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Multi-allele genotyping platform for the simultaneous detection of mutations in the Wilson disease related *ATP7B* gene

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ABSTRACT

Wilson's disease is an inherited disorder of copper transport in the hepatocytes with a wide range of genotype and phenotype characteristics. Mutations in the *ATP7B* gene are responsible for the disease. Approximately, over 500 mutations in the *ATP7B* gene have been described to date. We report a method for the simultaneous detection of the ten most common *ATP7B* gene mutations in Greek patients. The method comprises 3 simple steps: (i) multiplex PCR amplification of fragments in the *ATP7B* gene flanking the mutations (ii) multiplex primer extension reaction of the unpurified amplification products using allele-specific primers and (iii) visual detection of the primer extension reaction products within minutes by means of dry-reagent multi-allele dipstick assay using anti-biotin conjugated gold nanoparticles. Optimization studies on the efficiency and specificity of the PEXT reaction were performed. The method was evaluated by genotyping 46 DNA samples of known genotype and 34 blind samples. The results were fully concordant with those obtained by reference methods. The method is simple, rapid, cost-effective and it does not require specialized instrumentation or highly qualified personnel.

1. Introduction

Wilson disease (WD) is a disorder of copper transport associated with progressive liver disease and is inherited in an autosomal recessive pattern. The disease is characterized by reduced incorporation of copper into ceruloplasmin and decreased excretion of the metal in the bile. Copper accumulation occurs principally in the liver and brain, causing progressive damage of these organs. WD is observed with a prevalence of approximately 1:30,000, with a gene frequency of 0.56% and a carrier frequency of 1 in 90 and is similar among many ethnic groups [1]. The *ATP7B* gene (ATP7B; MIM# 277900), responsible for WD, encodes a copper transporting P-type ATPase which is the major regulatory protein of copper homeostasis. Alterations of protein structure and function, followed by copper accumulation in the liver, are associated with various mutations in the *ATP7B* gene causing changes of the amino acid sequence. Numerous mutational studies of WD have revealed that the mutation spectrum of the *ATP7B* gene is population specific [2].

Diagnosis of WD is based on a combination of clinical symptoms and biochemical tests. However, in case of asymptomatic patients with inconclusive biochemical markers, it is difficult to diagnose WD. For this reason, genetic testing has become the method of choice to establish a precise diagnosis [3]. Genetic studies become very important for validation of diagnosis and identification of affected but presymptomatic family members. Brothers and sisters of a WD patient should be checked as far as they have a 1 in 4 chance of also having the disease. Genetic screening of patients' children, that have a one in 200 chance of developing the disease, is generally not recommended. Molecular genetic studies include haplotype analyses, and direct sequencing of *ATP7B* gene for disease-specific mutations [4]. Numerous PCR-based methods including PCR amplification-single stranded conformational polymorphism (PCR-SSCP), denaturing gradient gel electrophoresis (DDGE), real-time amplification refractory mutation system (ARMS), denaturing high performance liquid chromatography (DHPLC), bidirectional PCR amplification of specific alleles (BIPASA), SYBR green intercalator method based on the ARMS, high

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resolution melting analysis (HRMA) etc., have also been developed to detect *ATP7B* gene mutations [5–11]. Most of these methods require confirmation of the positive findings by another method, commonly sequencing, whereas others target usually a specific mutation each time and represent a time-consuming strategy or use expensive specialized equipment or hazardous chemicals.

Technological advances in recent years (such as DNA microarrays) have led to the development of sophisticated high-throughput systems that enable genotyping of hundreds of thousands of mutations/polymorphisms in parallel [12]. While these systems are essential for carrying out large-scale association studies, it is anticipated that eventually only a few mutations per patient will be assessed routinely in the molecular diagnosis laboratory. As a result, the development of DNA biosensors has received considerable attention. During the last few years, dry-reagent dipstick-type biosensors in the form of lateral flow strips have been developed for the genotyping of single nucleotide polymorphisms (SNP). The key advantage of these sensors is that they enable visual detection within minutes without the use of instruments. In addition, they are disposable and the dry-reagent dipstick format eliminates several pipetting and washing steps because the reagents form an integral part of the sensor. Hybridization with allele-specific oligonucleotides, primer extension reaction and oligonucleotide ligation reaction have been used for allele discrimination in combination with dipstick-type biosensors [13–15].

In this paper, we report the development, optimization and validation of a simple, rapid, multiplexed and low-cost genotyping assay in a lateral flow 'dipstick' format which precludes the need of specialized instrumentation, and even highly trained technical personnel. The assay was developed to detect ten of the most common *ATP7B* gene mutations accounting for about 80% of Greek WD chromosomes [1]. The mutations included in this study were as follows: p.His1069Gln (c.3207C > A), p.Arg969Gln (c.2906G > A), p.Leu936X (c.2807T > A), p.Gln289X (c.865C > T), c.2530delA, p.Ter1466Arg (c.4396 T > C), c.845delT, c.2299insC, p.Ile1148Thr (c.3443T > C) and c.1708-1G > A.

2. Materials and methods

2.1. Materials

Agarose and ultra-pure 2-deoxyribonucleoside 5-triphosphates (dNTPs) were from HT Biotechnology (Cambridge, UK). Ethidium bromide was purchased from Research Organics (Cleveland, OH, USA). Multiplex PCR Master Mix was from Qiagen (Hilden, Germany). Bovine serum albumin was purchased from Serva (Heidelberg, Germany). EDC (1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide), MES (2-(N-morpholino) ethanesulfonic acid) and biotin-11-dUTP (B-dUTP) were from Applichem (Darmstadt, Germany). Immunopure goat anti-biotin was purchased from Pierce (Rockford, IL). Vent (exo-) DNA polymerase was from New England Biolabs (Beverly, MA), ΦX174 DNA/BsuRI marker, GeneRuler 1 kb DNA ladder and terminal deoxynucleotidyl transferase (TdT) were from MBI Fermentas (Vilnius, Lithuania). Gold nanoparticles (40 nm, 9×10^{10} particles per ml) were obtained from British Biocell (BB International, Cardiff, UK). Immunopore FP membrane (5-μm pore size) was from Whatman (Florham Park, NJ) and the plastic adhesive backing, wicking pad, glass-fiber conjugate pad, and absorbent pad were from Schleicher & Schuell (Dassel, Germany). Carboxylate-modified white microspheres 2 μm in diameter, were purchased from Polysciences (Warrington, PA, USA). Formamide was from Roth (Karlsruhe, Germany). All common reagents were purchased from Sigma (St Louis, MO, USA) or Fluka (Buchs, Switzerland).

2.2. DNA samples

Genomic DNA samples including patients and healthy controls were obtained from the Department of Medical Genetics, Athens University Medical School, "Aghia Sophia" Children's Hospital. Genomic DNA was isolated from whole blood using the automated robotic system Biorobot M48 Workstation (Qiagen, Hilden, Germany) and the commercially available kit MagAttract DNA blood midi M48 kit (Qiagen). The *ATP7B* gene mutations were characterized using denaturing gradient gel electrophoresis (DGGE) and all positive findings were confirmed by bidirectional sequencing on an automated sequencer ABI PRISM® 3500 Genetic Analyzer (Applied Biosystems, Life Technologies Corporation, USA).

2.3. Primer design

All oligonucleotides used in this study as primers for multiplex PCR and primer extension genotyping reactions as well as capture probes for the dipstick assay were designed *in-silico* using Primer-Premier 5 and PrimerPlex 2 software (Premier Biosoft International, Palo Alto, CA). Oligonucleotides were synthesized by VBC Genomics (Wien, Austria).

2.4. Genotyping of 10 mutations (20 alleles) in ATP7B gene

Genotyping consists of (i) Two 5-plex PCRs for the amplification of the *ATP7B* gene fragments (Gene Bank Accession Number NM_000053.3) flanking the mutations, (ii) two 10-plex PEXT reactions of unpurified PCR product in the presence of allele-specific primers (two primers per mutation, one for the wild type and the second for the variant allele) and biotin-modified nucleotide and (iii) visual detection of the PEXT products by two multi-allele dipstick type biosensors.

2.4.1. PCR amplification of the ATP7B gene fragments

Two 5-plex PCRs for the amplification of *ATP7B* gene fragments were carried out in a total volume of 50 μL containing 1 × Qiagen Multiplex PCR Master Mix (Hilden, Germany) including preoptimized concentrations of HotStarTaq DNA Polymerase, $MgCl_2$, plus dNTPs and a PCR buffer specially developed for multiplex PCR, 1 × Q Buffer, 1 μM (2 μM for the second PCR) of each primer (Table 1) and 2 μL of genomic DNA. The PCR conditions for the first multiplex PCR were as follows: Initial denaturation at 95 °C for 15 min and 35 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 45 s, and a final extension step at 72 °C for 5 min. The PCR conditions for the second multiplex PCR were: Initial denaturation at 95 °C for 15 min and 36 cycles of 94 °C for 45 s, 55 °C for 90 s, 72 °C for 60 s, and a final extension step at 72 °C for 8 min. PCR amplification and primer extension reactions were carried out in the TC-512 thermal cycler from Techne Inc (Burlington, NJ) and PTC-0150 thermal cycler from MJ Research (Watertown, MA).

2.4.2. Multiplex primer extension reaction

Two 10-plex PEXT reactions were performed in a total volume of 20 μL containing 20 mM Tris–HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0,1% Triton X-100 pH 8.8, 1 U Vent(exo-) DNA polymerase, 0.4 μL PCR product, 5 pmol each of the ten corresponding allele-specific primers (Table 2), 4 mM $MgSO_4$ and 10 μM dATP, dCTP, dGTP, 5 μM dTTP and 5 μM biotin-dUTP. The thermal cycling conditions for both PEXT reactions were as follows: initial denaturation at 95 °C for 5 min, followed by ten cycles of 95 °C for 15 s, 65 °C for 15 s, 72 °C for 15 s. All primer extension products were subjected to a final denaturation step at 95 °C for 10 min and placed immediately on ice before the dipstick assay.

Table 1
Oligonucleotide sequences used as primers in multiplex PCRs.

Name	Sequence (5′ → 3′)	Length (nt)	Amplicon (bp)/Exon	Mutation
1st 5-plex PCR				
LSW2iiF*	AAAGAGACCTTTATCTTCTGCTAAC	25	306/2	p.Gln289X
LSW2iiR*	CTCCCTTCGGCTCCATCA	18		
LSW10F	AGTGGCCATGTGAGTGATAA	20	206/10	c.2530delA
LSW10R	CTATGATATCCTCCTGAGGGAAC	23		
LSW14F	AGGAGTGTGACTATGGAAGC	20	316/14	p.His1069Gln
LSW14R	AGAAGGACATGGTGAGGAATAA	22		
LSW12F	TGTGGTGTTTTATTTCTTCATAGGT	25	360/12	p.Leu936X
LSW12R	AAGAACAGGATCAATGTCAGTAG	23		
LSW13F	GAGCTTCCTTATTGAACTCTCAAC	24	314/13	p.Arg969Gln
LSW13R	CAATGTGAAATAGTAAACAGATACTACTT	29		
2nd 5-plex PCR				
LSW2iiF	AAAGAGACCTTTATCTTCTGCTAAC	25	306/2	c.845delT
LSW2iiR	CTCCCTTCGGCTCCATCA	18		
LSW5F	GCTTTCCTTGATCCTGGG	18	281/5	c.1708-1G > A
LSW5R	CATTTTTCTTCACTGATTATATATTACTGT	30		
LSW8F	CATTGAACTCTCCTCCCTAC	20	349/8	c.2299insC
LSW8R	AGAGGAAGTGAGATTTGTTTACTG	24		
LSW16F	TCACAGTGAAATTGGACCATTTAG	24	241/16	p.Ile1148Thr
LSW16R	AAACTGTATTTCTGAGAGAGCG	22		
LSW21F	TAGAATGGCTCAGATGCTGTT	21	360/21	p.Ter1466Arg
LSW21R	GTGGTGAGTGGAGGCAA	17		

*F = Forward primer, *R = Reverse primer.

Table 2
Oligonucleotide sequences used as allele-specific primers and capture probes.

[a]Oligonucleotides	Name	Sequence (5′ → 3′)	Size (nt)	Sequence (5′ → 3′) [b]Capture probes
Allelle-specific primers and capture probes for the 1st 10-plex PEXT—dipstick assay				
p.His1069Gln	WTagged39	*CTTTCTATCTTTCTACTCAATAAT*	43	ATTATTGAGTAGAAAGATAGAAAG
(c.3207C > A) (N)	(5′ primer)	**GGAGGCCAGCAGTGAACAC**		
p.His1069Gln	WTagged40	*CTTCTCATTAACTTACTTCATAAT*	43	ATTATGAAGTAAGTTAATGAGAAG
(c.3207C > A) (M)	(5′ primer)	**GGAGGCCAGCAGTGAACAA**		
p.Arg969Gln	WTagged35	*CAATTAACTACATACAATACATAC*	45	GTATGTATTGTATGTAGTTAATTG
(c.2906G > A) (N)	(5′ primer)	**CCAGACAGAGGTGATCATCCG**		
p.Arg969Gln	WTagged36	*AATCCTTTTACATTCATTACTTAC*	46	GTAAGTAATGAATGTAAAAGGATT
(c.2906G > A) (M)	(5′ primer)	**CCCAGACAGAGGTGATCATCCA**		
p.Gln289X	WTagged5	*AATCAATCTTCATTCAAATCATCA*	49	TGATGATTTGAATGAAGATTGATT
(c.865C > T) (N)	(3′ primer)	**CAGTTTTGTTCTCCAAGGACACTTG**		
p.Gln289X	WTagged6	*TCAATCAATTACTTACTCAAATAC*	50	GTATTTGAGTAAGTAATTGATTGA
(c.865C > T) (M)	(3′ primer)	**GCAGTTTTGTTCTCCAAGGACACTTA**		
p.Leu936X	WTagged31	*AATCTACACTAACAATTTCATAAC*	50	GTTATGAAATTGTTAGTGTAGATT
(c.2807T > A) (N)	(5′ primer)	**ATCATCATCATGTCAACTTTGACGTT**		
p.Leu936X	WTagged32	*CTATCTTTAAACTACAAATCTAAC*	50	GTTAGATTTGTAGTTTAAAGATAG
(c.2807T > A) (M)	(5′ primer)	**ATCATCATCATGTCAACTTTGACGTA**		
c.2530delA (N)	WTagged27	*CTTTAATCTACACTTTCTAACAAT*	47	ATTGTTAGAAAGTGTAGATTAAAG
	(3′ primer)	**TGGTATTGCCTTCCAGGACTTTC**		
c.2530delA (M)	WTagged28	*ATACTACATCATAATCAAACATCA*	46	TGATGTTTGATTATGATGTAGTAT
	(3′ primer)	**TGGTATTGCCTTCCAGGACTTC**		
Allelle-specific primers and capture probes for the 2nd 10-plex PEXT—dipstick assay				
p.Ile1148Thr	WTagged43	*TACATTACCAATAATCTTCAAATC*	46	GATTTGAAGATTATTGGTAATGTA
(c.3443T > C) (N)	(5′ primer)	**CCCAGACCTTCTCTGTGCTGAT**		
p.Ile1148Thr	WTagged44	*CTTTCAAATCAATACTCAACTTT*	45	AAAGTTGAGTATTGATTTGAAAAG
(c.3443T > C) (M)	(5′ primer)	**CCAGACCTTCTCTGTGCTGAC**		
c.2299insC (N)	WTagged23	*TTACTCAAAATCTACACTTTTTCA*	42	TGAAAAAGTGTAGATTTTGAGTAA
	(3′ primer)	**ACAAAGAGCATGGGGGGC**		
c.2299insC (M)	WTagged24	*CAATTTCATCATTCATTCATTTCA*	42	TGAAATGAATGAATGATGAAATTG
	(3′ primer)	**CAAAGAGCATGGGGGGGC**		
c.845delT (N)	WTagged3	*CTTTAATCTCAATCAATACAAATC*	50	GATTTGTATTGATTGAGATTAAAG
	(3′ primer)	**CACTTGAATACTTTGAACCCCTAGGA**		
c.845delT (M)	WTagged4	*TACACTTTATCAAATCTTACAATC*	50	GATTGTAAGATTTGATAAAGTGTA
	(3′ primer)	**CACTTGAATACTTTGAACCCCTAGGG**		
c.1708-1G > A (N)	WTagged9	*TCAAAATCTCAAATACTCAAATCA*	46	TGATTTGAGTATTTGAGATTTTGA
	(5′ primer)	**TTCTTGCCATCCTGTGTTGCAG**		
c.1708-1G > A (M)	WTagged10	*TCATCAATCAATCTTTTTCACTTT*	46	AAAGTGAAAAAGATTGATTGATGA
	(5′ primer)	**TTCTTGCCATCCTGTGTTGCAA**		
p.Ter1466Arg	WTagged49	*ATCATACATACATACAAATCTACA*	43	TGTAGATTTGTATGTATGTATGAT
(N) (c.4396 T > C)	(3′ primer)	**CGCCTGCCTGAAGTCATCA**		
p.Ter1466Arg	WTagged50	*TACACTTTCTTTCTTTCTTTCTTT*	43	AAAGAAAGAAAGAAAGAAAGTGTA
(M) (c.4396 T > C)	(3′ primer)	**CGCCTGCCTGAAGTCATCG**		

[a] Recognition sequences of each primer are in italics; allele-specific sequences are in bold.
[b] Capture oligonucleotides (complementary to recognition sequences) carry an-NH_2 group at their 5′ end via a 6-carbon spacer arm to be conjugated to the carboxylate-modified microspheres.

2.4.3. Preparation of the multi-allele DNA strip

Each multi-allele (10 alleles per strip) dry-reagent biosensor (4 mm × 70 mm) comprising a wicking pad, a glass-fiber conjugate pad, a nitrocellulose membrane, an absorbent pad, and a plastic adhesive backing was constructed as described previously [15].

2.4.4. Visual detection of the extension products by lateral flow dipstick test

A 3 μL aliquot of the denatured primer extension reaction product was mixed with an equal volume of developing solution (2 × SSC, pH 7.0, 250 mL/L formamide, 30 g/L BSA, 20 mL/L glycerol, 5 mL/L Tween-20 and 5 g/L SDS). The mixture was applied to the upper part of the conjugate pad close to the functionalized gold nanoparticles (i.e., closer to the membrane). The wicking pad was then immersed into a tube containing 300 μL of developing solution which migrates along the length of the strip. The visual detection of PEXT products was completed within 15 min.

2.4.5. Interpretation of the results

Capture probes corresponding to the wild-type (normal) alleles were spotted on the left side of the diagnostic membrane, whereas capture probes specific for the mutant alleles (Table 2) were spotted on the right side of the membrane. For any particular mutation, a normal (wild-type) individual will give red colour at the left spot only, whereas a homozygous mutant will give colour only at the right spot of the pair. Both spots will be red if the sample is heterozygous for the mutation.

3. Results and discussion

3.1. Assay principle

The principle of the proposed genotyping method is illustrated in Fig. 1. The genomic DNA fragments that span the loci of interest are first amplified by two 5-plex PCRs. First 5-plex PCR includes fragments flanking 5 mutations that account for 68% of WD chromosomes in Greek population (p.His1069Gln (c.3207C > A), p.Arg969Gln (c.2906G > A), p.Leu936X (c.2807T > A), p.Gln289X (c.865C > T), c.2530delA), whereas the second 5-plex PCR includes fragments flanking 5 rare mutations, (p.Ter1466Arg (c.4396 T > C), c.845delT, c.2299insC, p.Ile1148Thr (c.3443T > C) and c.1708-1G > A) that account for the 9.7% of the WD chromosomes [1]. Subsequently, each of the 5-plex PCR products was subjected to a 10-plex PEXT reaction and the products were analysed by a multi-allele dipstick assay (10 alleles per strip). Two allele-specific primers (normal and mutant) that hybridize with the target DNA adjacent to the mutation and have at the 3′ end a nucleotide complementary to the allelic variant, are used in PEXT reactions. The distinction of genotypes is based on the high accuracy of nucleotide incorporation by DNA polymerase that lacks any 5′ > 3′ and 3′ > 5′ exonuclease activity. Only primers with perfectly matched 3′ ends are extended. Biotin is incorporated in the extended primer through the use of biotin-dUTP along with the dNTPs. All genotyping primers have at their 5′ end a unique sequence that enables subsequent capture of the primer on the test zone of the dipstick through hybridization with complementary immobilized capture oligonucleotide sequences (Fig. 1A). The PEXT reaction product is applied to the dipstick, which is then immersed into the appropriate buffer. As the buffer migrates along the strip, the products are captured by immobilized complementary oligonucleotides at the appropriate test spots and detected by antibiotin-functionalized gold nanoparticles. The nanoparticles bind only to the extended products through biotin/antibiotin interaction and the hybrids produce characteristic red spots denoting the presence of the corresponding allele in the sample. The excess of gold nanoparticles is captured at the control zone of the dipstick forming another red spot that indicates the proper performance of the system (Fig. 1B).

3.2. Optimization studies of the PEXT reaction

Various parameters were tested in order to optimize the PEXT reaction, including the annealing temperature, concentration of Mg^{2+}, dNTPs and that of allele-specific primers.

3.2.1. Effect of the annealing temperature

The annealing temperature of the PEXT reaction determines the stringency of primer hybridization and, therefore, is a critical parameter for the specificity of the extension reaction. The annealing temperature was studied in the range of 55–68 °C using sample normal for the mutations (Fig. 2A) and sample mutant for the 1708-1G > A mutation (Fig. 2B). We observed that the specificity of the PEXT reaction (i.e. absence of non specific spots) is not influenced by the annealing temperature in the temperature range studied, whereas the intensity of the spots (i.e. efficiency of the PEXT reaction) is slightly increased with increasing annealing temperature up to 60 °C and remain practically constant with the further increase up to 68 °C. The optimum annealing temperature selected was 65 °C.

3.2.2. Effect of Mg^{2+} concentration

Since the specificity of DNA polymerase depends strongly on the concentration of Mg^{2+}, we studied the effect of Mg^{2+} in the range of 2–4 mM using sample normal for all mutations (Fig. 2C), sample heterozygous for the Gln289X mutation (Fig. 2C'), and sample mutant for the 1708-1G > A mutation (Fig. 2D). As it is observed, the intensity of the spots is increased with increasing the Mg^{2+} concentration. As the optimum concentration for discrimination of the alleles, the 4 mM Mg^{2+} was selected. Higher Mg^{2+} concentration was not used in order to prevent the formation of any nonspecific primer extension which would complicate the assay.

3.2.3. Effect of dNTPs concentration

We also tested the effect of dNTPs concentration in the range 2–20 μM on the efficiency of PEXT reaction and the results are shown in Fig. 3A and 3A'. The intensity of the spots is increased with increasing the concentration of dNTPs up to 10 μM. However, at higher dNTPs concentration (20 μM) the formation of non-specific products is observed. Therefore, a 10 μM dNTPs concentration was selected as optimum.

3.2.4. Effect of the primers concentration

Optimization of the amount of allele-specific primers was performed in order to achieve the highest performance of PEXT reaction and to prevent the formation of non-specific products. A series of PEXT reaction was performed in the presence of equimolar amounts of all 10 primers in the range of 1–7.5 pmol per reaction. According to the results, the intensity of the specific signals remains practically stable up to the amount of 7.5 pmol (Fig. 3B and B'), whereas, the intensity of the non-specific signals reduces until it disappears completely. The latest was attributed to the competition between non-specifically extended primers and the non-extended ones which are in excess, for the hybridization to the immobilized capture-probes. The 5 pmol amount of each primer (0.25 μM final concentration) was selected as optimum.

3.3. Optimization of the visual detection by dry-reagent dipstick biosensor

3.3.1. Effect of formamide concentration

The developing solution is an important factor for the proper functioning of the dry-reagent strip. The role of formamide in

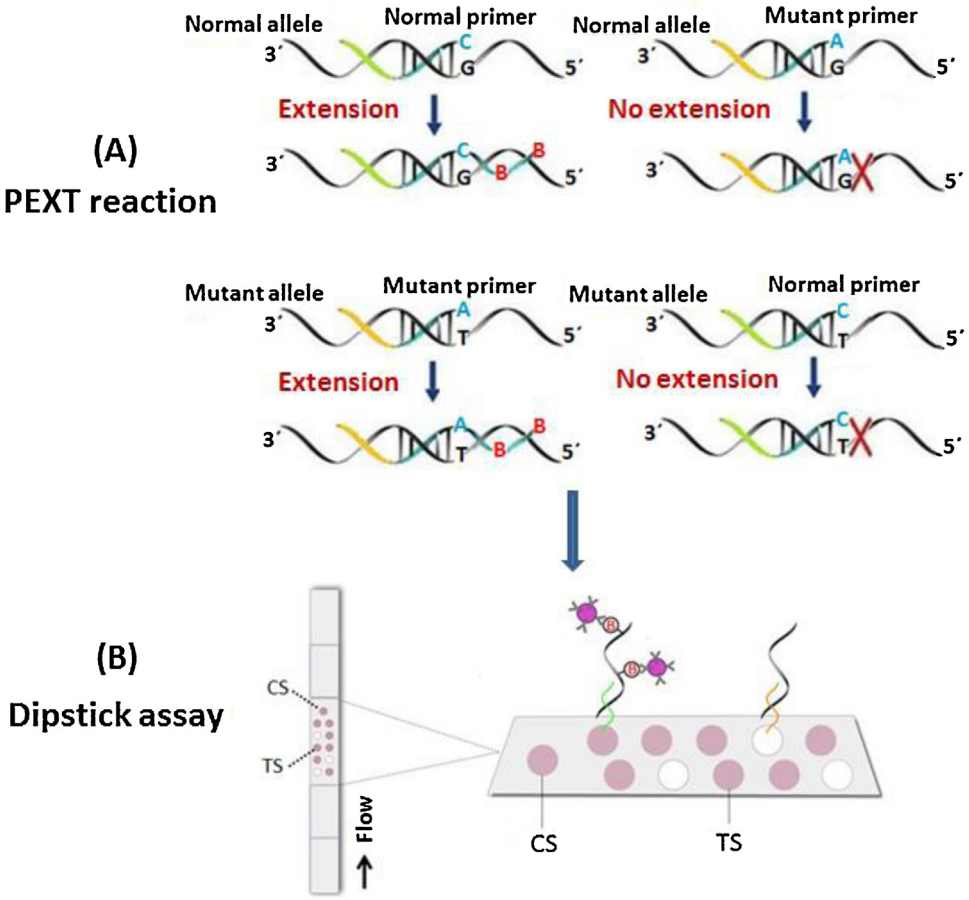

Fig. 1. Schematic presentation of the multi-allele genotyping assay for 10 *ATP7B* gene mutations. Genomic DNA fragments that span the loci of interest are first amplified by two 5-plex PCRs. (A) Aliquot of each non-purified amplification product is subjected to a 10-plex PEXT reaction in the presence of allele-specific primers (normal and mutant for each mutation) and biotin-dUTP. Only primers with perfectly matched 3′ ends are extended and biotin is incorporated in the extended primer. (B) An aliquot of the PEXT reaction product is applied to the conjugate pad and the sensor is immersed in the developing solution. As the buffer migrates along the strip, via capillary action, it rehydrates the antibiotin conjugated gold nanoparticles that hybridize with the biotin incorporated to the extended allele-specific primer. The hybrids are captured through the 5′ end tag of the allele-specific primer by immobilized anti-tag sequence, thereby forming a characteristic red color at the test spot of the sensor. If the allele-specific primer is not extended, then the antibiotin-nanoparticle conjugates are not captured at the test spot. The excess of nanoparticle conjugates form hybrids with immobilized biotinylated oligonucleotides at the control zone of the sensor, giving a second red spot. A red line at the control spot confirms the proper functioning of the sensor. A result is positive when a red color appears both in the test and control spot. TS: test spot; CS: control spot. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the developing solution is very important because of its ability (i) to destabilize double-stranded DNA molecules, increasing thus the number of molecules available for hybridization and (ii) to eliminate the non-specific hybridizations, increasing thus the specificity of the detection. In this study, we tested the effect of formamide concentration in the developing solution in the range of 0–250 mL/L and the results are presented in Fig. 3C. According to the results, non-specific signals observed in the absence or at low (up to 100 mL/L) formamide concentration. By increasing the concentration of formamide in the developing solution, the non-specific signals disappear. The formamide concentration, selected as optimum, was 200 mL/L. At higher formamide concentrations, the intensity of specific signals decreases.

3.3.2. *Effect of the amount of PCR product*

A series of PEXT reactions were performed using different amount of PCR product in the range of 0.05–2 μL. PEXT reaction products were analyzed by the dry-reagent dipstick assay. According to the results (Fig. 3D), the intensity of the specific signals increases with increasing the amount of PCR product up to 1 μL. At higher product amounts (>1 μL), however, the appearance of non-specific signals is observed. An amount of PCR product of 0.5 μL was selected as optimum. It is of interest to notice that the sample genotype could be obtained using as little as 0.05 μL of the PCR product.

3.3.3. *Effect of the amount of PEXT reaction product*

In this study, different amounts of a PEXT reaction product in the range 0.5–3 μL were analyzed by the dipstick assay and the results are shown in Fig. 3E. The intensity of the spots increases with increasing the amount of the PEXT reaction product up to 3 μL because larger amounts of extended primers are available to be immobilized on the diagnostic membrane of the strip. An amount of 3 μL was used as optimum. The results shown the remarkable sensitivity of the visual detection by dipstick assay as far as the genotype could be clearly defined even by analyzing 0.5 μL of the PEXT reaction product. According to the above findings, the minimum quantity of the PCR product producing visible genotyping result could be calculated as follows:$\mathrm{PCR\,(fmol)} = 0.05\mu\mathrm{L} \times \frac{100\mathrm{fmol}}{\mu\mathrm{L}} \times 3\mu\mathrm{L}/20\mu\mathrm{L} \approx 1\mathrm{fmol}\left(10^{-15}\mathrm{mol}\right)$, where 0.05 μL—the lowest volume of PCR product subjected to 10 cycles of PEXT reaction, 20 μL—the PEXT reaction volume, 100fmol/μL—the concentration of the amplicon and 3 μL—the aliquot of the extended product analyzed by the proposed dipstick assay.

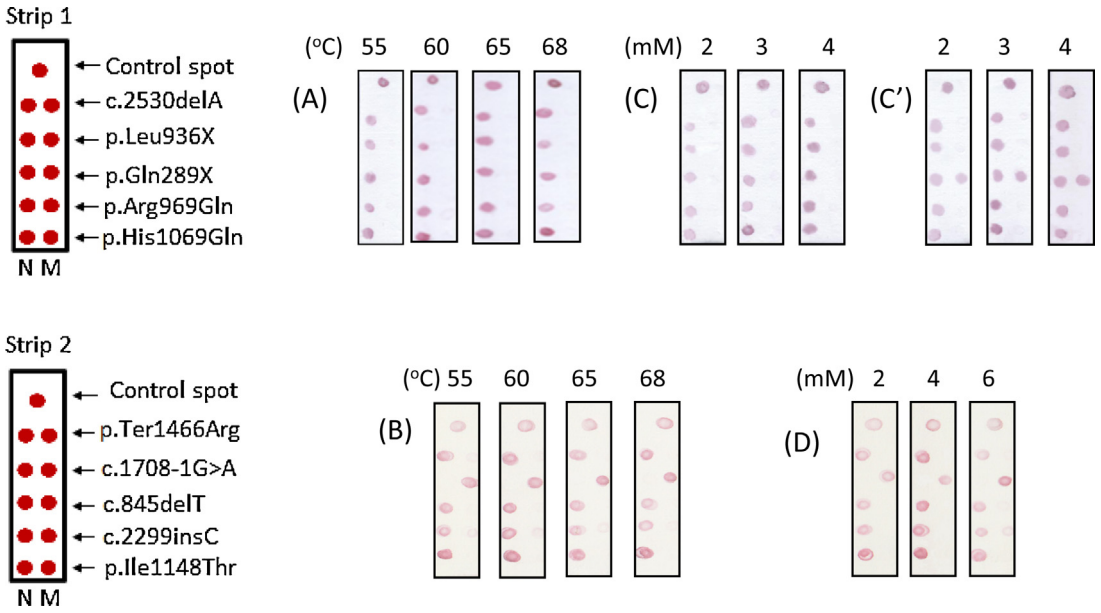

Fig. 2. (A, B) Effect of annealing temperature in the PEXT reaction 1 (A) and PEXT reaction 2 (B). The annealing temperature (°C) is indicated above each strip. Effect of Mg^{2+} concentration in the PEXT reaction 1 (C,C′) and PEXT reaction 2 (D). Samples: (C) 'normal', (C′) "heterozygote', (D) 'mutant'. The Mg^{2+} concentration is written above the strips. For the position of each mutation-specific probe see cartoon on the left side of the figure.

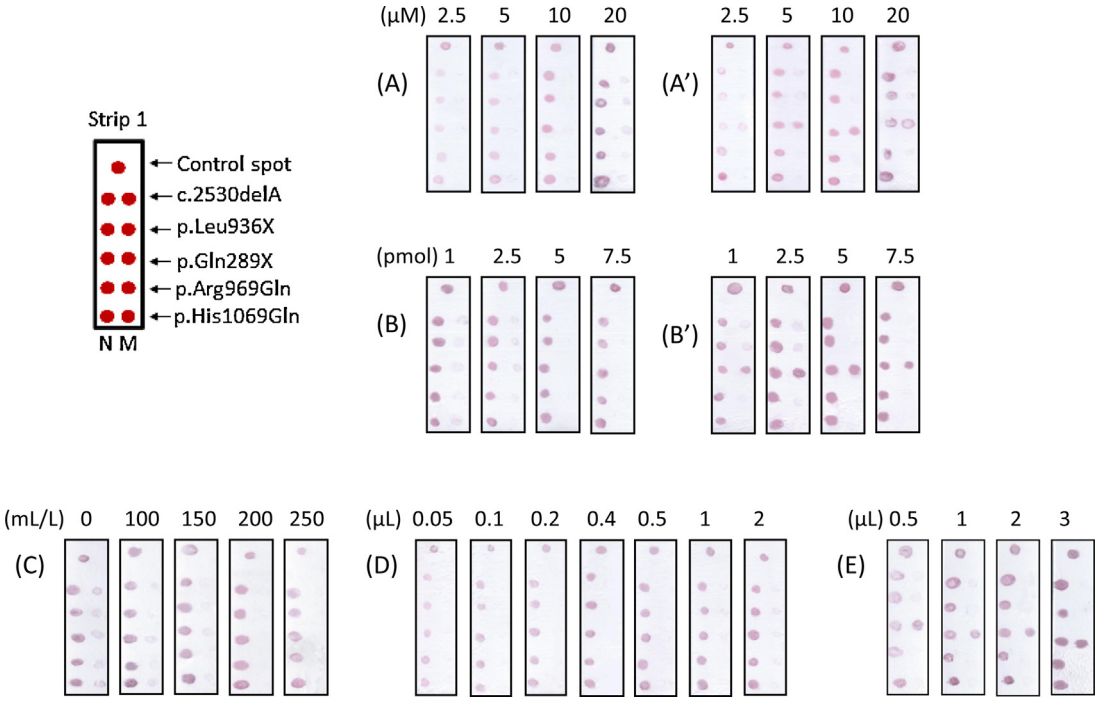

Fig. 3. (A, A′) Effect of dNTPs concentration. (B, B′) Effect of the amount of allele-specific primers. (C) Effect of the formamide concentration in developing solution. (D) Effect of the amount of PCR product used for extension reaction. (E) Effect of the amount of PEXT reaction used for the dipstick assay. All concentrations and amounts are indicated above the strips. Samples: (A–D) 'normal', (A′, B′, E) 'heterozygote'.

3.3.4. *Specificity of the dipstick assay*

In order to confirm that each extended primer hybridizes only to the corresponding capture probe on the membrane and not to any other immobilized probes, we performed the following experiments: three PCR amplified DNA samples of different genotype (normal for the mutations, heterozygote for all mutations and double heterozygote) were subjected to three PEXT reactions in the presence of three sets of allele-specific primers: one set of 5 primers corresponding to the normal alleles, one set of 5 primers corresponding to the mutant alleles and a set of all 10 primers corresponding to all alleles. The products were analyzed by the dipstick assay and the results are presented in Fig. 4A. As it is observed, red spots appeared only at positions that contained the corresponding capture probes for normal alleles, either in the presence of homozygous normal or heterozygous samples. Red spots at positions containing capture probes for mutant alleles appeared only when the double heterozygous and the synthetic heterozygous sample were tested. The results obtained confirm the specificity of the proposed genotyping assay.

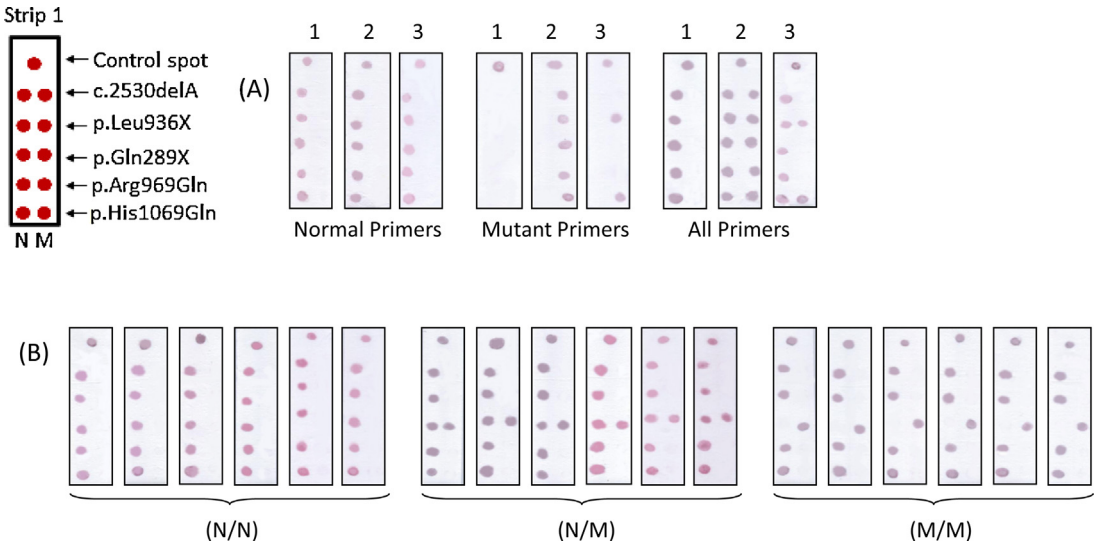

Fig. 4. Specificity (A) and reproducibility (B) of the multiplex PEXT dipstick genotyping assay. (A) Sample normal (1), synthetic heterozygote (2) for all mutations and double heterozygote (3) for the p.Leu936X/p.His1069Gln mutations were subjected to three PEXT reactions. The first PEXT reaction was performed in the presence of the five normal primers, the second in the presence of the five mutant primers and the third in the presence of all ten primers. The synthetic sample was a mixture of equal amounts of PCR products from samples that contain at least one of the 5 mutations. Therefore, the synthetic sample represented a heterozygous template for all primers. Normal primers are extended in the presence of all three samples whereas mutant primers are extended and give signals only in the presence of sample carrying the mutation. (B) Overall reproducibility (within run and between run) of the multi-allele genotyping assay includes PEXT reaction and the dipstick assay. Three individuals of different genotype (wild type, heterozygous and homozygous mutant for the p.Gln289X mutation) were used in the study.

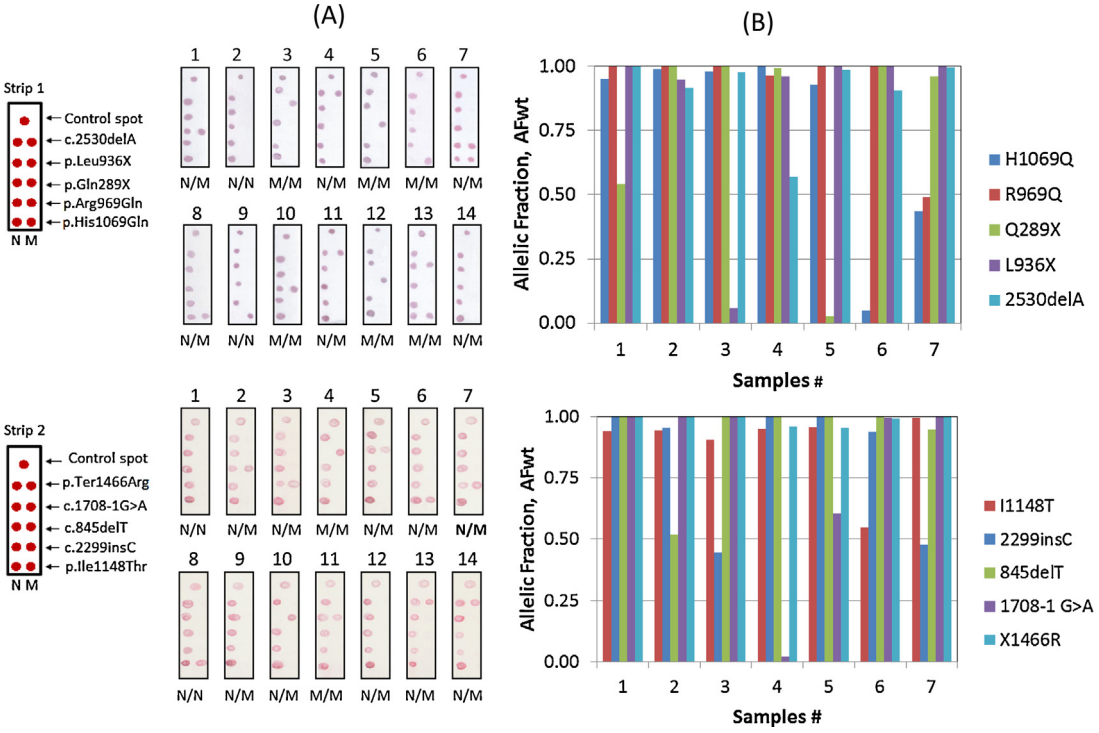

Fig. 5. (A) Genotyping results obtained from 14 clinical samples by the proposed method. For the position of each mutation-specific probe see cartoon on the left side of the sample pictures. The visual genotype is assigned by observing each pair of spots. For any particular mutation, a wild-type individual will give red color at the left spot only, whereas a mutant homozygote will only give color at the right spot of the pair. Both spots will appear red if the sample comes from a heterozygote for this mutation. (B) The allelic fractions for each of the 10 loci (included in strip 1 and strip 2) calculated for 7 (upper row) out of 14 samples versus the sample number. The genotype of each sample is presented by 5 colored bars for strip 1 and strip 2, respectively. Each colored bar corresponds to a specific locus as shown on the right side of the Fig. 5B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.3.5. *Reproducibility*

We tested the overall reproducibility of the multi-allele genotyping assay (including PEXT reaction and the dipstick assay) and the results are shown in Fig. 4B. Three samples (normal, heterozygote and homozygous mutant for p.Gln289X) were subjected to two separate PEXT reactions each and the products were analyzed in triplicate by dipstick assay. As it is observed the reproducibility of the proposed genotyping method is very satisfactory.

3.3.6. Evaluation of the method

The proposed method was evaluated by genotyping 46 DNA samples with previously defined genotypes for all the 10 *ATP7B* gene mutations and 34 samples with unknown genotype (blind samples). The genotyping results obtained by the proposed PEXT-dipstick assay were in full concordance with those obtained by the reference method. Characteristic genotyping results obtained from 14 genomic DNA samples are shown in Fig. 5A, where N/N refers to normal sample, M/N refers to a heterozygous or double heterozygous sample and M/M refers to a homozygous mutant sample. For easy interpretation of the results, the dry-reagent strip was configured in such a way that the capture probes corresponding to the wild-type (normal) alleles were spotted on the left side of the membrane, whereas capture probes specific for the mutant alleles were immobilized on the right side of the membrane. The genotype is assigned by observing each pair of spots. For any particular mutation, a wild-type individual will give red color at the left spot only, whereas a mutant homozygote will only give color at the right spot of the pair. Both spots will appear red if the sample comes from a heterozygote for this mutation. The proposed strip sensor could also provide quantitative information on target DNA. For this purpose, the strips were scanned with a common scanner and the intensity of the spots was estimated densitometrically using the GelAnalyzer 2010a image analysis software (www.gelanalyser.com). Quantitative measure of the sample genotype is given by the calculation of the normal (wild type) allelic fraction (AF_{wt}) as follows: AFwt = wt/(wt + mut), where wild type (wt) and mutant (mut) correspond to the densitometric signals obtained (for each mutation) from primer extension reaction with normal and mutant allele-specific primers, respectively [14]. Heterozygotes for any mutation are expected to have allelic fractions near 0.5 (a 1:1 ratio of the two alleles), whereas homozygotes for wt and mut are expected to give AFwt values that are higher than 0.8 and lower than 0.2, respectively. As an example, the allelic fractions calculated for each of the 10 loci (included in strip 1 and strip 2) for the 7 out of the 14 samples presented in Fig. 5 (upper row) are shown in Fig. 5B. The results clearly demonstrated that the calculated allelic fractions are indicative of the sample genotype obtained by visual detection.

4. Conclusions

Today, a variety of methods has been developed for the detection of WD chromosomes. They include screening methodologies such as PCR-SSCP, DGGE, DHPLC, and high resolution melting analysis (HRMA) in which nucleotide variations are located or excluded by evaluating conformational differences (under certain experimental conditions) or melting behaviour of selected PCR amplicons within a gene region. Most of these methods, even though highly accurate and sensitive, are costly, complex, time consuming and require extensive sample preparation. Moreover, they require confirmation of the positive findings by another method, commonly sequencing.

On the other hand, methods such as real-time amplification refractory mutation system (ARMS), bidirectional PCR amplification of specific alleles (BI-PASA), SYBR green intercalator method based on the ARMS, restriction fragment length polymorphism (RFLP) etc., target usually a specific mutation each time and represent a time-consuming strategy or use expensive specialized equipment or hazardous chemicals.

In this paper we report the development of a dry-reagent lateral-flow dipstick assay for the rapid genotyping of the 10 mutations in the Wilson disease related *ATP7B* gene most common in Greek population. The method is based on three simple steps including (i) multiplex PCR amplification of the *ATP7B* gene fragments flanking the mutations, (ii) multiplex PEXT reactions of unpurified PCR products in the presence of allelic-specific primers and biotin-dUTP which is incorporated into extended primers with perfectly matched 3′ ends and (iii) visual detection of PEXT reaction products by multi-allele dipstick type biosensor using anti-biotin conjugated gold nanoparticles. Due to the high detectability offered by gold nanoparticles, only a few (10) PEXT reaction cycles were required, thus achieving high allele specificity. The entire procedure lasts on average about 2 h. The method was validated by analyzing 46 patients' samples of known genotype and 34 blind samples and the results were fully concordant with those obtained by reference methods (DGGE and bidirectional sequencing).

To conclude, the proposed assay is very simple and rapid, does not require multiple pipettings, incubations and washings. Detection of alleles is accomplished by naked eye and therefore there is no need for highly trained personnel and sophisticated equipment except for a common thermocycler. It can serve as the first approach to characterize about 80% of Wilson disease chromosomes in patients of Greek origin, limiting the need for extensive mutation screening only to the patients that prove negative. Finally, the method could be easily adapted to support rapid genotyping of any other population specific *ATP7B* gene mutation spectrum in countries with high frequency of Wilson disease and is particularly suited for small molecular-diagnostic laboratories with a limited budget and a small-to-medium sample volume.

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