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Detection of four β-thalassemia point mutations in Iranians using a PCR-ELISA genotyping system

Pooria Gill[a,*], Mehdi Forouzandeh[b], Naser Eshraghi[c], Mostafa Ghalami[d], Majid Safa[e], Mohammad-Reza Noori-Daloii[f]

[a]*Research Center for Molecular Biology, Baqyiatallah Medical Sciences University, Tehran, Iran*
[b]*Department of Medical Biotechnology, Faculty of Medical Sciences, Tarbiat Modarres University, Tehran, Iran*
[c]*Deputy of Health, Ministry of Health Care and Medical Education, Tehran, Iran*
[d]*Laboratory Part of Center of Disease Control (CDC), Deputy of Health, Ministry of Health Care and Medical Education, Tehran, Iran*
[e]*Department of Hematology, Iran University of Medical Sciences and Health Care, Tehran, Iran*
[f]*Department of Medical Genetic, Faculty of Medical Sciences, Tehran University of Medical Sciences and Health Care, Tehran, Iran*

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Abstract

Development of molecular techniques with analytical capability of mutation detection can realize the medical diagnosis of diseases and improve people's health. β-Thalassemia is one of the most prevalent genetic disorders in Iran and using a simple and rapid test in laboratories for the mass screening and prenatal diagnosis is essential. Here, we described a simple method for rapid detection of four common β-thalassemia point mutations in Iranians (IVS-II-1 (G→A), IVS-I-5 (G→C), FSC 8/9 (+G), IVS-I-110 (G→A)) using a PCR-ELISA genotyping system. After DNA isolation from whole blood, a segment of β-globin gene was amplified by DIG-labeling PCR. The DIG-labeled PCR amplicons were denatured and added to biotinylated normal probe (for normal gene allele) and mutant probe (for mutant gene allele). The hybrids were detected by colorimetric ELISA method. The optical densities obtained using normal and mutant probes with heterozygous PCR products were very similar. The optical densities obtained using mutant probes were higher than normal probes with homozygous PCR products. In vice versa, the optical densities obtained using normal probes were higher than mutant probes with normal PCR products. All the results demonstrated that the PCR-ELISA has similar specificity in comparison to the amplification refractory mutation system.

Keywords: PCR-ELISA; Point mutations; β-Thalassemia; ARMS

1. Introduction

Early methods for the detection of point mutations were based on restriction fragment length polymorphisms (RFLP) analysis [1] and the effects of base-pair changes on DNA fragment melting temperatures (denaturing gradient gel electrophoresis) [2]. More recently, detection techniques have utilized allele-specific oligonucleotide (ASO) hybridization [3,4], the single-stranded conformational polymorphism [5], amplification refractory mutation system (ARMS) [6,7], primer-guided nucleotide incorporation assays [8,9], oligonucleotide ligation assays [10,11], real-time PCR [12,13] and DNA microarray technology [14,15].

The technique of differential hybridization to ASO probes has been widely used. Under high stringency conditions, synthetic DNA probes will only anneal to their complementary target sequences in the sample DNA if they are perfectly matched, with a single base-pair mismatch sufficient to prevent formation of a stable probe-target duplex [16–18]. PCR-amplified genomic DNA products are applied to nylon filters as a series of "dot-blot" to which radiolabeled ASOs are hybridized [4]. The reverse approach can also be used with ASO probes fixed to membrane support [19] or in a microtiter plate format

*Corresponding author. Tel.: +98 2122944230; fax: +98 2122944230.
E-mail address: pooriagill@yahoo.com (P. Gill).

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using colorimetric ELISA-based detection was developed that provided highly sensitive and quantitative detection [20,21]. The "enzyme-linked immunosorbent assay of polymerase chain reaction" (PCR-ELISA) method amplifies the number of copies of a DNA segment from a sample of genomic DNA by PCR with the incorporation of digoxigenin-11-dUTP. Samples are analyzed in a microtiter plate by denaturation and hybridization to biotinylated allele-specific capture probes bound to streptavidin-coated plates. The hybridized DNA is detected by ELISA, using anti-digoxigenin antibody horseradish proxidase conjugate and the colorimetric substrate, 2,2′-azino-di-(3-ethyl-benzthiazulinsulfonate) (ABTS) [21].

Here, we described an evaluation of a PCR-ELISA genotyping system in comparison to traditional methods (DNA sequencing and ARMS) to detect the four common (nearly 60%) β-thalassemia point mutations (IVS-II-1 (G→A), IVS-I-5 (G→C), FSC 8/9 (+G), IVS-I-110 (G→A)) in Iranians [22].

2. Materials and methods

2.1. Clinical specimens

The whole blood of the donor patients (heterozygote and homozygote) were provided from Central Laboratory of Deputy of Health (Tehran University of Medical Sciences and Health Care, Tehran, Iran) in accordance with the ethical standards of the Helsinki Declaration of 1975 as reflected in the guidelines of the Medical Ethics Committee, Ministry of Health, I.R. Iran.

2.2. DNA extraction

DNA extraction was performed using DNG™-plus solution (CinnaGen Inc.) protocol. Briefly the DNG™-plus solution was pre-warmed by placing it in 37 °C for 20 min. One hundred microliter of whole blood was mixed with 700 μl of DNG™-plus and vortexed for 15–20 s. Subsequently, 500 μl of isopropanol was added and gently mixed by inversion (10 times). Then the sample was centrifuged (Genfuge, Eppendorf, Germany) at 10,000 × *g* for 10 min. The tube was gently inverted and placed on a tissue paper for 2–3 s. One milliliter of 75% solution of ethanol was added to the pellet, mixed gently by about 10 times inversions and centrifuged (Genfuge, Eppendorf, Germany) at 10,000 × *g* for 5 min (this step could be repeated once more). The ethanol was poured off completely and the pellet was dried at 65 °C for 5 min. The DNA was dissolved in 50 μl of sterile distilled water by gentle shakes and placed at 65 °C for 5 min. The materials were pelletted by spin (Genfuge, Eppendorf, Germany) for 30 s at 1000 × *g* and the supernatant containing purified DNA was employed for polymerase chain reactions.

2.3. Oligonucleotides

The sequences of the primers [23] used in this study were as follows: BU6, 5′-GGCAGGAGCCAGGGCTG-3′ and BD7, 5′-CCCATTCTAAACTGTACCCTG-3′. The primers were synthesized by M.W.G Biotech Company (Germany). This pair of primers amplifies a 480 bp fragment of the β-globin gene that includes the region in which the mutations have been described (Fig. 1). The sequences of the allele-specific probes were as Table 1 oligomers. These oligomers were synthesized by M.W.G Biotech Company (Germany) and modified with 5′ Biotin-nucleotides [24].

2.4. Amplification refractory mutation system

Mutant and normal alleles of the four β-thalassemia mutations (IVS-II-1 (G→A), FSC 8/9 (+G), IVS-I-5 (G→C), IVS-I-110 (G→A)) were confirmed by using ARMS from 10 normal individuals and 80 patients with heterozygous or homozygous β-thalassemia (Table 1) [25,26].

2.5. DIG-labeling PCR

PCR amplification was carried out in a total of 25 μl final volume containing 0.25 μM BU6 and 0.25 μM BD7; 1 × Taq DNA polymerase buffer (Biotools); 0.2 mM concentrations each of dATP, dCTP, dGTP, 0.19 mM dTTP and 0.01 mM digoxigenin-11-dUTP (Roche); 2.5 U of Taq DNA polymerase (Biotools); and 150 ng of prepared DNA. Amplification was initiated by denaturing the sample for 5 min at 95 °C, followed by 33 cycles of 30 s at 94 °C, 30 s at 57 °C and 1 min at 72 °C. Finally, samples were held at 72 °C for 10 min to ensure complete extension of all PCR products [27].

2.6. PCR-ELISA DIG detection

The feasibility of the hybridization and enzyme-linked immunosorbent assay was demonstrated, using duplicate samples of DIG-labeled PCR product from a normal individual, a patient homozygous for the IVS-II-1 (G→A) mutation, an IVS-II-1 (G→A) heterozygote carrier person, a patient homozygous for the FSC 8/9 (+G) mutation, an FSC 8/9 (+G) heterozygote carrier person, a patient homozygous for the IVS-I-5 (G→C) mutation, an IVS-I-5 (G→C) heterozygote carrier person, a patient homozygous for the IVS-I-110 (G→A) mutation, and an IVS-I-110 (G→A) heterozygote carrier person as the control cases. These samples had been confirmed using DNA sequencing (as gold standard) and ARMS technique. The DIG-labeled PCR products were detected according to the protocols received from Roche Applied Sciences (a PCR-ELISA DIG detection kit, Roche, Germay). In detail, 2.5 μl of each DIG-labeled PCR products was added to 10 μl denaturing solution and incubated at room temperature for 10 min. The denatured PCR product was hybridized in solution phase with 112.5 μl of hybridization solution contained 5 μM of specific 5′-biotinylated probe (Table 1). The mixture was then

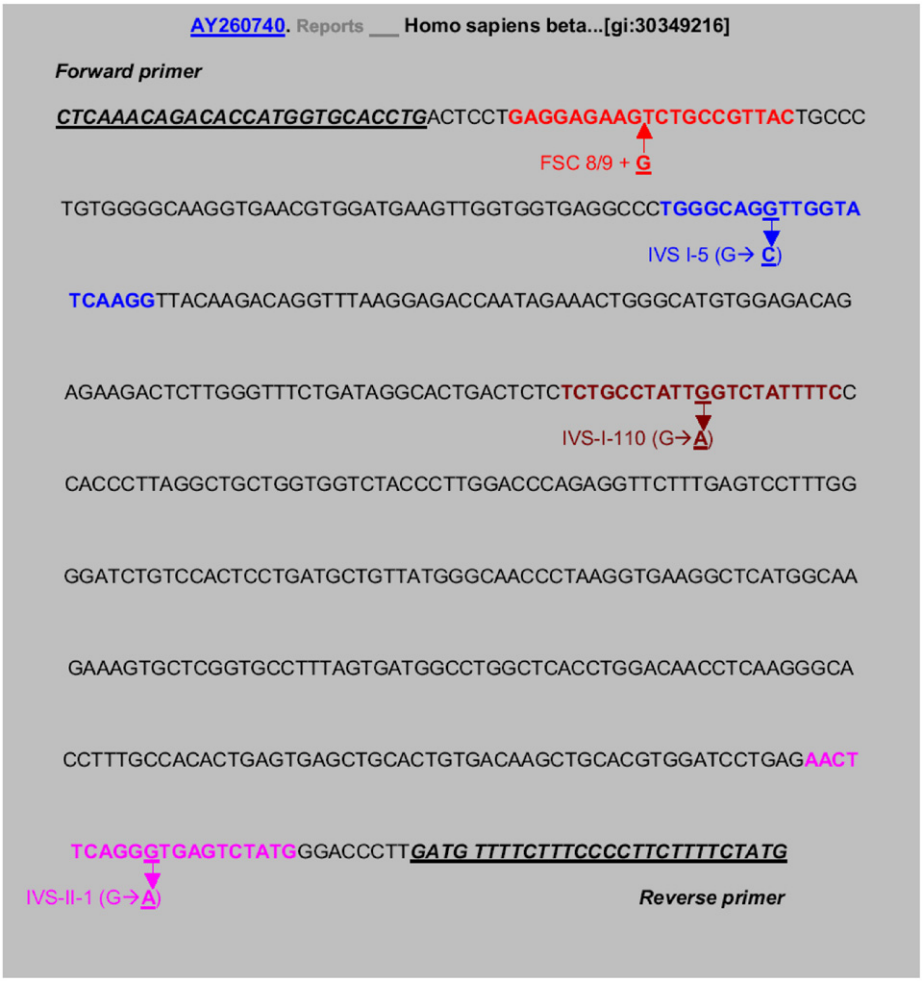


Fig. 1. Human β-globin gene segment amplified with forward and reverse primers and the position of four most common Iranian β-thalassemia point mutations. BM: mutant probe, BN: normal probe, G, C, A, A: the position of point mutation according to data of Table 1.

Table 1
The allele-specific β-globin gene oligomer probes

Name	Sequence	T_m [a]	T_h [b]	Name of mutation
BN2	AACTTCAGGGTGAGTCTATG	55.3	53	IVS-II-1 (G→A)
BM2	AACTTCAGGATGAGTCTATG	53.2		
BN3	TCTGCCTATTGGTCTATTTTC	58.8	56	IVS-I-110 (G→A)
BM3	TCTGCCTATTAGTCTATTTTC	56.7		
BN4	TGGGCAGGTTGGTATCAAGG	59.6	59	IVS-I-5 (G→C)
BM4	TGGGCAGGTTGCTATCAAGG	59.9		
BN5	GAGGAGAAGTCTGCCGTTAC	60.4	59	FSC 8/9 (+G)
BM5	GAGGAGAAGGTCTGCCGTTA	61.4		

[a] T_h: hybridization temperature obtained in practice from the 2.7 section protocol and Fig. 2 plots.

[b] T_m: melting temperature (the temperature at which 50% of the oligonucleotides are duplexes) of the oligonucleotide calculated using the nearest-neighbor thermodynamic values for DNA [26]: $T_m = \Delta H/(A + \Delta S + R \times \ln[C/4]) - 273.5 + 16.6 \times \log[\text{salt}]$. ΔH (cal/mole) is the sum of the nearest-neighbor enthalpy changes for DNA helix formation (<0). A (cal/°C/mole) is a constant for helix initiation, which is equal to −10.8 cal/°C/mole for non-self-complementary sequences and −12.4 for self-complementary sequences. ΔS (cal/°C/mole) is the sum of the nearest-neighbor entropy changes for helix formation (<0). R is the molar gas constant (1.987 cal/°C/mole). C is the oligonucleotide concentration. [salt] is the salt concentration.

immediately transferred to streptavidin-coated microtiter plates. In order to increase the stringency of the probe attachment to the single-stranded PCR amplicons, the incubation was performed at the specific hybridization temperature in a hybridizer oven (Techne, USA) for 1 h. Non-specific binding was measured by adding an equal volume of distilled water instead of denatures PCR product to the hybridization solution contained the probe. For removing the extra PCR-probe complexes, the wells were washed five times with washing solution. Then the immobilized PCR products were labeled by incubating 0.2 mU of anti-digoxigenin antibody HRP conjugate in 100 μl conjugate buffer for 30 min at 37 °C. After removing the unbound antibodies by washing, the PCR products were visualized with the ABTS substrate solution (15 min at 37 °C). The optical densities were measured at 405 nm with ELISA reader instrument [28–30].

2.7. Specificity optimization of allele-specific probes

The optimal-hybridization temperature for the specific attachment of each normal probe and mutant probe (Table 1) to a specific gene allele was examined by heating

from 37 °C to the highest possible temperature (up to probe melting temperature) in the hybridization step of PCR-ELISA DIG detection procedure [29,30].

2.8. Specificity assessments of PCR-ELISA of homozygote and heterozygote samples

After setting the PCR-ELISA system, the specificity of the system was evaluated by repeating the tests on 10 samples of each normal, heterozygous, and homozygous β-thalassemia mutations (IVS-II-1 (G→A), FSC 8/9 (+G), IVS-I-5 (G→C), IVS-I-110 (G→A)).

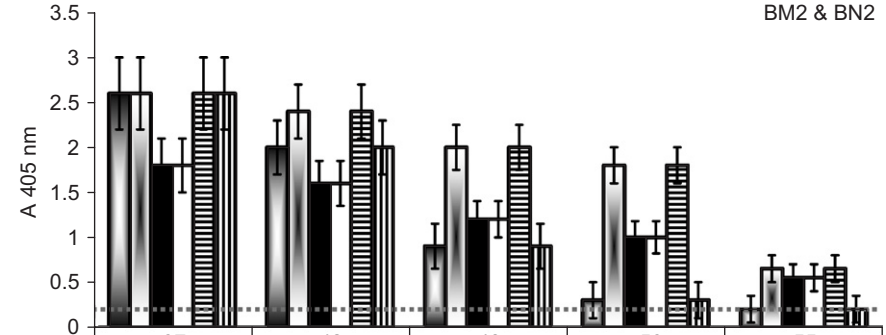
3. Results

3.1. Specificity of primers in DIG-labeling PCR

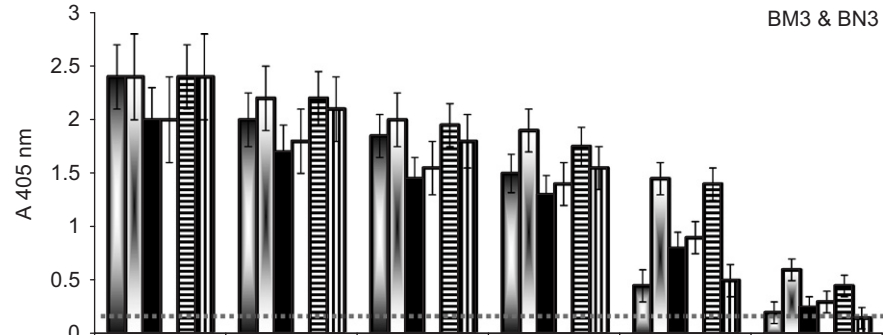
The 480 bp fragment of β-globin gene sequence was amplified specifically by employing DIG-labeling PCR. The results of 90 samples from normal individuals, patients with the homozygote known mutations and heterozygote carriers with known mutations demonstrated that the reactions were efficiently performed.

3.2. Specific temperature for probes

The results showed that the specific temperature for the detection of mutant and normal alleles was similar to the melting temperature (T_m) of the specific oligomer probes (Fig. 2). The results indicated that the optical densities obtained from mutant probes (BM2, BM3, BM4 and BM5) were very similar to the probes of normal alleles (BN2, BN3, BN4 and BN5) in the carriers with heterozygote mutations at the specific hybridization temperature. The optical densities of mutant probes were higher than normal probes in the patients with homozygote mutations. In normal individuals, the optical densities of normal probes were higher than mutant probes. The non-specific-binding-index

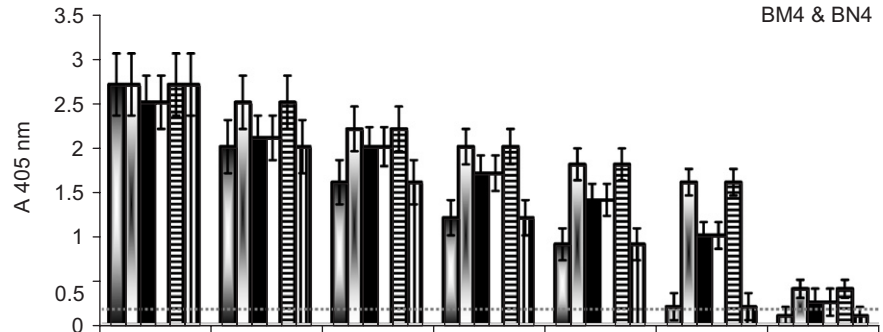


Temperature	37	42	48	53	55
Series1	2.6	2	0.9	0.3	0.2
Series2	2.6	2.4	2	1.8	0.65
Series3	1.8	1.6	1.2	1	0.55
Series4	1.8	1.6	1.2	1	0.55
Series5	2.6	2.4	2	1.8	0.65
Series6	2.6	2	0.9	0.3	0.2

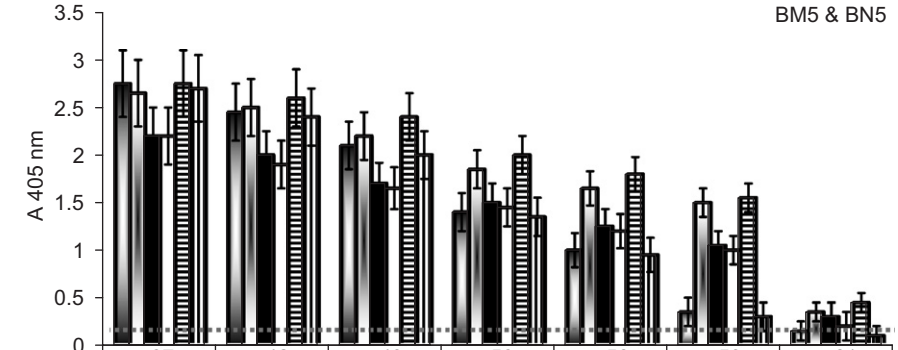


Temperature	37	42	46	53	56	59
Series1	2.4	2	1.85	1.5	0.45	0.2
Series2	2.4	2.2	2	1.9	1.45	0.6
Series3	2	1.7	1.45	1.3	0.8	0.25
Series4	2	1.8	1.55	1.4	0.9	0.3
Series5	2.4	2.2	1.95	1.75	1.4	0.45
Series6	2.4	2.1	1.8	1.55	0.5	0.15

Fig. 2. Various heat temperatures for specific hybridization of the probes. (- - -) Non-specific binding (NSB); series 1, 2→normal sample; series 3, 4→heterozygote carrier sample; series 5, 6→homozygote patient sample. The plotted results are the averages of duplication analysis.



Temprature	37	42	46	52	56	59	61
Series1	2.7	2	1.6	1.2	0.9	0.2	0.1
Series2	2.7	2.5	2.2	2	1.8	1.6	0.4
Series3	2.5	2.1	2	1.7	1.4	1	0.25
Series4	2.5	2.1	2	1.7	1.4	1	0.25
Series5	2.7	2.5	2.2	2	1.8	1.6	0.4
Series6	2.7	2	1.6	1.2	0.9	0.2	0.1



Temperature	37	42	49	53	56	59	61
Series1	2.75	2.45	2.1	1.4	1	0.35	0.15
Series2	2.65	2.5	2.2	1.85	1.65	1.5	00.35
Series3	2.2	2	1.7	1.5	1.25	1.05	0.3
Series4	2.2	1.9	1.65	1.45	1.2	1	0.2
Series5	2.75	2.6	2.4	2	1.8	1.55	0.45
Series6	2.7	2.4	2	1.35	0.95	0.3	0.1

Fig. 2. (*Continued*)

(NSB) value for the PCR-ELISA system was calculated and expressed as mean of four optical density values from negative wells. The NSB value was generally within the range of 0.2 ± 0.1.

3.3. Specificity of PCR-ELISA

According to the results described in the previous section, the hybridization specific temperature for the mutant and wild probes were determined. The specificity was tested with 80 samples of known mutations and 10 samples with normal gene alleles (ARMS detected) by the PCR-ELISA genotyping system (with the optimal T_h for specific hybridization of the probes). The results of the test were 100% in accord with earlier reports of ARMS-typed system and the duplicates of each test were perfectly reproducible (mean $\pm$ S.E.M. and P-value < 0.05). The mean optical density ratios of mutant to normal probes were higher than 5.49 ± 1.25 OD units for the samples with homozygote mutations and the mean optical density ratios of mutant to normal probes were similar to 0.98 ± 0.08 OD units for the carrier samples with heterozygote mutations. The mean optical density ratios of mutant to normal probes were lower than 0.20 ± 0.06 OD units for normal samples.

4. Discussion and conclusions

Molecular biology tools are needed to improve current methodologies and develop new approaches for detecting gene mutation in human diseases [31]. Differential hybridization with sequence-specific oligonucleotide probe has become a widely used technique for the detection of genetic mutations and polymorphisms [32–34]. PCR-ELISA is one of the approaches of hybridization phenomenon that can be used for specific detection of point mutations [33]. This study reports the use of PCR-ELISA genotyping system, using biotinylated probes, to detect four point mutations responsible for β-thalassemia in Iranians. We examined 90 whole bloods with known point mutations (ARMS

detected) using a PCR-ELISA. The assay has many advantages when compared to the other PCR-based methods. The assay can be run and the results obtained within 3 h compared to approximately 5 h with the PCR-RFLP and overnight with the DNA sequencing method. Another major advantage of this assay is that the PCR amplicons are analyzed using a colorimetric assay rather than gel electrophoresis and ethidium bromide staining (e.g., in PCR-RFLP and ARMS), thus significantly reducing the risk of mutagen-staining materials and DNA contamination through laboratory contamination. However, to avoid potential carryover contamination, careful adherence to the protocol, including bleach decontamination of reaction tubes and work surfaces, is required. The PCR-ELISA combines the sensitivity of nucleic acid amplification and the high specificity of hybridization protocols with a non-isotopic quantification method that is itself highly sensitive and specific. Obtained results demonstrated the feasibility of determining the genotype of the DNA sample by increasing the hybridization temperature for the probe attachment to specific target gene allele. The technique was developed to genotype four β-thalassemia point mutations within homozygote, heterozygote and normal samples and the specificity of the test was determined by comparison to the results of ARMS. This showed that the typing method was as specific as the ARMS for detection of point mutations in the β-globin gene region and it could be used simply and rapidly rather than ARMS for analysis more samples. The PCR-ELISA genotyping system that we described, has utilizes commonly available reagents and equipment and is simple to perform, requiring only a basic understanding of molecular techniques. The entire procedure, including DNA extraction, PCR amplification, and ELISA detection, can be performed in a single working day, and the results can be analyzed objectively, thereby eliminating the possibility of misinterpretation by different investigators [34]. The system provides a rapid means of screening for the common β-thalassemia mutations in Iranians and will therefore have an important impact in the future control of β-thalassemia within Iranian population [35–37].

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