Asian Biomedicine Vol. 6 No. 1 February 2012; 105-109 DOI: 10.5372/1905-7415.0601.134

Brief communication (Original)

Limit of sensitivity for melting curve screening for α-thalassemia

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Background: Thalassemia is a major inherited disease in Thailand, with a large number of people being either directly affected or carrying the trait. The Southeast Asian α-thalassemia 1 deletion (--^{SEA}) is the most common α-thalassemia 1 type, with a high number of carriers in Thai population. Individuals who carry the deletion have the potential for having offspring with Hb Bart's hydrops fetalis, the most severe thalassemia syndrome. Given the incidence of this disease, screening for thalassemia is an important tool in diagnosis and many methods have been developed.

***Objectives*:** A single tube real-time PCR methodology with a melting curve analysis method has been recently developed, and this work sought to determine the limit of sensitivity of this technique to determine whether it is possible to optimize this methodology and apply it to the single cell level for application in preimplantation genetic diagnosis screening programs.

***Methods*:** DNA was extracted from whole blood or isolated peripheral blood mononuclear cells of normal volunteers as well as α-thalassemia 1 (--^{SEA}) carriers. Sensitivity of the melting curve analysis was established with serial dilutions of DNA down to the picogram (pg) level.

***Results*:** The melting curve analysis as previously established was sensitive down to 2 ng of DNA. Further optimization increased sensitivity down to 200 pg, but discrimination of a normal allele from an α-thalassemia 1 (--^{SEA}) allele below this level of DNA was not achieved.

***Conclusion*:** While advantageous in routine screening programs, real time PCR coupled with a melting curve analysis is not currently suitable for adaptation to pre-implantation diagnosis for alpha thalassemia.

***Keywords*:** Hb Bart's hydrops fetalis, PCR, PGD, thalassemia

Thalassemia is a hereditary hematological disorder characterized by the defective production of globin chains as a consequence of a wide range of underlying genetic abnormalities and is characterized by a mild to severe anemia [1]. Thalassemia is extremely common in many parts of Southeast Asia, including Thailand where as much as 1% of the population is affected with thalassemic diseases [2]. Thalassemia can be classified into two major types depending on the globin chains that are defective, namely α-thalassemia and β-thalassemia, and α-thalassemia is a heterogeneous group of inherited disorders characterized by the reduced or absent synthesis of α-globin chains [1]. The majority of α-thalassemia results from α-globin gene deletions, and α-thalassemia is further divided into α-thalassemia 1 and α-thalassemia 2 based on the number of α-globin genes that are missing. Thus α-thalassemia 1 is characterized by the deletion or loss of both of the two α-globin genes on chromosome 16, while α-thalassemia 2 is characterized by the loss of only one α-globin gene. The prevalence of α-thalassemia in Thailand ranges from 3 to 30% [3] and the most common type of α-thalassemia 1 in Asian populations is the Southeast Asian type (--^{SEA}) in which a deletion of approximately 20 kb removes both α-globin genes but leaves the ζ- and ψζ-globin genes intact [4]. The most severe form of α-thalassemia disease is the homozygous genotype

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of α-thalassemia 1 (--/--) called Hb Bart's hydrops fetalis, in which no functioning α-globin genes are present [1]. This is a lethal disease in which the fetuses will die either in utero or soon after birth and 75% of mothers will develop toxemia during pregnancy. Couples both carrying α-thalassemia 1 have a 25 % chances that conception will result in a case of Hb Bart's hydrops fetalis.

To prevent such occurrences, several strategies have been developed, such as genetic counseling, prenatal diagnosis (PND) that results in the termination of the affected fetus, and more recently pre-implantation genetic diagnosis (PGD) that avoids the need to terminate affected pregnancies through the identification and transfer of only unaffected embryos established from in vitro fertilization (IVF) programs [5]. Despite the widespread prevalence of the α-thalassemia 1 trait in Southeast Asia there are few reports in the literature of PGD methodologies for screening for α-thalassemia [6-9]. Those studies that do report on PDG screening for α-thalassemia are undertaken primarily with either multiplex PCR or fluorescent gap PCR on single cell blastomeres and all methodologies require multiple handling steps for analysis. A recent report [10] presented a novel melting curve analysis methodology for diagnosis of α-thalassemia which eliminates the requirement for post-PCR gel electrophoresis, reducing the number of steps required for analysis. This study sought to determine the limit of sensitivity of this technique for detection of the common, α-thalassemia 1 $--^{SEA}$ deletion type, to determine its potential applicability to PGD of α-thalassemia.

Materials and methods

Samples

Following Ethics Committee approval from the Mahidol University Institutional Review Board and individual informed consent, 3 ml of peripheral blood was taken from known genotype α-thalassemia 1 trait and normal volunteers and genomic DNA was prepared using the Puregene Blood kit (QIAGEN, Valencia, CA) and serially diluted down to picogram (pg) DNA levels. Human peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Histopaque-1077 (density 1.077±0.001 g/ml) and the negative fraction from a direct $CD34^+$ progenitor cell isolation kit with MACS isolation system were collected as PBMCs. DNA was extracted from a defined number of cells using the Puregene Blood kit (QIAGEN, Valencia, CA) according to manufacturer's protocol.

Conventional PCR

Monoplex amplification of normal α2-globin gene was undertaken using the alpha-F (5'-TCCTTGCACC GGCCCTTCCT-3') and alpha-R (5'-GTCCTTGGT CTGAGACAGGTAA-3') primers as previously described [10]. The reaction was undertaken in a final volume of 10 µl containing 1x PCR buffer (Promega), 2.5 mM $MgCl_2$, 10 mM each dNTP, 200 nM α2-F and α2–R and 1 unit Taq DNA polymerase (Promega). Amplification was performed using the Applied Biosystems Veriti□ 96-Well Thermal Cycler detection system. The mixture was preheated to 94°C for 5 min and then the PCR was cycled 55 times at 94°C for 30 sec, 60°C for 20 sec and 72°C for 30 sec with a final extension at 72°C for 5 min. DNA amplicons were run on 1.5 % agarose gels containing 0.5 x TBE buffer and DNA visualized by staining gels with ethidium bromide and viewing gels under UV light.

Real-time PCR and melting curve analysis method

PCR primers for the real time PCR and melting curve analysis were as previously described [10] and were designed according to the principle of the gap-PCR methodology to specifically co-amplify the $--^{SEA}$ deletion and the normal allele. Initial DNA amplification was carried out in a 25 µl reaction volume containing 1x EvaGreen□ PCR master mix (Bio-Rad Laboratories, Hercules, CA) 800 nM 3'ψζ-F (5'-CTCTGTGTTCTCAGTATTGGAG-3'), 400 nM 3'ψζ-R (5'-GTTCCCTGAGCCCCGACACG-3'; Normal-R) and SEA-R (5'-GAGTGCAGTGTTGT AGTCATGG-3'). The reaction was performed using the iQ5 real-time PCR detection system (Bio-Rad Laboratories). The mixture was preheated to 95°C for 3 min and then the PCR reaction was cycled 55 times at 94°C for 30 sec, 62°C for 20 sec and 72°C for 20 sec. The amplification cycles were followed by a melting cycle from 85□C to 95°C at a rate of 0.5°C per 10 sec. Reaction was subsequently optimized by varying the annealing temperature and primer concentrations.

Results and discussion

Real-time PCR is the continuous collection of fluorescence signal from one or more PCR amplicons over a range of cycles. A fluor dye fluoresces upon

incorporation into the newly synthesized double-stranded DNA (dsDNA), while unbound dye produces undetectable levels of fluorescence emission. Although primer dimers or nonspecific amplified products can cause an increase of fluorescence emission, the melting curve analysis is used to monitor the specificity of the PCR product [11]. A melting curve analysis is an assessment of the dissociation characteristics of dsDNA during heating, which is measurable by the large reduction in fluorescence that results. These altered melting properties give rise to changes in the shape of the melting curve [12] that is specific with melting temperature (Tm) of each amplicon when plotting the derivative data between fluorescence relative to the temperature by the real-time PCR instrument. In this study three primers were used, one forward primer and two reverse primers, with the first reverse primer used to amplify the 307 bp normal fragment and the second reverse primer used to amplify the 212 bp --^{SEA} deletion fragment.

We initially assessed the ability of normal, monoplex PCR to amplify the α-globin gene using serial dilutions of genomic DNA extracted from bulk preparations of DNA from blood of normal volunteers and DNA extracted directly from known numbers of PBMCs purified from blood of normal volunteers. Results show that a clear amplicon was produced from DNA dilutions down to 6.25 ng (**Figure 1A**). The amplicon from 10^4 PBMCs (equivalent to 60 ng of DNA) was comparable in intensity to the 50 ng DNA lane, showing relatively good agreement. Further serial dilutions of DNA showed a faint amplicon in 6.25 pg lane, which corresponds approximately to the DNA in a single cell (**Figure 1B**).

In multiplex real-time PCR at annealing temperature 62^{O}C using 3 primers, 800 nM 3'ψζ-F, 400 nM 3'ψζ-R (Normal-R) and SEA-R (SEA-R) followed by a melting curve analysis of serial dilutions of genomic DNA from α-thalassemia 1, --^{SEA}, heterozygote (--^{SEA}/αα) two separate peaks of the normal fragment and the --^{SEA} deletion fragment were clearly detected at Tms of 92.5^{O}C and 85.5^{O}C, respectively. The minimum amount of DNA that produced two peaks corresponding to two distinct amplicons was 2 ng (**Figure 2A**), which was confirmed by agarose gel electrophoresis (**Figure 2B**). Below this level of template, only one amplicon was seen. Interestingly, it was the normal allele that proved not to be amplified as seen with both the melting curve analysis and by directly running the amplification produces on agarose gels.

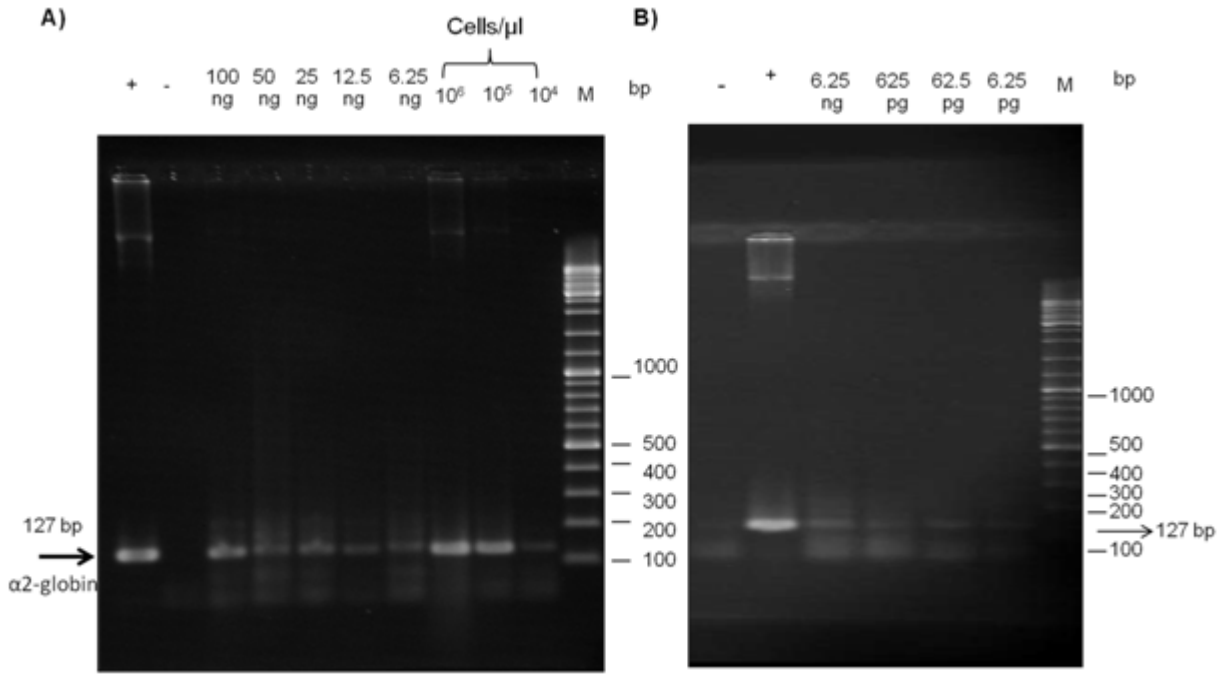

Figure 1. Sensitivity of monoplex PCR analysis. DNA extracted either directly from blood and serially diluted or from known numbers of PBMCs was used as the template in monoplex PCR reactions to amplify a portion of the α-globin gene. Positive control was --^{SEA} heterozygote genomic DNA and M represents a DNA marker. The expected amplified product of 127 bp is arrowed.

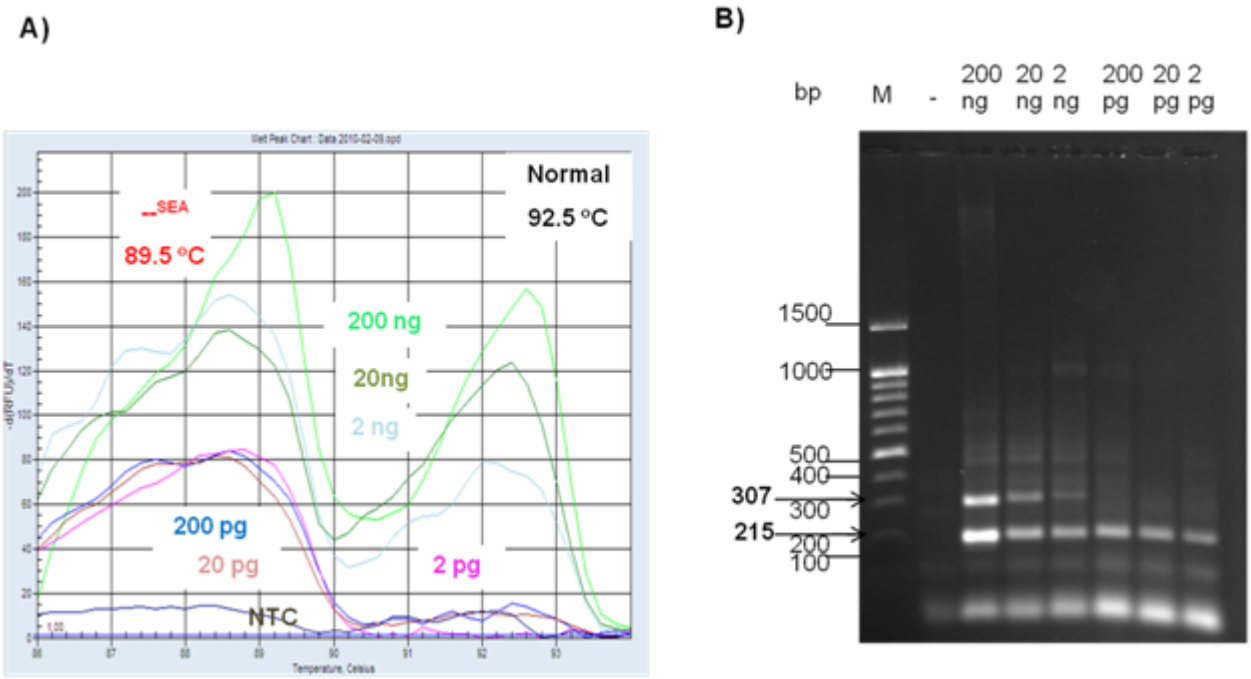

Figure 2. Sensitivity of melting curve analysis. Serial dilutions (200 ng to 2 pg) of DNA from $--^{SEA}$ heterozygote ($--^{SEA}/\alpha\alpha$) were subjected to a multiplex PCR and melting curve analysis. **A:** Two peaks of Tm 89.5°C (the $--^{SEA}$ amplicon) and 92.5°C (normal amplicon) are observed in the melting curve analysis at dilutions down to 2 ng. **B:** An agarose gel analysis of the amplicons from the multiplex PCR reaction using different DNA dilutions.

After a process of varying both primer concentration and annealing conditions, it was found that adjusting the annealing temperature to 64°C and reducing the primer concentration to 400 nM 3'ψζ-F, 400 nM 3'ψζ-R (Normal-R) and 200 nM SEA-R (SEA-R) two separate peaks of the normal fragment and the $--^{SEA}$ deletion fragment were clearly detected at the minimum DNA template level of 200 pg, but below this level even with further alterations in reaction conditions it was not possible to generate two amplicons (data not shown).

Overall our results show that the melting curve analysis for diagnosis of α-thalassemia is not suitable to be directly applied to single cell PGD in the management of α-thalassemia as the current limit for sensitivity corresponds to roughly the DNA from 30 cells. However, future technological advances may well bring the development of a suitable fast, easy and accurate technique that can be widely employed in the management of this common trait in Southeast Asia as part of PGD programs.

Acknowledgements

This work was supported by a Research Chair Grant from the National Science and Technology Development Agency (NSTDA) and Mahidol University. S.Sr. was supported by the Faculty of Graduate Studies, Mahidol University. A.L. was supported by the Office of the Higher Education Commission, and P.L. was supported by a Thai Royal Golden Jubilee Research Scholarship. The authors declare no conflict of interest in conducting this study.

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