Clinica Chimica Acta 446 (2015) 241–247

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Multi-allele DNA biosensor for the rapid genotyping of 'nondeletion' alpha thalassaemia mutations in *HBA1* and *HBA2* genes by means of multiplex primer extension reaction

Margarita Petropoulou [a,1], Amalia Poula [a,1], Jan Traeger-Synodinos [b], Emmanuel Kanavakis [b], Theodore K. Christopoulos [c,d], Penelope C. Ioannou [a,*]

[a] Laboratory of Analytical Chemistry, Department of Chemistry, Athens University, Athens 15771, Greece
[b] Laboratory of Medical Genetics, Athens University, Athens 11527, Greece
[c] Department of Chemistry, University of Patras, Patras 26500, Greece
[d] Foundation for Research and Technology Hellas, Institute of Chemical Engineering and High Temperature Chemical Processes (FORTH/ICE-HT), Patras 26504, Greece

ARTICLE INFO

Article history:
Received 6 February 2015
Accepted 10 April 2015
Available online 17 April 2015

Keywords:
Nondeletion mutations
HBA1 and *HBA2* genes
Genotyping
Primer extension reaction
Multi-allele DNA biosensor

ABSTRACT

Background: Alpha-thalassaemia is an autosomal recessive disorder characterized by defective production of the alpha chain of haemoglobin. It is caused mainly by deletions of one or both of the duplicated alpha-globin genes on chromosome 16, and/or by nucleotide variations, known as "nondeletion" mutations. Definition of the alpha globin genotype in carriers supports genetic counselling, and in patients with Hb H disease is useful to predict prognosis and management options. Here, we report a method that facilitates direct detection by naked eye of the 13 most common "nondeletion" alpha-globin gene mutations in populations around the Mediterranean and Middle East.

Methods and results: The method comprises (i) PCR amplification of a single 1087 bp fragment for each *HBA1* and *HBA2* gene (separately); (ii) multiplex primer extension reaction of just 10 cycles, using unpurified amplification product as a template, to incorporate biotin into those allele-specific primers that extend and, finally, (iii) visual detection of the reaction products within minutes by the dipstick biosensor. The method was evaluated by analysing 105 samples of known genotypes and the results were found fully concordant with those obtained by the reference methods.

Conclusions: The proposed assay is particularly suited for small molecular-diagnostic laboratories with a limited budget and a low-to-medium sample volume. In addition this platform represents a very simple and useful genotyping tool to support gene scanning methods whenever nucleotide variations have to be specified.

1. Introduction

Alpha-thalassaemia, one of the most monogenic disorders in humans, is the result of defective production of the alpha chain of haemoglobin [1]. The primary causes of alpha-thalassaemia involve deletions of one or both of the alpha-globin genes in the telomeric region of chromosome 16 (16p 13.3) on chromosome 16 [2]. Deletions leading to alpha-thalassaemia are more common than nucleotide variations, otherwise known as "nondeletion" alpha-thalassaemia variants. Defects due to either type of mutation that leads to dysfunction of one or two alpha genes may cause slight reduction in erythrocyte volume and size but are not clinically relevant. Loss of function of three alpha genes leads to Hb H disease, a chronic moderate anaemia associated with a picture of alpha-thalassaemia intermedia [3]. Complete deletion of all four alpha-globin genes results in severe anaemia in utero and a condition known as Hb Barts hydrops foetalis. Definition of alpha globin genotypes in potential carriers is useful to predict prognosis and management options, due to the strong correlation of phenotype and genotype in Hb H disease patients [4–7]. For identification of nondeletion variants in alpha globin gene, Sanger sequencing is the reference method. However, in routine laboratories, the detection of known thalassaemia mutations and globin chain variants in populations with specific mutation spectrums is mainly performed using traditional well-established methods such as restriction enzyme analysis of PCR amplicons (RE-PCR), allele-specific amplification using the amplification refractory mutation system (ARMS), allele-specific mutation detection of amplified DNA based on hybridization of PCR products to allele-specific oligonucleotide probes (ASO) in either forward or reverse ASO approach [8]. More recently, pyrosequencing, involving sequencing by synthesis [9] and high resolution melting analysis without the need for post-PCR sample manipulation [10], have been proposed for the rapid genotyping or screening of "nondeletion" *HBA* mutations.

* Corresponding author. Tel.: +30 210 7274574; fax: +30 210 7274750.
E-mail address: ioannou@chem.uoa.gr (P.C. Ioannou).
[1] M.P. and A.P. contributed equally to this work.

http://dx.doi.org/10.1016/j.cca.2015.04.016
0009-8981/

In this paper, we report a method that facilitates direct detection by the naked eye of the 13 most common "nondeletion" alpha-globin gene mutations in populations around the Mediterranean and Middle East using a dipstick biosensor in a dry-reagent format. The method comprises PCR amplification of a single fragment for each *HBA1* and *HBA2* gene flanking all mutations, using any conventional thermal cycler, multiplex primer extension (PEXT) reaction of just 10 cycles followed by the visual detection of the reaction products within minutes by the multi-allele dipstick biosensor. Specialized instrumentation is precluded, and the requirements for highly qualified technical personnel are minimized.

2. Materials and methods

2.1. Materials and instrumentation

Materials and instrumentation are provided in supporting information.

2.2. DNA samples

105 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). Genotyping of *HBA1* and *HBA2* nondeletion variants in samples was performed by amplification refractory mutation system-ARMS, real-time PCR genotyping and/or direct sequencing as previously described [4]. The "nondeletion" variants included in this study are shown in Table 1.

2.3. Primer design

All PCR primers and allele-specific primers for the multiplex primer extension genotyping reactions used in this study were designed in-silico using PrimerPremier 5 and PrimerPlex 2 software (Premier Biosoft International, Palo Alto, CA). Since α-chain haemoglobin (Hb) mutations can be located in either *HBA1* and/or *HBA2* genes (NG_000006), two different primer pairs were used to selectively amplify each of the highly homologous *HBA1* and *HBA2* genes. The amplified *HBA1* and *HBA2* gene fragments (1087 bp) flanking all 13 mutations were subsequently used as templates for genotyping by primer extension reaction in the presence of allele-specific primers. Oligonucleotides used as primers and probes in the course of this study were synthesized by VBC Genomics (Wien, Austria). *HBA1* and *HBA2* gene allele-specific primer sequences are available on request to the authors.

Table 1
List of α-chain nondeletion variants included in this study.

Gene/strip*	Genotype (traditional names)	HGVS nomenclature of mutations
HBA1/strip 1	Hb Questembert	c.394T > C
	Hb Aghia Sophia	c.187_189delGTG
	Hb Adana	c.179G > A
	Hb Taybe	c.118_120delACC
	Hb Heraklion	c.112_114delCCC
HBA2/strip 1	Hb Adana	c.179G > A
	Hb Icaria	c.427T > A
	Hb Agrinio	c.89T > C
	IVS1 (Hph)	c.95 + 2_95 + 6delTGAGG
	PolyA	c.*+94A > G
HBA2/strip 2	Initiation codon	c.2T > C
	IVS1-116	c.96-2A > G
	Hb Questembert	c.394T > C
	Turkish polyA	c.*+92A > G
	Constant spring	c.427T > C

* The reference sequence for the *HBA* genes is NCBI, NG_000006.1.

2.4. Genotyping of 5 mutations in HBA1 gene (Hb Heraklion, Hb Taybe, Hb Adana, Hb Aghia Sophia and Hb Questembert) by PEXT reaction and visual multi-allele dipstick assay

Genotyping consists of (i) PCR amplification of a 1087 bp *HBA1* gene fragment (Gene Bank Accession Number NG_000006.1) flanking the nondeletion mutations in *HBA1* gene, (ii) a 10-plex PEXT reaction 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 multi-allele dipstick type biosensor. *HBA1* and *HBA2* gene allele-specific primer sequences are available on request to the authors.

2.4.1. PCR Amplification of HBA1 gene fragment

PCR was carried out in a total volume of 50 μL containing 1 U of Kapa 2G Fast DNA polymerase (Kapabiosystems, Cambridge, US), 1 × PCR buffer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.25 μM of each 5′-TGGA GGGTGGAGACGTCCTG-3′ (forward) and 5′-CTGGCACGTTTGCTGAGG GAA-3′ (reverse) primer, 7.5% DMSO and 100 ng of genomic DNA. The PCR conditions were as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 2 min. PCR and primer extension reactions were carried out in the TaKaRa Dice Gradient thermal cycler (Otsu, Shiga, Japan).

2.4.2. Multiplex primer extension reaction

Multiplex (10-plex) PEXT reaction was performed in a total volume of 20 μL containing 1 U of AmpliTaq DNA polymerase, Stoffel fragment (Applied Biosystems, Foster City, CA), 1 × PCR buffer (10 mM Tris–HCl, 10 mM KCl, pH 8.3), 4 mM $MgCl_2$, 0.1 pmol of amplified DNA, 1 pmol each of 10 allele-specific primers, 10 μM each of dATP, dCTP, dGTP and 5 μM dTTP and biotin-11-dUTP. The cycling conditions for the extension reactions were as follows: initial denaturation 98 °C for 2 min, followed by 10 cycles of 98 °C for 15 s, 65 °C for 15 s, 72 °C for 15 s. All PEXT reaction products were subjected to a final denaturation step at 98 °C for 10 min and placed immediately on ice before the dipstick assay.

2.4.3. Preparation of the multi-allele DNA strip

The multi-allele dry reagent strip (4 mm × 70 mm) comprising a wicking pad, a glass-fibre conjugate pad, a nitrocellulose membrane, an absorbent pad, and a plastic adhesive backing was constructed as described previously [11]. For more details see supporting information.

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 a developing solution (2 × SSC, pH 7.0, 250 mL L^{-1} formamide, 30 g L^{-1} BSA, 20 mL L^{-1} glycerol, 5 mL L^{-1} Tween-20 and 5 g L^{-1} 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 five wild-type (normal) alleles were spotted on the left side of the diagnostic membrane, whereas capture probes specific for the five variant (mutant) alleles were spotted on the right side of the membrane. The genotype is assigned by observing each pair of spots. 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.

2.5. Genotyping of 10 mutations in HBA2 gene (Initiation Codon, Hb Agrinio, IVS1 (Hph), IVS1-116, Hb Adana, Hb Questembert, Hb Icaria, Hb Constant Spring, polyA, Turkish PolyA) by multiplex PEXT reaction and visual multi-allele dipstick assay

Genotyping consists of the same four steps as described above for *HBA1* gene.

2.5.1. PCR amplification

PCR amplification of a 1087 bp fragment in *HBA2* gene (Gene Bank Accession Number NG_000006.1) flanking the 10 most common nondeletion mutations was carried out using primer pair 5′-TGGAGGGT GGAGACGTCCTG-3′ (forward) and 5′-CTCCATTGTTGGCACATTCCG-3′ (reverse) and the same cycling protocol as for HBA1 fragment.

2.5.2. Multiplex primer extension reaction

Detection of the 10 HBA2 mutations was performed using two 10-plex PEXT reactions covering 5 of the mutations (10 alleles) each. Multiplex 10-plex primer extension reactions were performed in a total volume of 20 μL containing 1 U of Amplitaq DNA polymerase, Stoffel fragment, 1 × PCR buffer (10 mM Tris–HCl, 10 mM KCl, pH 8.3), 4 mM $MgCl_2$, 0.1 pmol of amplified DNA, 1 pmol each of 10 allele-specific primers, 10 μM each of dATP, dCTP, dGTP and 5 μM dTTP and biotin-11-dUTP. The cycling conditions for the extension reactions were as follows: initial denaturation 95 °C for 2 min, followed by 10 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 15 s. All primer extension reaction products were subjected to a final denaturation step at 95 °C for 10 min and placed immediately on ice before the dipstick assay.

2.5.3. Visual detection of the PEXT products by lateral flow dipstick test

Detection of the two 10-plex PEXT reaction products (20 alleles) was performed by means of two 10-plex dipstick strips as described above (2.4.4.).

2.5.4. Interpretation of the results

As described above (2.4.5.).

2.6. Construction of the mutant DNA fragments

Synthetic mutant DNA fragments were constructed for Hb Heraklion, Hb Adana, Hb Questembert (in α1) and Hb Questembert (in α2) variants due to the lack of representative clinical samples. All mutant DNA fragments were created by a three-step PCR (PCR-A, PCR-B and PCR-AB) using genomic DNA as template as previously described [12]. The amplified mutant DNA fragments were cloned into the pDrive Cloning Vector using PCR cloning kit from Qiagen according to the manufacturer's instructions.

3. Results

3.1. Assay principle

Schematics of the proposed multi-allele genotyping platform are shown in Fig. 1. Non purified PCR amplified (1087 bp) fragment of either *HBA1* and *HBA2* gene is subjected to few (10) cycles of multiplex PEXT reaction in the presence of allele-specific primers (two primers per mutation, one for the wild type and the second for the mutant allele) and biotin-modified nucleotide that is incorporated in the extended product. Allele-specific primers consist of two parts, the 3′ end that is complementary to the particular HBA1 or HBA2 allele and the 5′ end containing a unique segment that enables subsequent capture of the primer on the test zone of the dipstick through hybridization with complementary immobilized capture oligonucleotides. Extension by DNA polymerase, lacking 5′ → 3′ exonuclease activity occurs only if primer is perfectly complementary to the target sequence. Finally, the extension products are detected within 15 min by means of dry reagent dipstick assay. The PEXT reaction mixture is applied to the dipstick and the latter is immersed in the developing solution. As the solution flows along the strip, the products are captured by immobilized complementary oligonucleotides at the appropriate test spots and detected by antibiotin-functionilized gold nanoparticles. Extended products carrying biotin are bound to the nanoparticles through biotin-antibiotin interactions 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 by immobilized biotinylated oligonucleotides forming another red spot that indicates the proper performance of the system.

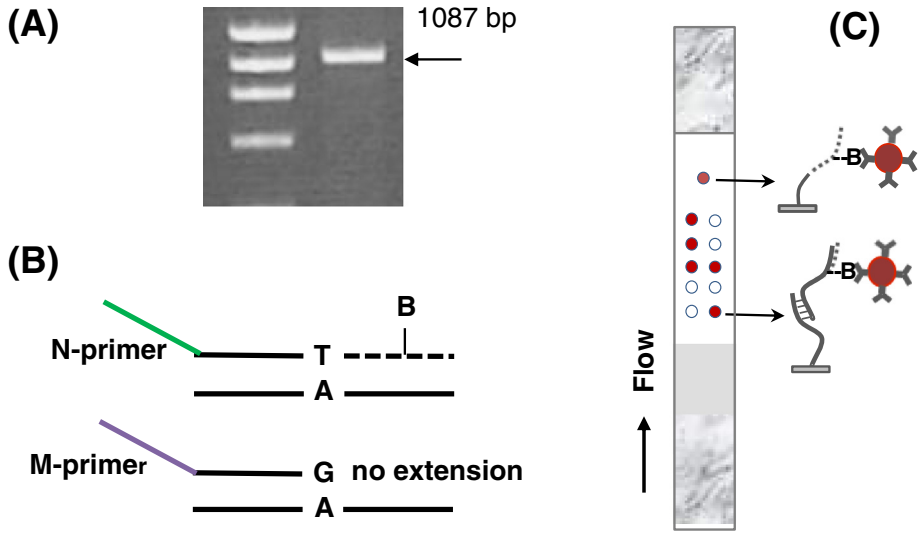

Fig. 1. Schematics of the multi-allele genotyping assay. (A) PCR amplification of a 1087 bp fragment of both *HBA1* and *HBA2* gene flanking the mutations. (B) Multiplex PEXT reaction (10 cycles) in the presence of allele-specific primers (two primers per mutation) and biotin-modified nucleotide that is incorporated in the extended product only if primer is perfectly complementary to the target sequence. (C) Detection of the extension products within 15 min by means of dry reagent dipstick assay. As the solution flows along the strip, the products are captured by immobilized complementary oligonucleotides at the appropriate test spots and detected by antibiotin-functionilized gold nanoparticles.

3.2. Optimization studies of PCR reaction and multiplex PEXT reaction

Extended optimization studies were performed to maximize PCR amplification efficiency of GC-rich 1087 bp fragments for both *HBA1* and *HBA2* genes, including Mg^{2+} and DMSO concentration, enzyme units, primer concentration and PCR cycling parameters. In addition, several parameters affecting the specificity and the yield of genotyping reaction were optimized. These include Mg^{2+} concentration, annealing temperature and time, concentration of primers and dNTPs, quantity of DNA template, etc. The effect of each parameter was studied in a series of PEXT reactions containing amplified target DNA from wild type samples or samples carrying mutations and varying amounts of the parameter under investigation. Some indicative results of the performed optimization studies are presented in Fig. 2. The optimized parameters selected for the multiplex PEXT reactions were as follows: Mg^{2+} concentration 4 mM, allele-specific primer concentration 1 pmol each, annealing temperature 65 °C (60 °C for *HBA2* gene), dNTPs concentration 10 μM and about 100 fmol (2 to 3 μL) of unpurified PCR product.

3.3. 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 experiment: two samples, one normal and the second synthetic mutant for all mutations, were subjected to two PEXT reactions, one in the presence of normal primers and the second in the presence of mutant primers. The synthetic sample was prepared by mixing equal amounts of PCR products from samples that contained at least one of the mutations. Therefore, the synthetic sample represented a heterozygous template for all primers. An example of specificity results is shown in Fig. 3A. Normal primers are extended in the presence of both samples while mutant primers are not extended in the presence of normal sample. The mutant primers are extended and give signal only in the presence of mutant sample.

3.4. Reproducibility

The overall reproducibility of the multi-allele genotyping assay (including extension reaction and the dipstick test) was assessed by analysing nine times (three PEXT reaction products analysed in triplicate) PCR products from genomic DNA obtained from three individuals of different genotype (wild type, herozygous and homozygous mutant for Hb Icaria). According to the results (Fig. 3B) the reproducibility of the allele's detection in each sample is very satisfactory.

3.5. Evaluation of the method

The proposed method was evaluated by investigating the presence of thirteen mutations (two variants are common for *HBA1* and *HBA2* genes) within the 1087 bp fragments of the *HBA1* and *HBA2* gene from DNA samples with previously defined genotypes. Samples for HBA1 mutations included 13 heterozygotes representing two of the five mutations (Aghia Sophia Hb and Taybe Hb), synthetic (homozygous and heterozygous) samples for Questembert, Adana and Heraklion Hb and 17 normal for HBA1 mutations samples; samples for HBA2 mutations included 54 homozygotes, heterozygotes or double heterozygotes representing all 10 mutations at least once each and 21 normal samples (containing only the normal alleles). The genotyping results obtained with the dipstick assay were fully concordant with those of the reference methods (ARMS-PCR, real-time PCR and/or direct sequencing) indicating the accuracy of this method. Typical genotyping results from a series of samples for HBA1 and HBA2 mutations are shown in Fig. 4. In addition, the proposed method was evaluated as an alternative (to Sanger sequencing) method that support definitive identification of the nucleotide variants in any sample that demonstrates behaviour that deviates from that of wild type when analysed by non-sequence specific methods (e.g. high resolution melting analysis, HRMA). More specifically, the proposed rapid PEXT-dipstick assay was adapted for genotyping of the nucleotide variants in our recently submitted work [unpublished

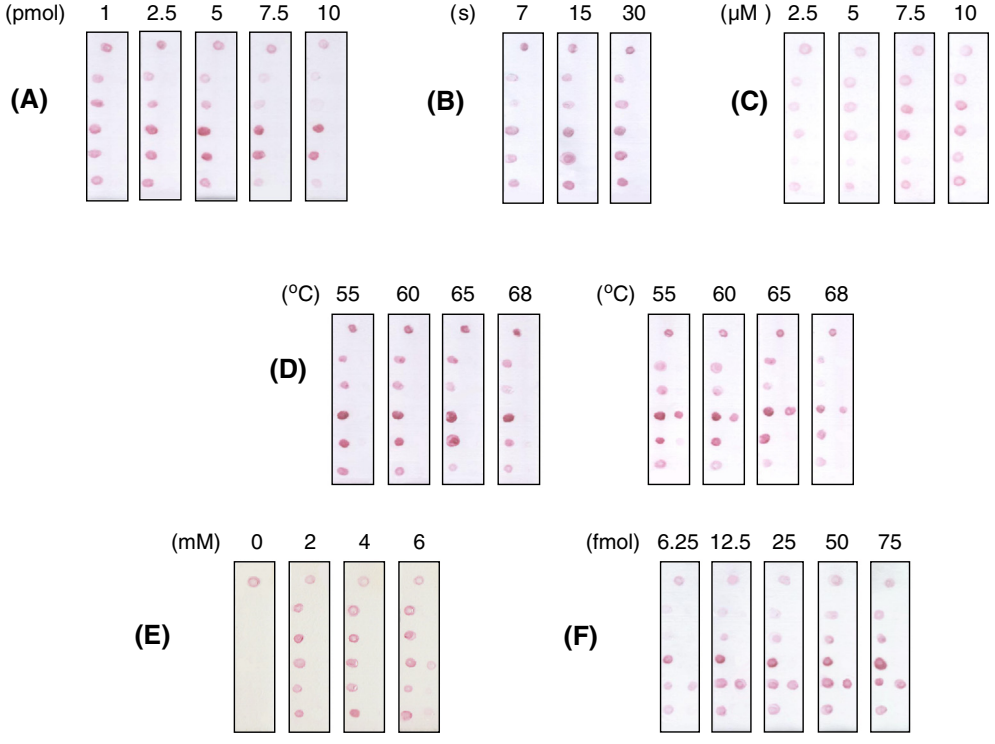

Fig. 2. Optimization studies of the multiplex PEXT reaction. (A) Concentration of allele-specific primers; (B) annealing time; (C) dNTPs concentration; (D) annealing temperature; (E) Mg^{2+} concentration; (F) quantity of DNA template. The selected optimized parameters are as follows: Mg^{2+} concentration 4 mM, allele-specific primer concentration 1 pmol each, annealing temperature 65 °C (60 °C for *HBA2* gene), dNTPs concentration 10 μM, about 100 fmol of PCR product (2 to 3 μL). Studies were performed using either wild-type or hetererozygous samples.

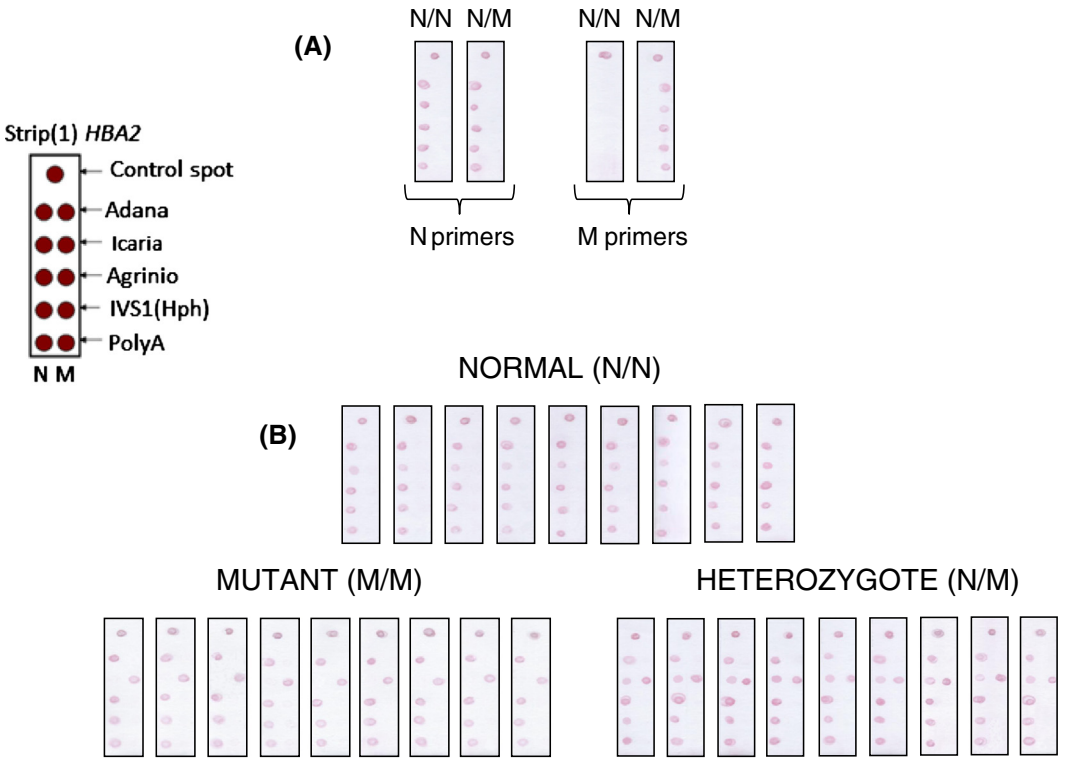

Fig. 3. Specificity (A) and reproducibility (B) of the multiplex PEXT dipstick genotyping assay. (A) Normal and synthetic mutant (for all mutations) samples were subjected to two PEXT reactions, one in the presence of normal primers and the second in the presence of mutant primers. The synthetic sample was a mixture of equal amounts of PCR products from samples that contain at least one of the 13 mutations. Therefore, the synthetic sample represented a heterozygous template for all primers. Normal primers are extended in the presence of both samples whereas mutant primers are extended and give signal 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, herozygous and homozygous mutant for Hb Icaria) were used in the study.

results] reporting on a HRMA method for screening nondeletion alpha-thalassaemia mutations in the *HBA1* and *HBA2* genes. Genotyping results obtained by analysing the same PCR fragments used for HRM analysis are presented in Fig. 5.

4. Discussion

In routine laboratories, the detection of "nondeletion" alpha globin chain variants in populations with specific mutation spectrum is mainly performed using traditional well-established PCR-based methods. These methods, however, target a specific mutation each time and represent a time-consuming strategy in most populations [7]. More advantageous in terms of time-saving and costs, especially, when genotyping samples with a large spectrum of globin gene mutations, are methods locating or excluding nucleotide variations by evaluating the melting behaviour of selected PCR amplicons within a gene region. These methods, however, do not provide a definitive diagnosis, and all samples must be characterized using another appropriate method to specify the nucleotide variation [8].

Here, we present a simple, less laborious and cost-effective genotyping platform for identifying 13 known "nondeletion" alpha globin gene mutations using as tools PCR and PEXT reaction combined with dipstick assay (biosensor). The method comprises PCR amplification of a single 1087 bp fragment for each *HBA1* and *HBA2* gene using any conventional thermal cycler; multiplex PEXT reaction of few (10) cycles, using unpurified amplification product as a template, to incorporate biotin into extended allele-specific primers and, finally, visual detection of the reaction products within minutes by the dipstick biosensor. The genotype is simply assigned by observing each pair of spots. For any particular mutation, a normal (wild-type) individual will produce red colour at the left spot only, whereas a case that is homozygous for a mutation will produce colour only at the right spot of the pair. Both spots will be red if the sample is heterozygous for the mutation. The entire protocol of the proposed method including PCR, primer extension reaction and the dipstick assay takes about 2 h.

5. Conclusions

The genotyping platform described in this work for the detection of "nondeletion" HBA1 and HBA2 variants resulting from point mutations or short deletions/insertions is characterized by the following advantages: (i) sequence specific allelic discrimination by means of primer extension reaction using exonuclease free DNA polymerase, (ii) spatial resolution of alleles on a solid support (diagnostic membrane) through hybridization (in flow) of generic sequences representing each allele, (iii) detection of alleles by naked eye, (iv) no need for multiple pipettings, incubations and washings and (v) no need for expensive instrumentation, consumables and highly trained personnel.

The proposed assay is particularly suited for small molecular-diagnostic laboratories with a limited budget and a small-to-medium sample volume. In addition this platform represents a very simple and useful genotyping tool to support gene scanning methods whenever nucleotide variations have to be specifically identified.

Funding

None.

Conflict of interest

The authors declare no conflict of interest.

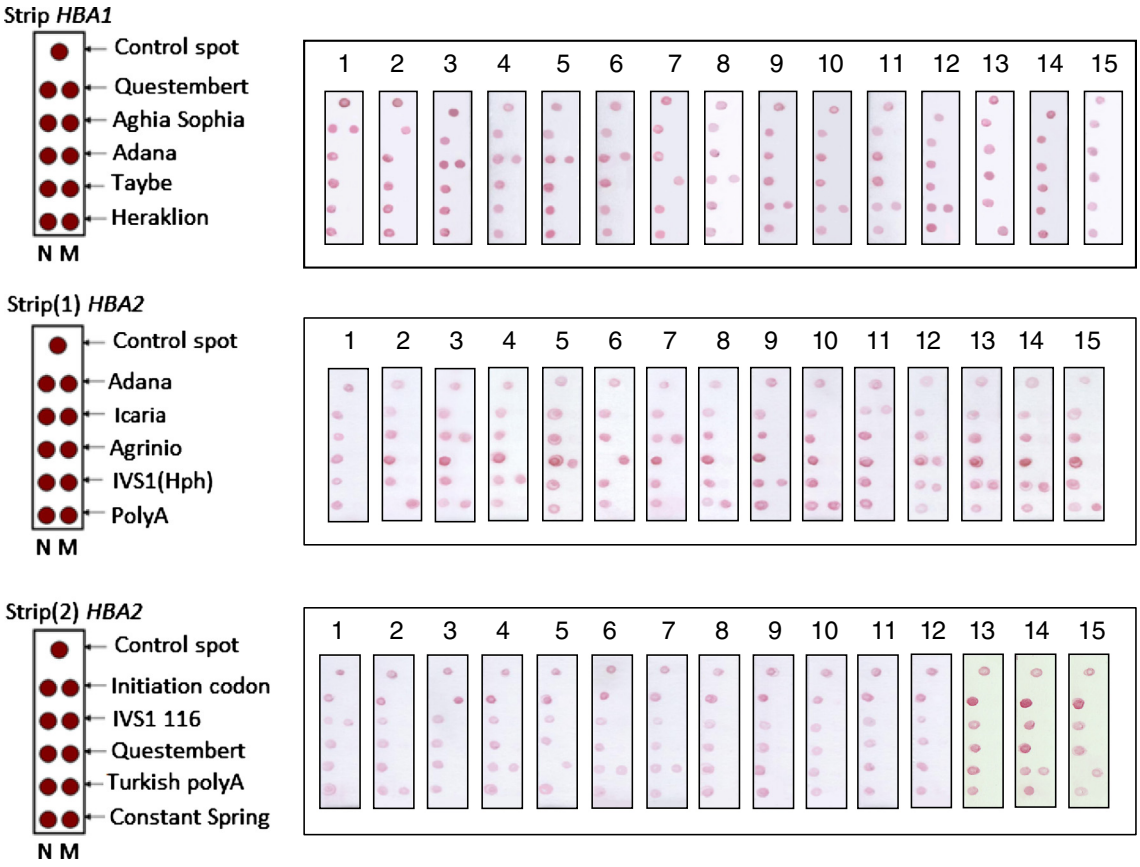

Fig. 4. Typical genotyping results from a series of samples for HBA1 and HBA2 mutations using three 10-plex primer extension reactions. The genotype for *HBA1* gene requires a 10-plex primer extension reaction and 1 strip (5 mutations, 10 alleles per strip). The full genotype for *HBA2* gene requires two 10-plex primer extension reactions and 2 strips (10 mutations, 20 alleles). For the position of each mutation-specific probe set in all subsequent samples see cartoon on the left side of the sample pictures. The 5 spots on the left side correspond to the normal alleles. The 5 spots on the right side correspond to the mutant alleles. The single spot at the top centre of the strip is a control spot that indicates the proper performance of the strip.

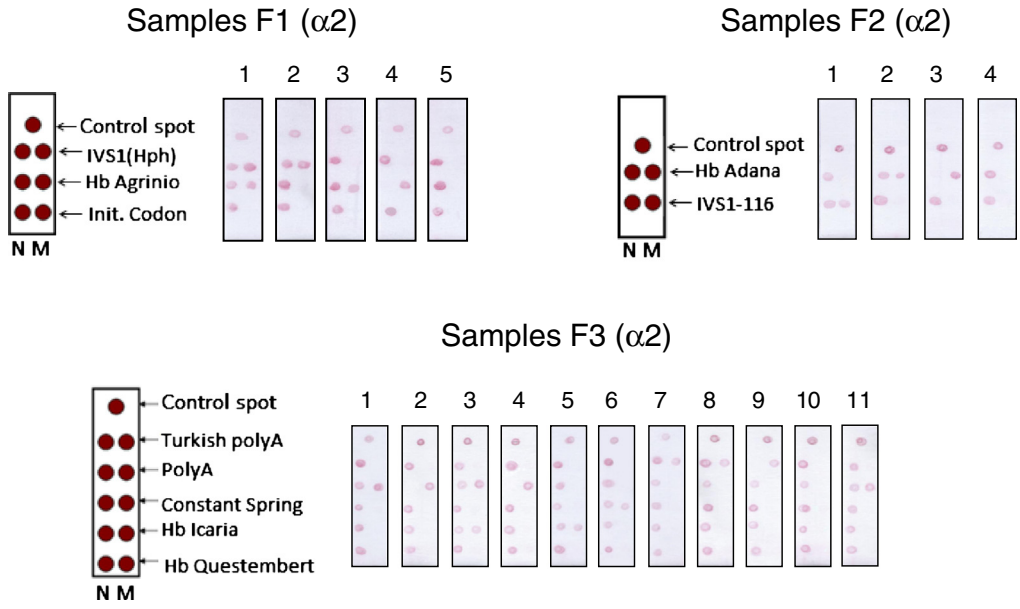

Fig. 5. The amplified *HBA2* gene fragment (1087 bp) was used as template for nested amplification of three separate fragments (F1, F2 and F3) flanking all of the 10 "nondeletion" α-thalassaemia HBA2 mutations. The length of the fragments was kept at around 200 bp and the HRM assays were performed using LightScanner Instrument (Idaho Technology). The same three fragments (for each sample) were genotyped by the proposed PEXT-dipstick assay using the corresponding allele-specific primers and the dipstick platform for nucleotide variations included in each fragment.

Appendix A. Supplementary data

Supplementary information is available at the Clinica Chimica Acta website.

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