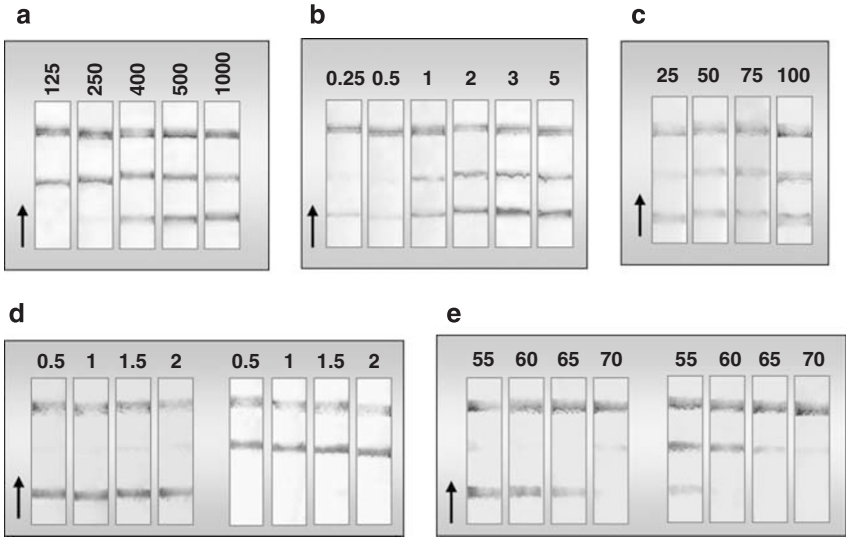

Figure 2 (**a**) Study of the effect of the amount of anti-digoxigenin antibody on the intensity of the test zone of the strip. The sample was a heterozygote for the TLR4-399 polymorphism. The amount of anti-digoxigenin antibody (ng per strip) is written above the strips. (**b**) Dependence of the intensity of zones T1 and T2 on the amount of PEXT reaction product applied to the strip. Each PEXT product (μl) is written above the strips. The sample was a heterozygote for TLR4-399 polymorphism. (**c**) Study of the effect of biotin-dUTP/dTTP and Dig-dUTP/dTTP molar ratio in the PEXT reaction mixture. The ratios are written as percentages above the strips. The sample was a heterozygote for the TLR4-299 polymorphism. (**d**) Effect of Mg^{2+} concentration in the PEXT reaction mixture. The Mg^{2+} concentrations (mM) are written above the strips. The samples were: TPMT*2 'normal' (left panel) and 'mutant' (right panel). (**e**) Study of the effect of annealing temperature in the PEXT reaction. The annealing temperatures (°C) are written above the strips. The samples were: CYP2C19*3 'normal' (left panel), and 'mutant' (right panel).

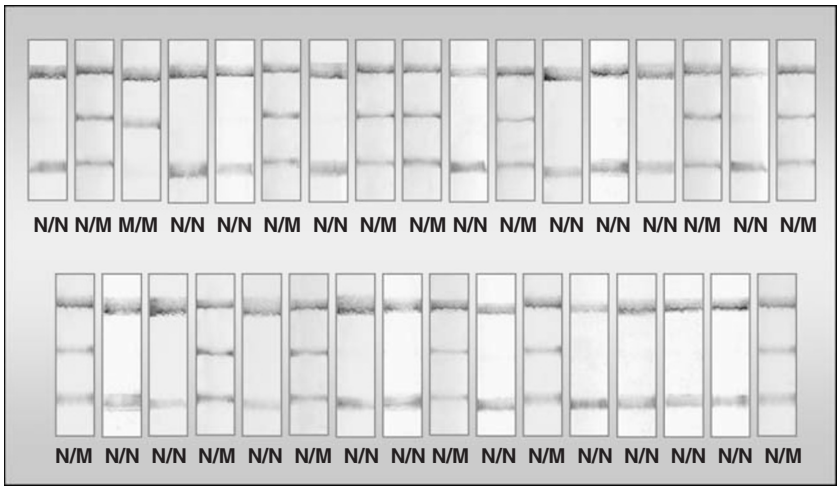

Figure 3 Application of the proposed method to rapid genotyping of TLR4-299 polymorphism.

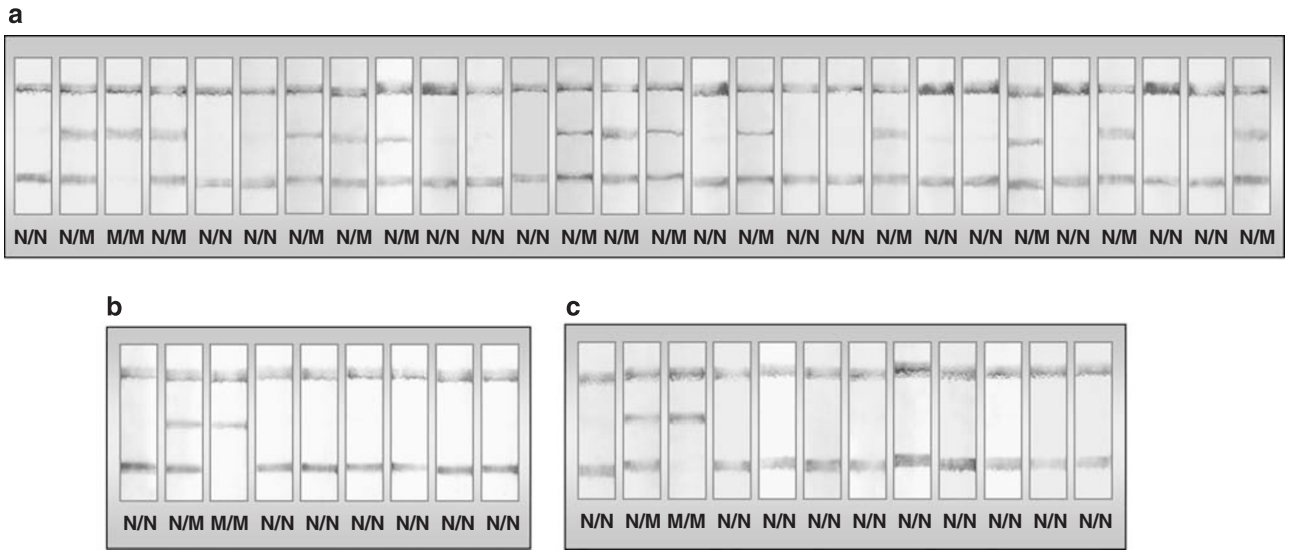

Figure 4 Application of the proposed method to rapid genotyping of: (**a**) TLR4-399, (**b**) TPMT*2 and (**c**) CYP2C19*3 polymorphisms.

studied, it was observed that nonspecific extension occurs at low annealing temperatures. The specificity increases with the temperature. However, at high temperatures the specific signal drops to decreases annealing of the primer to the template strand. The annealing temperatures chosen as optimal were 40, 55, 60 and 55°C for TLR4-299, TLR4-399, CYP2C19*3 and TPMT*2, respectively.

Application to clinical specimens

The proposed dual-allele dry-reagent dipstick test was evaluated by genotyping four SNPs as follows: 33 samples for SNP TLR4-299, 28 samples for TLR4-399, 9 samples for TPMT*2 and 12 samples for CYP2C19*3. The results for each SNP are presented in Figures 3 and 4. The genotyping results showed 100% concordance with direct DNA sequencing.

The overall reproducibility (% CV) of the proposed method including PEXT reaction and the dual-allele dry-reagent dipstick assay was assessed by analyzing, 4 ×, PCR products from genomic DNA and was found to be 11 and 19% for the test zones T1 and T2, respectively.

Conclusions

The proposed dual-allele dry-reagent dipstick test offers the following distinct advantages: (1) It enables rapid and low-cost genotyping of SNPs by visual detection of both PEXT products on the same strip. (2) Contrary to most

genotyping methods, the proposed test does not require specialized equipment for detection of PEXT products. (3) The PCR product is pipetted directly into the PEXT reaction mixture without prior purification from the excess of primers and dNTPs. (4) In contrast to microtiter well-based methods, the proposed assay does not require incubation and washing steps. (5) The high sensitivity of the strip allows completion of the PEXT reaction in three cycles only (7 min in total). The visual detection of both alleles is complete in 15 min. Therefore, PCR constitutes the lengthiest step of the assay. However, the time required for PCR amplification could be reduced substantially by employing microfabricated devices (PCR chips), which allow rapid heat transfer due to the large surface-to-volume ratio.[21](6) The dry-reagent dipstick format minimizes the requirements for highly qualified personnel for performing the test and interpreting the results.

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