

Validation of a reverse-hybridization StripAssay for the simultaneous analysis of common α -thalassemia point mutations and deletions

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Abstract

Background: α -Thalassemia is a worldwide disease and considered to be a major public health problem in countries within the so-called thalassemia belt. The complex genetics of α -thalassemias requires diagnostic methods with the capacity to screen rapidly and accurately for common causative mutations.

Methods: We developed and validated a reverse-hybridization assay (Alpha-Globin StripAssay) for the rapid and simultaneous detection of 21 α -globin mutations: two single gene deletions ($-\alpha^{3,7}$; $-\alpha^{4,2}$), five double gene deletions [$--_{MED}$; $--_{SEA}$; $--_{THAI}$; $--_{FIL}$; $-(\alpha)^{20,5}$], $\alpha\alpha\alpha^{anti-3,7}$ gene triplication, two point mutations in the $\alpha 1$ gene (cd 14 G>A; Hb Adana) and 11 point mutations in the $\alpha 2$ gene (initiation cd T>C; cd 19 -G; IVS1 -5nt; cd 59 G>A; Hb Quong Sze; Hb Constant Spring; Hb Icaria; Hb Pakse; Hb Koya Dora; polyA-1; polyA-2).

Results: Reliable genotyping of recombinant mutant clones and reference DNA samples was achieved by means of two corresponding test strips presenting parallel arrays of allele-specific oligonucleotides. The

entire procedure from blood sampling to the identification of mutations required less than 6 h, and hybridization/detection was manual or automated. The diagnostic potential of this Alpha-Globin StripAssay was carefully evaluated on 272 pre-typed samples in a multicenter validation study. In 96.14% of the cases, StripAssay typing was completely concordant with the reference methods.

Conclusions: The Alpha-Globin StripAssay proved to be a fast, easy-to-perform and reliable screening method to identify >90% of α -globin mutations in endemic areas worldwide.

Clin Chem Lab Med 2007;45:605–10.

Keywords: α -globin; genotyping; reverse hybridization; thalassemia.

Introduction

α -Thalassemia (α -thal) is characterized by a reduced or completely absent α -globin synthesis, which is mostly caused by the physical loss of one ($-\alpha$) or both ($--$) cis-linked α -globin genes, and less frequently by non-deletional mutations ($\alpha^T\alpha$ or $\alpha\alpha^T$). Carriers of α^+ -thal ($-\alpha$) or α^0 -thal ($--$) are at risk of having offspring with hemoglobin (Hb) H disease ($--/\alpha$) or the lethal Hb Bart's hydrops fetalis syndrome ($--/--$). Hb H disease demonstrates a marked genetic and phenotypic heterogeneity, ranging from mild anemia to a severe transfusion-dependent state (1–3). Its most common form is a combination of α^0 -thal with α^+ -thal, called the deletional type. In Southeast Asia, up to 35% of Hb H disease was reported to be of non-deletional type ($--/\alpha^T\alpha$), and such patients showed more pronounced anemia and reticulocytosis than those with three deleted α -globin genes (4, 5). Homozygosity or compound heterozygosity for non-deletional mutations in the $\alpha 2$ -globin gene ($\alpha^T\alpha/\alpha^T\alpha$) can lead to Hb H phenotypes with variable severity (6–8). The Middle East has a particularly high incidence of Hb H disease presenting with a mild to intermediate phenotype and involving homozygosity for the polyA-1 mutation or heterozygosity for polyA-1 combined with the $-\alpha^{3,7}$ single gene deletion (9, 10).

The technique most frequently used for identification of prevalent α -thal deletions in routine diagnosis is the single-tube multiplex gap-PCR (11–13). The rarer point mutations are typed either by reverse dot-blot analysis (RDB), by amplification refractory mutation systems (ARMS) or by restriction fragment length polymorphism (14–16). More recently, DNA arrays for α -globin mutations occurring in Southeast Asia have

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been developed, but are still far from being in common use (17, 18). The major pitfalls of using direct sequencing of specifically amplified DNA as a routine diagnostic technique are the cost and time of analysis.

As the spectrum and frequencies of mutations within populations can vary substantially, each molecular diagnostic laboratory has its own repertoire of techniques covering the important mutations encountered in its region. However, increasing migration and the resulting multi-ethnic mix in many cosmopolitan cities argue for testing a broader spectrum of mutations. Moreover, accurate and comprehensive diagnosis of α -globin genotypes is required for genetic counseling and, when necessary, for prenatal diagnosis.

We have designed a reverse-hybridization assay for the simultaneous screening of 21 deletional and non-deletional α -globin mutations in a single procedure. The Alpha-Globin StripAssay is based on a three-tube multiplex PCR for specific amplification of the full-length $\alpha 1$ and $\alpha 2$ genes, as well as common single and double gene deletions, followed by hybridization of the biotinylated amplification products to allele-specific oligonucleotide probes arrayed on test strips.

Materials and methods

DNA extraction and cloning

Genomic DNA was extracted from 50 μ L of anticoagulated peripheral blood using a commercially available spin-column-based DNA extraction system (Spin Micro DNA Extraction System, ViennaLab Diagnostics, Vienna, Austria). Alternatively, DNA from blood, chorionic villus sampling (CVS) or cord blood was extracted by a salting-out method (19). A total of 21 PCR-amplified mutant alleles of pretyped DNA samples were cloned into TA-cloning vectors (TOPO TA Cloning Kit, Invitrogen, Carlsbad, USA) and the presence of the respective mutation in the recombinant clones was confirmed by DNA sequencing. Plasmid DNAs were used as homozygous references for assay set-up and for quality control of the test strips.

Multiplex PCR

For each reaction, 5 μ L of DNA (~10–50 ng) was added to 20 μ L of amplification mix containing 75 mmol/L Tris-HCl (pH 8.8), 20 mmol/L $(\text{NH}_4)_2\text{SO}_4$, 0.01% Tween 20, 200 μ mol/L dNTPs, 2.5 mmol/L MgCl_2 and 1.33 U of Taq polymerase (SuperTaq, HT Biotechnology, Cambridge, UK). Concentrations of betaine (1.0–1.5 mol/L) and biotinylated primers were optimized empirically for each of three separate amplification mixes. Mix A1 comprised primers for specific amplification of the $\alpha 1$ gene and the $-\alpha^{3.7}$ deletion (20). Mix A2 contained primers for amplification of the $-\alpha^{4.2}$, $-\alpha^{20.5}$, $-\alpha^{\text{SEA}}$, $-\alpha^{\text{THAI}}$ and $-\alpha^{\text{FIL}}$ deletions (21). Mix B included primers for specific amplification of the $\alpha 2$ gene and the $\alpha\alpha^{\text{anti-3.7}}$ gene triplication (20). Primers for PCR control A in mix A2 and PCR control B in mix B acted as internal amplification controls (A, nt 3678–3973 of GenBank sequence Z69706; B, nt 3205–3506 of GenBank sequence Z84721). PCR was performed in 0.2-mL thin-walled tubes under the following cycling conditions: initial denaturation at 95°C for 5 min; three cycles at 97°C for 40 s, 64°C for 40 s and 72°C for 1 min 30 s; 37 cycles at 97°C for 40 s, 58°C for 40 s and 72°C for 1 min 30 s; and a final extension step at 72°C for 5 min.

Test strip preparation, reverse hybridization and detection

Preparation of test strips, reverse hybridization under stringent conditions ($45 \pm 0.5^\circ\text{C}$) and detection of specifically bound biotinylated DNA were carried out as previously described (22). A set of oligonucleotide probes that unambiguously discriminates between mutant and wild-type alleles or captures the breakpoint of deletions, as well as PCR controls A and B, were immobilized in parallel lines on two test strips, as shown in Figure 1. For genotyping, 10 μ L each of the biotinylated amplification products of mixes A1 and A2 were hybridized to test strip A, and 10 μ L of mix B products were hybridized to test strip B. The hybridization and detection procedure was performed either manually using thin-walled plastic trays and a shaking water bath at 45°C, or fully automated in a temperature-controlled test-strip processor (profiBlot IIT, TECAN, Männedorf, Switzerland).

StripAssay validation

Eight thalassemia centers from different regions (Siriraj Hospital, Bangkok, Thailand; Chronic Care Center, Beirut, Lebanon; Hôpital Henri Mondor, Creteil, France; King Faisal University, Dammam, Saudi Arabia; Department of Health, Dubai, UAE; KK Women's and Children's Hospital, Singapore; Social Welfare and Rehabilitation Sciences University, Tehran, Iran; Rudolfstiftung Hospital, Vienna, Austria) participated in the evaluation of the Alpha-Globin StripAssay. The centers contributed a total of 272 DNA samples randomly chosen from patients with low mean corpuscular volume (MCV; <85 fL), low mean cell hemoglobin (MCH; <27 pg), normal or slightly reduced Hb levels and normal HbA₂. Patient samples were tested in parallel using the StripAssay and in-house methods (multiplex gap-PCR, home-made RDB, ARMS-PCR, DNA sequencing, and mD_x^{TM} Alpha Gene 1 Assay, BioRad, Hercules, CA, USA) routinely used at the different thalassemia centers.

Results

Assay development

For simultaneous screening of 21 deletions and point mutations covered by the Alpha-Globin StripAssay, three multiplex PCRs were established. Amplicons of mix A1 comprised the entire coding region of the $\alpha 1$ gene and, in case of the presence of the $-\alpha^{3.7}$ deletion, an $\alpha 2/\alpha 1$ hybrid fragment specific for this mutation. Mix A2 amplified fragments across the breakpoints generated by the following deletions: $-\alpha^{4.2}$, $-\alpha^{\text{MED}}$, $-(\alpha)^{20.5}$, $-\alpha^{\text{SEA}}$, $-\alpha^{\text{THAI}}$ and $-\alpha^{\text{FIL}}$. Mix B covered all mutations along the $\alpha 2$ gene and produced an $\alpha 1/\alpha 2$ hybrid fragment in the presence of the $\alpha\alpha^{\text{anti-3.7}}$ mutation. In the case of homozygous or compound heterozygous deletions (e.g., $-\alpha^{3.7}/-\alpha^{3.7}$; $-\alpha^{4.2}/-\alpha^{\text{MED}}$), no α -globin PCR product was generated with mix B (Figure 1B, test strips 2 and 7). In samples negative for the $-\alpha^{4.2}$ deletion and double gene deletions, no junction fragments were produced with mix A2. To avoid unrecognized PCR drop-out in such cases, primers amplifying a small segment at the distal or proximal end of the α -globin gene cluster were included as PCR performance controls in mixes A2 and B, respectively. Matching oligonucleotide probes on test strips A and

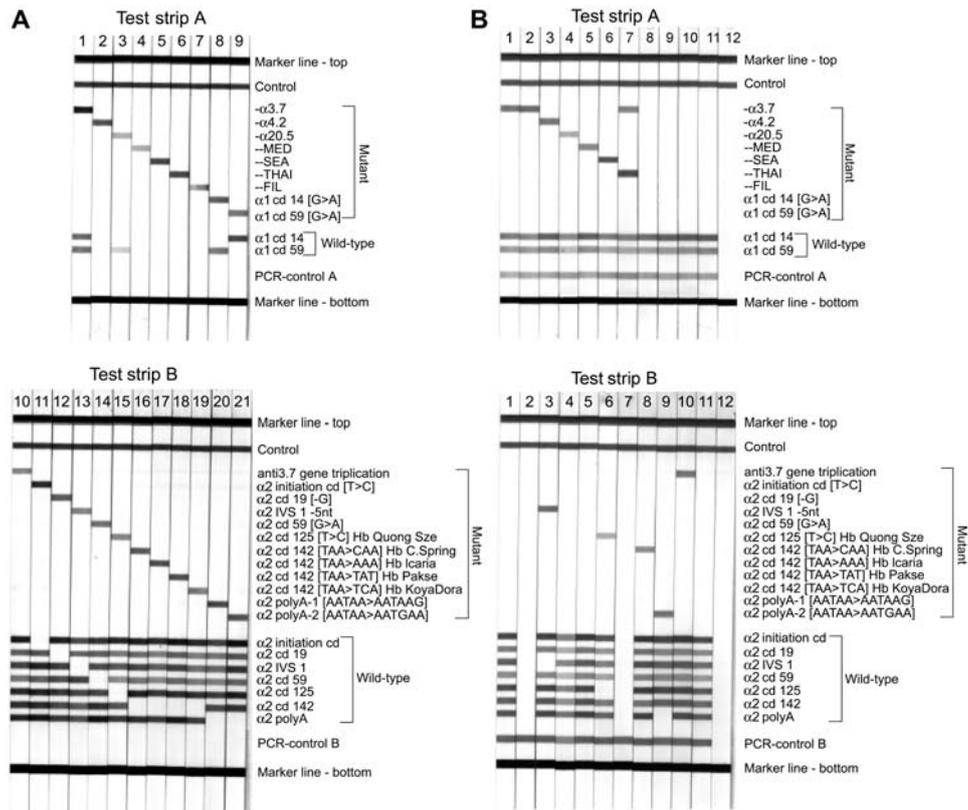


Figure 1 Staining patterns for test strips A and B after hybridization to various PCR products and subsequent color development.

(A) Results for 21 plasmid clones. Test strips A 1–9 show plasmid clones containing the $-\alpha^{3.7}$ and $-\alpha^{4.2}$ single gene deletions, the $-(\alpha)^{20.5}$, $--_{MED}$, $--_{SEA}$, $--_{THAI}$, $--_{FIL}$ double gene deletions, and the cd 14 G>A and Hb Adana point mutations in the α^1 gene, respectively. Clones for the $-\alpha^{4.2}$, $-(\alpha)^{20.5}$ (3'-breakpoint upstream of cd 59, thus staining positive for the wild-type probe), $--_{MED}$, $--_{SEA}$, $--_{THAI}$ and $--_{FIL}$ deletions contain junction fragments only. Test strips B 10–21 show plasmid clones for the $\alpha\alpha\alpha^{anti-3.7}$ gene triplication, the init cd T>C, cd 19 -G, IVS 1 -5nt, cd 59 G>A, Hb Quong Sze, Hb Constant Spring, Hb Icaria, Hb Pakse, Hb Koya Dora, polyA-1 Saudi type and polyA-2 Turkish type mutation in the α^2 gene, respectively. Note that PCR controls on test strips A and B are missing, as plasmid clones contained no sequences from the 5'- or 3'-end of the α -globin gene cluster. (B) Results for 11 genomic DNA samples. On test strips A/B, samples tested positive for mutations as follows: 1) $-\alpha^{3.7}/\alpha\alpha$; 2) $-\alpha^{3.7}/-\alpha^{3.7}$; 3) $-\alpha^{4.2}/\alpha^{IVS1-5nt}$; 4) $-(\alpha)^{20.5}/\alpha\alpha$; 5) $--_{MED}/\alpha\alpha$; 6) $--_{SEA}/\alpha^{HbOS}$; 7) $-\alpha^{3.7}/--_{THAI}$; 8) $\alpha^{HbCS}/\alpha\alpha$; 9) $\alpha^{polyA-2}/\alpha^{polyA-2}$; 10) $\alpha\alpha\alpha^{anti-3.7}/\alpha\alpha$; 11) $\alpha\alpha/\alpha\alpha$. Sample 12 is the negative (no template) control. Assay performance control was positive in all cases, PCR controls A and B stained positively with all samples, except for the negative control on test strip 12.

B indicated the presence of the PCR control fragments (Figure 1B).

To distinguish between mutations occurring in the highly homologous α^1 and α^2 genes, amplification products A1/A2 and B had to be hybridized to separate test strips. Probes on test strip A were designed to detect point mutations in the α^1 gene (cd 14 TGG>TAG and cd 59: Hb Adana GGC>GAC) and all of the gap-PCR products derived from single and double gene deletions. Test strip B contained a capture probe to indicate the presence of $\alpha\alpha\alpha^{anti-3.7}$ and specific oligonucleotide probes for point mutations and small deletions in the α^2 gene (initiation cd: ATG>ACG; cd 19: del G; IVS1: del TGAGG; cd 59: GGC>GAC; cd 125: Hb Quong Sze CTG>CCG; cd 142: Hb Constant Spring TAA>CAA; cd 142: Hb Icaria TAA>AAA, cd 142: Hb Pakse TAA>TAT; cd 142: Hb Koya Dora TAA>TCA; polyA-1: Saudi type AATAAA>AATAAG; polyA-2: Turkish type AATAAA>AATGAA). For genotype interpretation, the results of test strips A and B had to be combined for each sample. During assay development, the dis-

criminative capabilities of all mutant and wild-type probes were optimized and tested for lack of cross-reactivity using amplification products from mutant plasmid clones (Figure 1A). Staining patterns of various α -globin genotypes detected during assay validation are depicted in Figure 1B. The analysis of results for point mutations and small deletions followed the usual rules for StripAssays: in the absence of a mutation, only its wild-type probe stained positively; in heterozygotes, the corresponding wild-type and mutant signals were both visible; and in homozygotes, only the respective mutant probe was positive (Figure 1B test strips 11, 8 and 9). In the presence of large deletions, interpretation of the staining patterns was more complex, as either one or both α -globin genes were missing in cis or in trans. Homozygous or compound heterozygous single-gene deletions (e.g., $-\alpha^{3.7}/-\alpha^{3.7}$; $-\alpha^{4.2}/-\alpha^{3.7}$), as well as compound heterozygous single- and double-gene deletions (e.g., $-\alpha^{3.7}/--_{THAI}$) stained positively for the respective mutant probe and all the wild-type probes on test strip A, but lacked those on test strip B (Figure

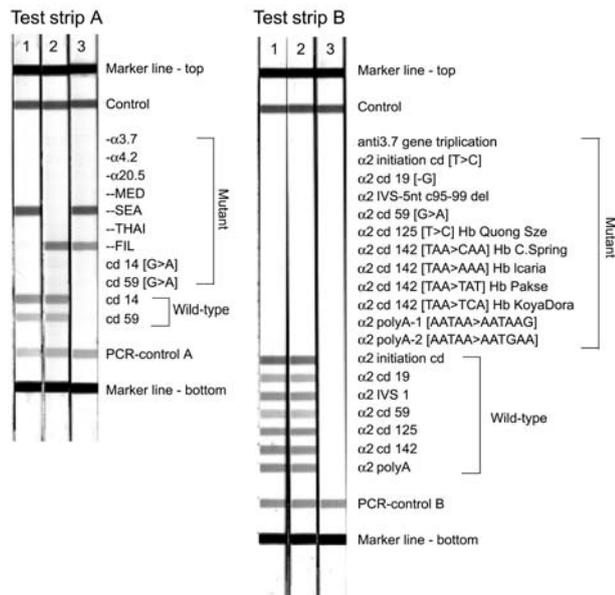


Figure 2 Genotyping results for fetal DNA and corresponding parental DNA samples.

Test strips A/B 1–2 show the staining patterns for parental blood samples ($--^{SEA}/\alpha\alpha$ and $--^{FIL}/\alpha\alpha$); test strips A/B 3 show the result for CVS DNA from the fetus ($--^{SEA}/--^{FIL}$).

1B, test strips 2 and 7). Compound heterozygous double-gene deletions missed all wild-type probes present on the two test strips (Figure 2, test strips 3). Since any of the single and double gene deletions covered by the StripAssay at least partially remove the $\alpha 2$ gene, the presence of α -globin signals on test strip B due to successful amplification of $\alpha 2$ served to indicate the heterozygous state when one of the deletions was present (Figure 1B, test strips 1 and 3–6; Figure 2, test strips 1 and 2). If combined with one of the gene deletions, heterozygous point mutations in the $\alpha 2$ gene appeared as hemizygous on test strip B (no wild-type signal; Figure 1B, test strips 3 and 6).

Assay validation

To evaluate the accuracy and practical utility of the StripAssay in α -thal genotyping, patient samples from the catchment areas of eight different thalassemia centers were investigated. A total of 272 DNA samples (268 derived from peripheral blood, 3 from CVS and 1 from cord blood), representing 61 different genotypes were tested in parallel using the Alpha-Globin StripAssay and the reference methods routinely used in these laboratories (Table 1). Among these DNA samples, all the 21 mutations covered by the Alpha-Globin StripAssay were observed.

Of the 544 wild-type or mutant α -globin alleles from our patient cohort, the results for 523 (96.14%) were completely concordant between the StripAssay and in-house methods. Discrepancies between the different methods were observed in 21 cases because: (a) 13 mutations could be detected by the StripAssay, but were not covered by in-house methods (10 \times IVS1–5nt, 1 \times Hb Constant Spring, 1 \times cd 19–G, 1 \times polyA-1); (b) oligonucleotide probes for four local mutations

were not present on the test strips; (c) rare nucleotide exchanges in the vicinity of the mutation site prevented binding of mutant and wild-type StripAssay probes in two samples; and (d) divergent genotyping results for two samples remained unresolved due to depletion of DNA.

Hb Q-Thailand ($\alpha 1$ cd 74 GAC>CAC), a mutation invariably linked to the $-\alpha^{4.2}$ deletion, could not be detected by the StripAssay due to the lack of an appropriate oligonucleotide probe on test strip A. This sample from Singapore was typed as being homozygous for $-\alpha^{4.2}$ instead of $-\alpha^{4.2}/-\alpha^{4.2Q}$. For the same reason, one Thai case of Hb Pak Num Po ($\alpha\alpha^{PNP}$, $\alpha 1$ cd 131+T) remained undetected by the StripAssay, and thus its genotype $--^{SEA}/\alpha\alpha^{PNP}$ was incompletely diagnosed as being heterozygous for the $--^{SEA}$ deletion only. Two DNAs carrying the $--^{SEA}$ deletion and either a mutation in the initiation codon ($\alpha^{A-G\alpha}$) or in the poly(A) site (α^{AATA--} α India type), both of which are not covered by the StripAssay, did not hybridize with mutant or wild-type probes specific for the initiation codon and the poly(A) region, respectively. A sample from Dubai, typed by an in-house method as heterozygous for $-\alpha^{3.7}$, was confirmed by the StripAssay, but lacked the cd 19 wild-type signal. Sequencing revealed a silent mutation leading to Hb Fontainebleau ($\alpha 2$ cd 21 GCT>CCT), which impaired hybridization, as it lies within the region encoded by the cd 19 capture probe. Sequencing of the $\alpha 2$ gene resolved the false-negative StripAssay result for a heterozygous polyA-2 mutation. In this sample from Singapore, recombination of the $\alpha 2$ 3'-end with the pseudo- α 3'-end resulted not only in an altered polyA site, but also in additional nucleotide exchanges in the binding site of the capture probes and hence in a loss of signal. Two ambiguously typed samples were both heterozygous for $-\alpha^{3.7}$ in the BioRad mD_x ™ Alpha Gene 1 Assay, but presented as homozygous $-\alpha^{3.7}$ and compound heterozygous $-\alpha^{3.7}/-\alpha^{4.2}$ in the Alpha-Globin StripAssay. The fact that we could specifically amplify the $\alpha 2$ gene in a heterozygous $-\alpha^{3.7}$ control sample but not in these two samples using a primer pair other than that of mix B corroborated the results of the StripAssay (data not shown).

The feasibility of using the Alpha-Globin StripAssay for prenatal diagnosis was examined using fetal DNA samples obtained from CVS and DNA extracted from cord blood. In all four cases investigated, both the in-house gap-PCR method and the StripAssay clearly identified the presence of Hb Bart's hydrops fetalis syndrome. Two cases were caused by a homozygous $--^{SEA}$ deletion, one case had a homozygous $-(\alpha)^{20.5}$ deletion, and one case revealed a compound heterozygous $--^{SEA}$ and $--^{FIL}$ deletion. Staining patterns for test strips hybridized to one of the CVS DNAs and its corresponding parental DNAs are shown in Figure 2.

Discussion

In this report we have described the set-up and multicenter performance validation of the Alpha-Globin

Table 1 Number of α -globin alleles identified with the Alpha-Globin StripAssay (SA) and in-house (IH) methods in 272 DNA samples.

Allele	StripAssay	In-house	Explanation and comments
$-\alpha^{3.7}$	154	153	One sample typed $\alpha\alpha$ by IH
$-\alpha^{4.2}$	31	29	One sample typed $\alpha\alpha$ by IH
$-\alpha^{4.2}$ HbQ-T	-	1	No mutant probe on test strip A, sample typed $-\alpha^{4.2}$ by SA
$-(\alpha)^{20.5}$	6	6	
..MED	9	9	
..SEA	51	51	
..THAI	5	5	
..FIL	3	3	
$\alpha\alpha\alpha^{\text{anti-3.7}}$	6	6	
$\alpha^{\text{initcdA}_G}\alpha$	1	1	No mutant probe on test strip B, loss of wt signal for initiation cd
$\alpha^{\text{initcdT}>C}\alpha$	1	1	
$\alpha^{\text{cd19}}\alpha$	2	1	One sample not tested for cd 19 -G by IH
$\alpha^{\text{cd19nd}}\alpha$	1	-	Unknown mutation determined by loss of wt signal for cd 19
$\alpha^{\text{IVS1-5nt}}\alpha$	14	4	10 samples not tested for IVS1 -5nt by IH
$\alpha^{\text{cd59}}\alpha$	4	4	
$\alpha^{\text{HbQS}}\alpha$	5	5	
$\alpha^{\text{HbCS}}\alpha$	11	10	One sample not tested for Hb CS by IH
$\alpha^{\text{HbIC}}\alpha$	1	1	
$\alpha^{\text{HbKD}}\alpha$	1	1	
$\alpha^{\text{HbPS}}\alpha$	3	3	
$\alpha^{\text{polyA-1}}\alpha$	2	1	One sample not tested for polyA-1 by IH
$\alpha^{\text{polyA-2}}\alpha$	6	7	One sample typed $\alpha\alpha$ by SA due to recombination of pseudo- α and $\alpha 2$ genes
$\alpha^{\text{polyA AATA-}}\alpha$	1	1	No mutant probe on test strip B, loss of wt signal for polyA
$\alpha\alpha^{\text{HbPNP}}$	-	1	No mutant probe on test strip A, sample typed $\alpha\alpha$ by SA
$\alpha\alpha^{\text{cd14}}$	1	1	
$\alpha\alpha^{\text{HbAD}}$	3	3	
$\alpha\alpha$	222	236	14 samples not tested for mutations by IH
Total	544	544	

Q-T, Q-Thailand; initcd, initiation codon; cd19nd, not determinable, mutation at or around cd 19; QS, Quong Sze; CS, Constant Spring; IC, Icaria; KD, Koya Dora; PS, Pakse; PNP, Pak Num Po; AD, Adana. Discrepancies in mutation detection between SA and IH are shown in bold.

StripAssay for the simultaneous screening of common α -globin gene deletions and point mutations. The high concordance of genotyping results (96.14%) with reference methods demonstrated the reliability and comprehensive coverage of the Alpha-Globin StripAssay needed to improve existing diagnostic strategies for α -thal. Based on published data, the new StripAssay covers approximately 90%–99% of α -globin mutations found in patients from Mediterranean, Middle Eastern and Southeast Asian countries (23–26). Particularly in regions with a high incidence of deletional and non-deletional mutations, this new assay represents a valuable tool as a rapid single test procedure in an otherwise often multi-step diagnostic approach.

Reverse-hybridization arrays for α -globin mutations spotted on membranes or glass slides have been reported in the past, but neither of these has been transferred into a commercially available assay format (14, 15, 17, 18). On the other hand, a commercially available kit (BioRad *mD_x*™ Alpha Gene 1 Assay) based on reverse hybridization in microtiter plates covers only two α -globin mutations ($-\alpha^{3.7}$, $\alpha\alpha\alpha^{\text{anti-3.7}}$) and thus is insufficient for the majority of thalassemia diagnostic centers. The Alpha-Globin StripAssay provides ready-to-use test strips and reagents, follows a simple protocol and relies on commonly available equipment, such as a thermocycler and water bath. Hybridization and enzymatic color detection can be

fully automated for increased sample throughput using commercial temperature-controlled test strip processors. The Alpha-Globin StripAssay requires only small amounts of DNA (approx. 50 ng), which is of particular importance for use in prenatal diagnosis.

Unlike more labor-intensive and technically demanding methods, such as denaturing HPLC and/or DNA sequencing, the Alpha-Globin StripAssay is designed exclusively for the detection of known mutations, which are represented on the test strips by suitable capture probes. Other α -globin deletions, rearrangements or point mutations cannot be diagnosed by this type of assay. At best, in combination with a deletion or in the homozygous state, the presence of an unconsidered mutation located within the sequence spanned by capture probes can be indicated by the loss of both mutant and wild-type signals on the test strip. The changes in staining patterns due to co-localizing mutations (e.g., initiation cd $\alpha^{\text{A-G}}\alpha$, polyA $\alpha^{\text{AATA-}}$) further proved the discriminative power of oligonucleotides selected as allele-specific probes for mutant and wild-type alleles. The absence of appropriate oligonucleotides for some rare mutations on the test strips led to incomplete or incorrect typing of DNA samples. An inherent limitation of the StripAssay is its inability to distinguish between homozygous and heterozygous α -globin gene triplication ($\alpha\alpha\alpha^{\text{anti-3.7}}$). In both conditions, the normal α -globin genes and the extra hybrid $\alpha 1/\alpha 2$ gene on the

crossover chromosome(s) will be amplified, and their PCR products will hybridize to the respective probes on the test strips. However, this ambiguity holds true for any PCR-based detection method. If required, discrimination between homo- and heterozygosity for $\alpha\alpha^{\text{anti-3,7}}$ can be achieved by Southern Blot analysis.

To conclude, the Alpha-Globin StripAssay has the potential to allow very efficient α -thal genotyping, especially in combination with a more comprehensive second-line technique such as DNA sequencing.

Acknowledgements

The authors are grateful to Bettina Rauscher (Vienna), Amir H. Taromchi (Tehran), Guek-Peng Tan (Singapore), Worrawut Chinchang (Bangkok), Iqbal Yousuf Jaber (Dubai) and to the many other technical assistants involved in this study for their excellent support.

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Received December 19, 2006, accepted February 15, 2007