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AN OPTIMIZED HRM METHOD FOR DIAGNOSIS OF G6PD DEFICIENCY IN KINH VIETNAMESE VIA VIANGCHAN MUTATION

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SUMMARY

With Glucose-6-phosphate dehydrogenase (G6PD) deficiency being the most common enzyme disorder in human, there have been 184 discovered point mutations and several methods that have been applied for diagnosing this disease. However, these techniques often pose several major problems such as being time-consuming, low sensitivity and high cost. Recently, the High Resolution Melting (HRM) has been studied and proven to be effective for DNA genotyping, mutation scanning and sequence matching. Therefore, HRM has been chosen for diagnosing G6PD deficiency via Viangchan mutation in this study. In this study, a total of 56 dried blood spot samples (including six control samples which were known the exact genotype by sequencing and fifty unknown samples) were collected and extracted DNA by using QIAamp DNA Blood Mini Kit. Primers for HRM analysis were designed through by the Umelt software. Then HRM optimization was carried out for annealing temperature of primers (Ta) and $MgCl_2$ concentration on six control samples. The optimized HRM protocol with 2.5 µM of $MgCl_2$ and Ta at 62^oC was applied for fifty G6PD samples and then comparing with ARMS-PCR genotyping results for the validation process. In the final step, genotyping results were confirmed by sequencing. In a results, both sensitivity and specificity of this technique reached 100%. Based on these favorable outcomes, this study has successfully optimized the HRM conditions for diagnosing fifty G6PD samples. It was such an essential precondition that showed HRM could be applied for other types of G6PD through other types of mutations such as Canton mutation or continues to be developed for HRM-Multiplex reactions.

Keywords**:** *G6PD Viangchan, HRM, Melting curves analysis, UmeltHets software, ARMS-PCR*

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency, an X-linked inherited disease, is one of the most common enzyme disorders in human (Beutler, 1994). This enzyme converts Glucose-6-phosphate into to 6-phospho D-gluconolactone and turns NADP to NADPH at the same time. In the red cells, this pathway is the only source of NADPH, which is a necessary substance for protecting the cell and its haemoglobin from reactive oxidation species (ROSs) such as fava beans, anti-malaria drug or fava plant (Efferth *et al.*, 2006).

There are more than 184 mutations that have been discovered and most of them are point mutations that result in the substitution of amino acids (Peters *et al.*, 2009). The frequency of G6PD deficiency and molecular techniques for diagnosis vary depending on the specific population and area. Specifically, in Africa, the mutation A^- (376A>G and 202G>A) – the most prevalent cause for G6PD – is tested by ARUP laboratory with PCR (ARUP-Laboratories, 2015). On the other hand, gene screening is applied in South East Asia to detect the Viangchan (871G>A), which is the main cause for the Cambodian (Louicharoen *et al.*, 2005). Moreover, Viangchan (871G>A) was discovered as the most common G6PD-related mutation in Thailand, occupying 54% of samples that were tested by PCR-RFLP method (Nuchprayoon *et al.*, 2002). In Vietnam, Viangchan mutation (871G>A) has been investigated as the main causative mutation of G6PD deficiency in Vietnamese Kinh by ARMS-PCR method (Hue *et al.*, 2013). Nevertheless, the fact that those techniques often suffer from substantial draw-

backs such as laborious procedure, low accuracy and high-cost calls for a novel, more rounded genotyping routine.

Recently in China, whose genetic relationship has been identified as particularly close to that of Vietnam, 9 out of 21 distinct point mutations (A95G, G392T, G487A, A493G, C592T, C1024T, C1360T, G1376T, and G1388A) with an occurrence rate up to 92.3% cases have been detected precisely by High-Resolution Melting Analysis (HRM) (Yan *et al.*, 2010). Being a new post-PCR analysis method, HRM has been used for detecting various genetic variations in nucleotide sequences with an extremely high sensitivity as well as specificity and cost advantages (Taylor, 2009). The principle of this method is simple, starting with PCR amplification of the target genes in the presence of a double strand DNA binding dye. This binding dye expresses high fluorescence when bounds to dsDNA and much lower fluorescence in the unbound state. Following the amplification step, the PCR products are melted and their fluorescence signals are recorded by a specialized instrument to generate melting curves with high resolution (ie. the temperature increment between each recorded point is less than 0.2^{o}C). By basing on the difference in melting temperature (Tm), the genetic variance(s) could be detected quickly and easily. Such advantages has made HRM a powerful method for DNA genotyping, mutation scanning and sequence matching with various applications in Salmonella, medium-chain acyl-CoA dehedrogenase, primary carnitine deficiency, RET, epidermal growth factor, gap-junction protein β1, G6PD … In addition, this method is not only fast but also inexpensive as an HRM procedure can be performed with relatively low cost reagents. Moreover, HRM does not require any processing or separation step after PCR performance, which reduces the risk of contamination. Therefore, the aim of this research is to optimize a HRM protocol for diagnosing G6PD Viangchan for the Kinh-Vietnamese population.

MATERIALS AND METHODS

Samples preparation

A total of 56 dried blood spot samples were collected from male and female infants from Tu Du Hospital (HCMC, Vietnam) from 2009 to 2012. Those collections were approved by Tu Du hospital and the NAFOSTED scientific board. The participants' parents took part in this study voluntarily and signed the consent forms. Three blood spots were taken from each newborn and one of them was used in the enzyme activity detection process. Two remaining spots were stored in room temperature condition before undergoing the DNA extraction procedure. The enzyme level of fifty G6PD deficiency patients (< 5.1 IU/gHb) was measured by Fluorescent Spot test and genotyped by ARMS-PCR method. It should be noted that the G6PD level of the remaining 6 control samples (that were known the exact genotype by sequencing and will be used as standard samples for optimization) was higher than 5.3 IU/gHb.

DNA extraction

All samples were extracted following QIAamp DNA Blood Mini Kit procedure (QIAGEN-company, 2015) carefully step by step. The purity and DNA concentration of the extractions were quantified and measured by NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) in a range of OD from 1.7 to 2.0.

Primer design and melting curve prediction

Using the Search tool on NCBI Genbank, the reference sequence for SNP rs2046210 can be obtained through the SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). Its sequence was taken from Homo sapiens chromosome X Genomic scaffold.

In order obtain the primer pairs for HRM, the Web-based Primer3plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) was used with these settings: product size 80 – 150bp, primer size 20 – 27bp, primer T_m around 60 – 65^{o}C. Primers creating undesired PCR's products would be eliminated by using NCBI Blast tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to test the specificity of these primers.

The optimal primer pair was chosen by the web tool UmeltHets (https://www.dna.utah.edu/hets/umh.php), a program that could predict the melting curve of PCR's product with different parameters such as: [Mg^{2+}] (mM) or %DMSO. The prediction based on adjusting those parameters in order to determine the best condition for each where the clearly discrimination between different genotypes and the best melting peak of each genotype could be formed.

HRM optimization

For HRM analysis, there are several components affect on the outcome melting curves such as DNA

concentration, DMSO, $MgCl_2$ concentration, primer concentration or annealing temperature (Ta) in HRM stage. Among those, DNA and DMSO concentration were proved not helpful for HRM analysis and the primer concentration was followed the guidance as 0.4 µM from the HRM kit. As a result, this study just focused on 2 elements: $MgCl_2$ concentration and Ta. To perform this step, 2 positive controls of each genotype (YG, GA and YA) that were sequenced and selected from the ARMS-PCR research (Hue *et al.*, 2013) were used to run with a dilution series of $MgCl_2$ and gradient temperature of Ta. The results were then immediately analyzed through by the melting curves, the cycle-threshold (*Ct*) in LightCycler 96 Software Version 1 and on gel electrophoresis (1.5%).

$MgCl_2$ concentration

LightCycler 96 Instrument (Roche Diagnostics, Germany) with a 96-well thermal block was used for PCR amplification and HRMA. All assays were performed in 10µL final volume reaction containing 5µL HRM Master Mix 2X Conc. (Roche Diagnostics, Germany), 0.4µL of each primer per reaction, 30ng of DNA, and an appropriate amount dH_2O would be added if the total volume did not reach 10µL. In the case of $MgCl_2$, real-time PCR with $MgCl_2$ concentrations ranged from 1 – 3 mM was conducted.

Annealing temperature (Ta)

PCR-HRM assay was performed by using the thermal cycle of 5 minutes pre-incubation at 95 °C. Then the amplification step consists of 40 cycles of 30 seconds at 95 °C, 30 second at 60 °C, 30 seconds at 72 °C and finally 3 min at 72 °C. Then the HRM stage was set up with 1 min 95°C, 1 min 40 °C. The annealing temperature in HRM stage was investigated in the range of 58 - 68°C to get the most appropriate temperature for further analysis.

HRM validation

The optimal HRM protocol in $MgCl_2$ concentration and annealing temperature was applied for genotyping 50 samples. The negative and positive controls were also included in each assay. HRM results were analyzed using fluorescence versus temperature graphs which were generated by Light Cycler® 96 Application Software Version 1.1 and then compared to results from ARMS-PCR. The different results between two methods would be validated by directly sequencing.

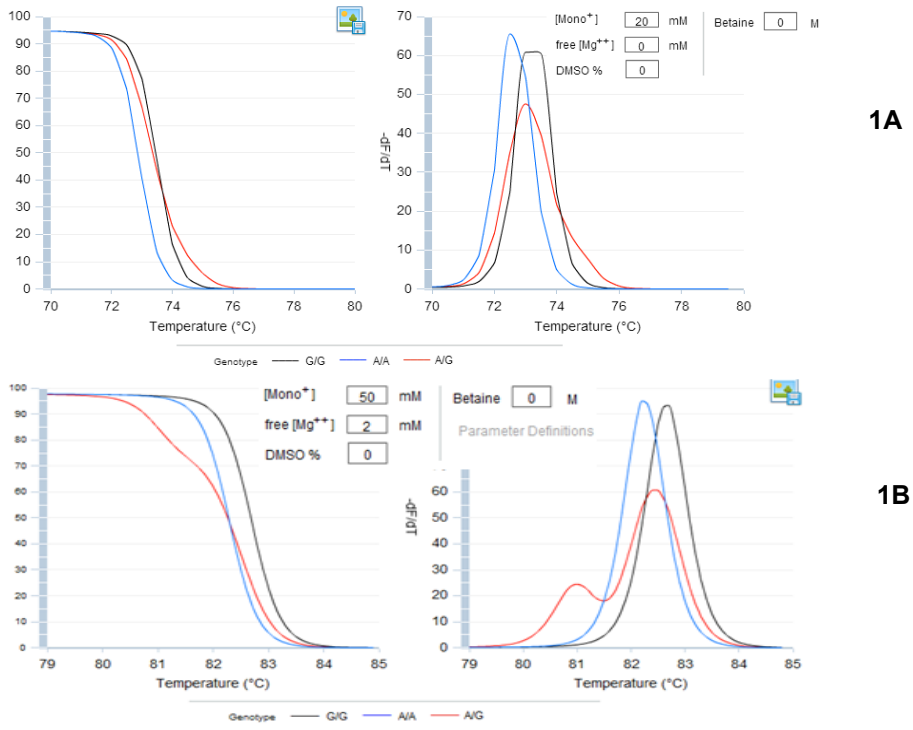


Figure 1. The effect of $MgCl_2$ on melting curves. 1A Melting curve prediction with 0 mM [$MgCl_2$]. 1B Melting curve prediction with 2 mM [$MgCl_2$]. Black, Blue and Red curves/peaks represent for melting curves/peaks of genotype YG, YA, GA, respectively.

RESULTS

Primer design

G6PD Viangchan sequence was taken from NCBI website with the accession number NC_000023.11. The primer pair- Forward-HRM (5'-ATCCCTGCACCCCAACTCAA-3') and Reverse_HRM (5'-TTGTTGGCCTGCACCTCTGA-3') - satisfied the sensitivity requirement for G6PD disease identification. In addition, the amplicon (80bps) was also checked by NCBI Blast with its high specificity result.

Before employing 2 primers in practical experiment, Umelt online program (The-Wittwer-Lab) was used to predict the optimal $MgCl_2$ concentration for HRM-PCR. Figure 1 showed the key role of $MgCl_2$ on the result melting curves. Figure 1A which indicated the concentration of 0 mM $MgCl_2$, the homomutant (YG) and wild type (YA) are uneven. In addition, the heterozygous (GA) genotype did not express two peaks for G and A which the difference with YG and YA curves could hardly be found. However, figure 1B (at 2.0 mM $MgCl_2$) provided three separated curves, with GA genotype being obviously recognized by the appearance of two peaks. Therefore, the range from 1mM to 3mM of $MgCl_2$ concentration was chosen for optimizing process.

HRM optimization

Annealing temperature (Ta)

The temperature gradient was performed in the range from 58 to 68°C for PCR. The bands at 58°C, 60°C and 62°C in Figure 2 showed that the desired PCR product had been amplified successfully. However, only the Ta at 62°C was chosen as a higher Ta often resulted in more specific PCR products.

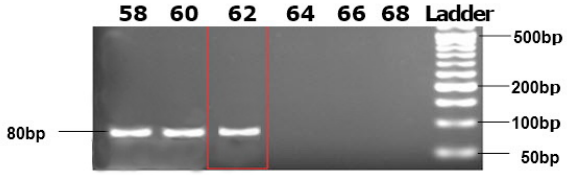


Figure 2. Annealing temperature gradient 58-68 °C.

$MgCl_2$ concentration

Following the HRM kit's guide and the Umelt's prediction above, $MgCl_2$ was performed with 5 different concentrations (1.0 mM, 1.5 mM, 2.0 mM, 2.5mM and 3.0 mM).

As can be seen in figure 3, at the concentrations 1.0 and 1.5 mM, the Melting Curves and Melting peaks of the wild type (YA) and homomutant (YG) are too close, therefore, they could hardly be recognized. In addition, the Tm difference between those genotypes seemed to be overlapped, just only about 0.3°C in disparity. At 2.0 mM, not only the melting curves of YG and YA were almost overlapped but also the heterozygous (GA) outcome did not show 2 clearly observable peaks. Both 2.5mM and 3.0 mM $MgCl_2$ expressed the best melting curves, which could be effortlessly identified 3 genotypes. However, the 3 melting curves at 3.0 mM $MgCl_2$ were marginally closer to each other than at 2.5 mM concentration. As a result, it was necessary to perform a further analysis on gel electrophoresis.

It could be immediately observed in figure 4 that the band at 1.5 mM MgCl2 was significantly blurred which possibly reflected the ineffective PCR amplification. Although three cases 2.0 mM, 2.5 mM, and 3.0 mM all expressed bright bands on the gel, the brightest band was at 2.5 mM. With the HRM optimization information above, it was reasonable to conclude that 2.5 mM would be the most appropriate MgCl2 concentration for this study.

Applying the initial optimization step for positive controls.

The optimized protocol applied for the 6 positive control samples were applied included Ta at 62°C and $MgCl_2$ concentration at 2.5 µM. Fortunately, all of the melting curves could be immediately categorized into a specific genotype (Figure 5) in addition to the fact that Cq values diverged faintly within 27 to 29 cycles. This also confirmed that the HRM protocol as well as the value of each component was successfully optimized. From this stage, this protocol would be applied massively for the remaining 50 samples.

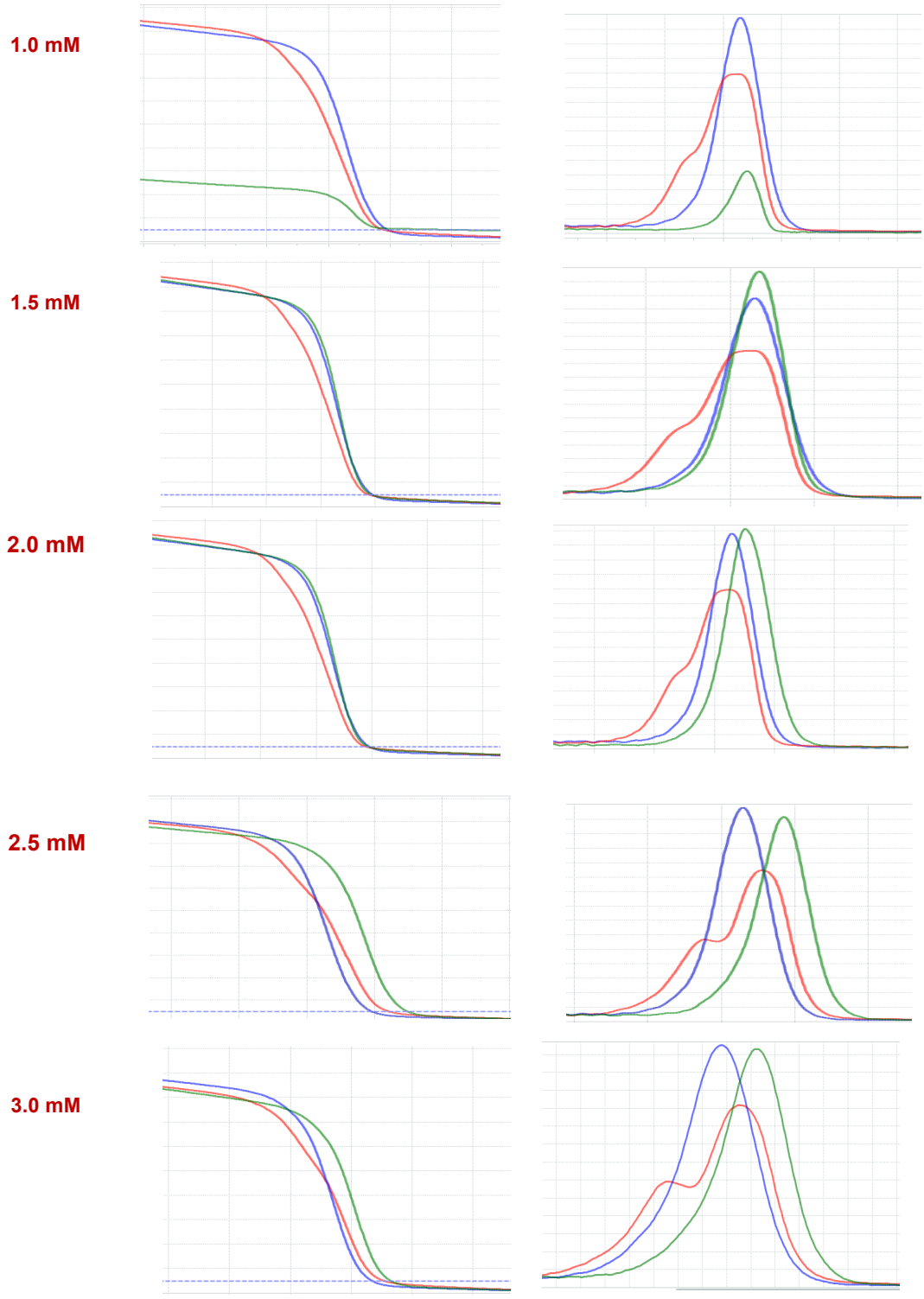


Figure 3. Affecting of $MgCl_2$ concentration on discrimination of 3 genotypes in Normalized Melting Curves and Normalized Melting Peaks. Green, Blue and Red curves/peaks represent for melting curves/peaks of genotype YG, YA, GA, respectively.

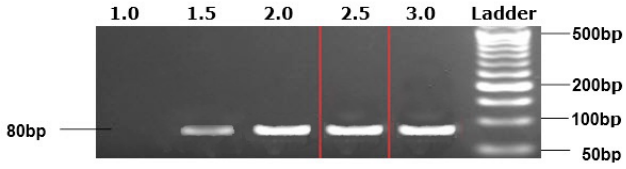
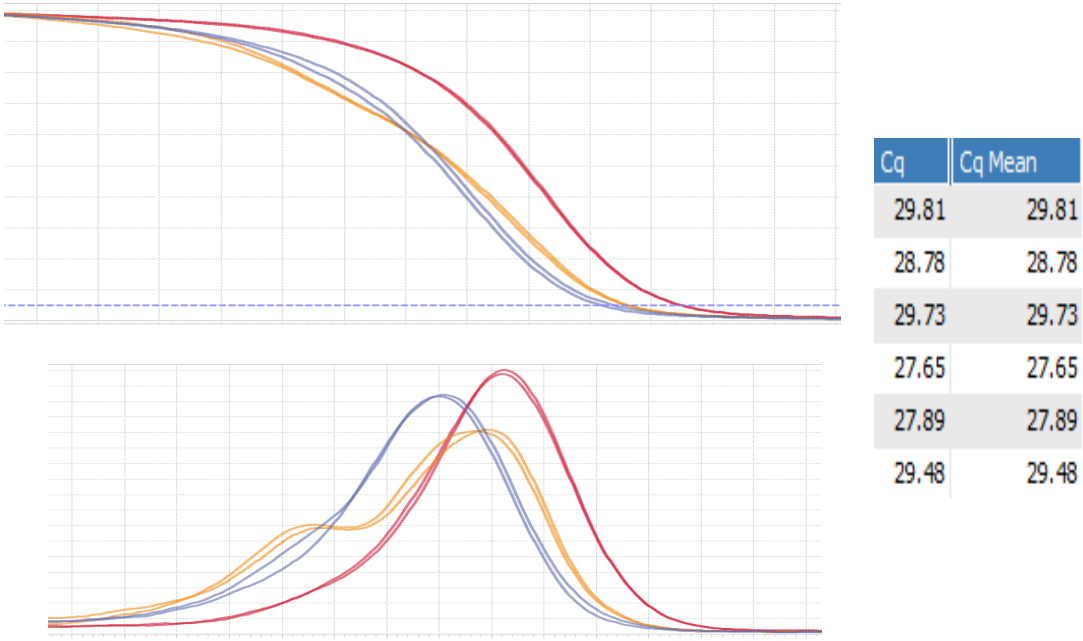


Figure 4. Optimizing MgCl2 concentration in the range [1.0, 3.0] mM on gel electrophoresis.



Cq	Cq Mean
29.81	29.81
28.78	28.78
29.73	29.73
27.65	27.65
27.89	27.89
29.48	29.48

Figure 5. The positive controls melting curve when applying the initial optimization. Red, Blue and Orange curves represent for melting curve of genotype YG, YA, GA, respectively. The table beside the picture shows the number of cycles needed to reach a set threshold fluorescence signal level).

HRM validation

The above optimal HRM protocol was applied to genotype fifty samples, whose genotypes had been previously determined by ARMS-PCR. The melting curves of three different genotypes (YA, YG, and GA) appeared clearly and separately from each other which is easy to distinguish (Figure 6).

Among these 50 samples, there were 3 of them that had dissimilar outcomes compared to ARMS-PCR method (as listed in table 1). For validating the HRM protocol, the sequencing process was carried out for all 3 these samples.

All of the unmatched genotype samples were performed HRM assay once again before the sequencing step. However, the results of those suspicious samples were unchanged so they were sent to OUCRU (Oxford University Research Unit, HCMC, Vietnam) for sequencing results (Figure 7).

For the GD130 sample, only one black peak represented the G nucleotide at the SNP position which reflected the YG genotype of this sample. On the other hand, the two other samples (GD207 and GD292) displayed two peaks for G and A at the same location, proving that those cases were GA genotype. This meant that the sequencing results had confirmed the accuracy of HRM assay for all 3 unmatched samples. In other word, both sensitivity and specificity of this method reached 100%.

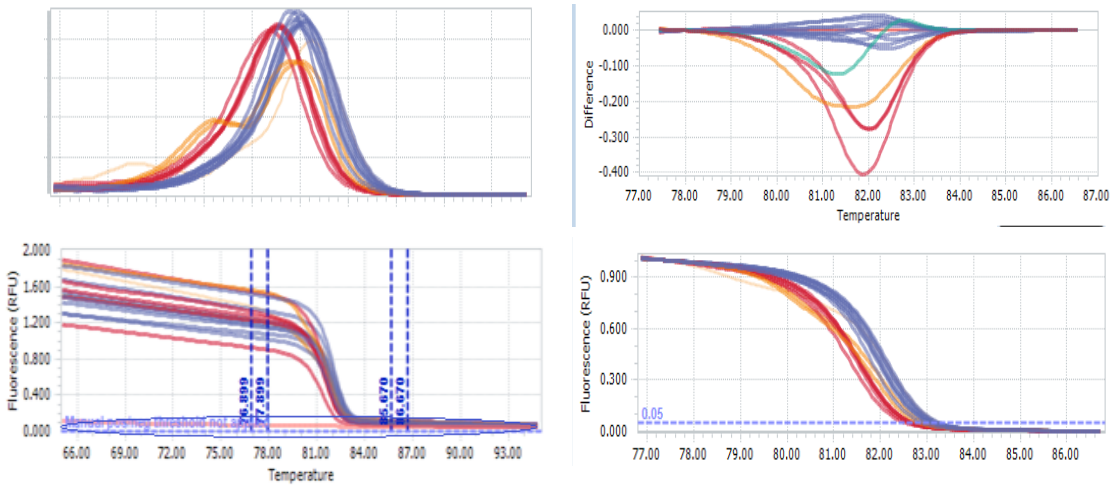


Figure 6. Massive genotyping for 50 samples. Blue, Red and Orange curves represent for melting curve of genotype YG, YA, GA, respectively.

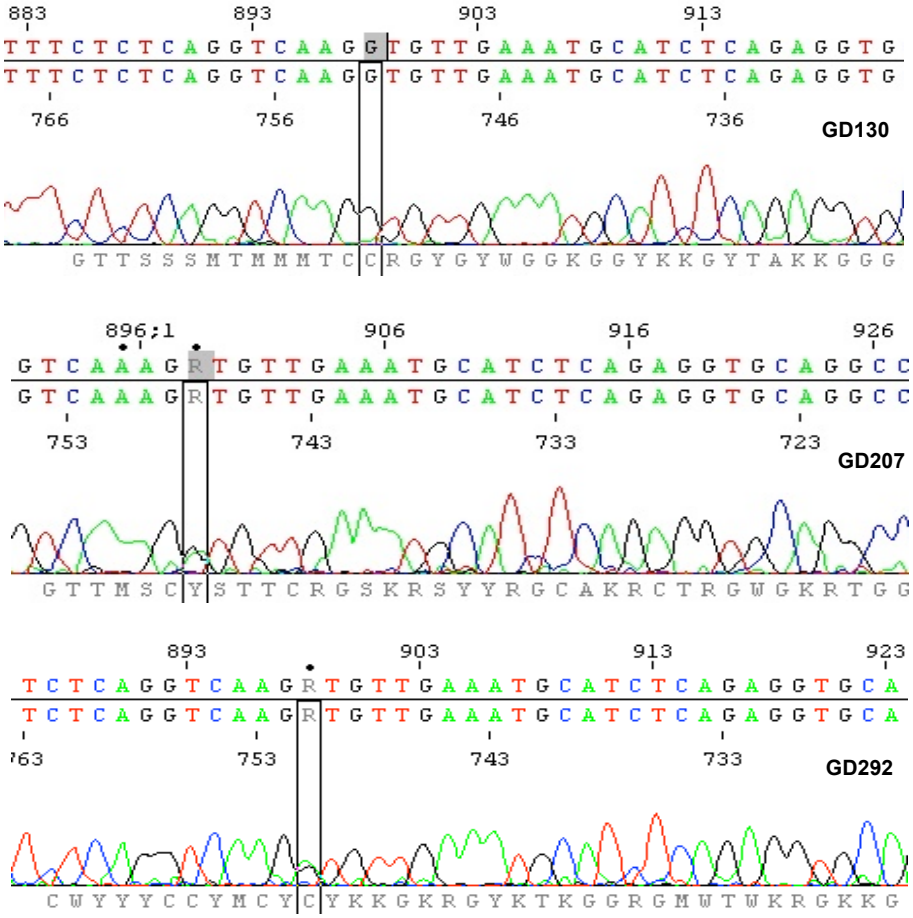


Figure 7. Sequencing results of 3 unmatched samples. Black panes indicate the position of Viangchan mutation on the DNA sequence. Letter R (in samples GD207 and GD292) represents for mix of G and A at this position that means samples are heterozygotes.

Table 1. Three samples were recorded with different result by using 2 techniques.

Sample ID	HRM results	ARMS-PCR results
GD130	YG	YA
GD292	GA	YG
GD207	GA	YG

DISCUSSION

Despite having been introduced since 2013 for genotyping Viangchan and Canton mutation in Vietnam, the time and the cost for more substances requirement are the disadvantages of ARMS-PCR. To be more detailed, ARMS-PCR required three primers, including one reverse primer and two forward primers, which leads to each sample undergoing two PCR reactions with different forward primers in two different PCR tubes. This could increase the chances of contamination thereby producing misleading final results. In addition, the gel electrophoresis process, which must be carried out with two wells per sample, was time-consuming and cost more reagents during performing. On the contrary, HRM is a closed-tube, simple, rapid and cost-effective method (Joly *et al.*, 2010; Pan *et al.*, 2013) which does not require any post-PCR step and as a consequence, the risk of contamination is significantly reduced.

The results of this study have also showed the advantages of HRM analysis in comparison with ARMS PCR. The sequencing step proved that HRM has successful genotyped the 3 unmatched samples. The error rate was only 3 out of 50 cases; however, it should be a concern if a large amount of sample was analyzed with ARMS-PCR. In contrast, the optimal HRM protocol is definitely more reliable by reducing the incorrect results on genotyping step than ARMS-PCR method.

In HRM reactions, PCR enhancers such as $MgCl_2$, DMSO are commonly included in order to reduce non-specific amplification, the number of required cycles and increase yield. However, the addition of DMSO only increases the amplification efficiency in case of GC-rich sequence (>=65%GC) (Varadaraj *et al.*, 1994; Jensen *et al.*, 2010) that not match to amplicon of this study. Moreover, according to the study of Chen Song in 2015, the use of DMSO with SYBR Green, the non-saturating dye that included in the HRM Master Mix 2X Conc - Roche Diagnostics, does not create clear improvement in HRM analysis (Song *et al.*, 2015). Therefore, DMSO was not included in HRM reactions of this study.

Another important factor that strongly affects on HRM reaction is the concentration of $MgCl_2$. At the optimal concentration, this factor has ability to reduce non-specific amplification and allow clearer distinction of sequence variations. Because the HRM Master Mix contained too small amount of $MgCl_2$, this study focused on optimization of $MgCl_2$ concentration. The results from figure 3, figure 4 showed that there was no PCR product would be seen at the low concentrations of $MgCl_2$ (at 1.0mM $MgCl_2$), and as the MgCl2 concentration is increased, the HRM difference graphs change distinctively (at 2.5mM MgCl2), but the discrimination of graphs is not better at higher concentration. Then we can conclude that 2.5mM MgCl2 is the optimal concentration for this HRM protocol.

Amplicon length may also influence the sensitivity of genotyping. The shorter the amplicon is, the clearer the discrimination of single base differences is created. For single base changes or SNPs, and for single base insertions/deletions, 50 – 300 bp is recommended for the amplicon length. With small changes of these variation types, as the amplicon size increases, the Tm differences among genotypes decreases, thus creating small differentiation between mutant and non-mutant samples (Liew *et al.*, 2004; Krypuy *et al.*, 2006; Yan *et al.*, 2010) and then making it more difficult to distinguish between single nucleotide variants. In addition, the shorter amplicon also help us to limit the possibility that the target single nucleotide polymorphism (SNP) is replaced by another unexpected SNP. And in case of some G6PD variants are too close to target point mutation, a direct sequencing should be carried out if the HRM curve of an unknown patient is not perfectly matched with one of the DNA controls in a repeatedly way and if there is a discordance in genotype and phenotype to avoid a misdiagnosis (Joly *et al.*, 2010).

As concerned the possible limitations of HRM, although HRM is the least expensive of the currently used screening methods (Yan *et al.*, 2010), HRM

requires an expensive equipment and YA - AA genotypes (or YG – GG) that expressed the similar outcome melting curves could only be distinguished by the patient's gender.

CONCLUSION

In conclusion, this study has successfully optimized the HRM conditions for genotyping G6PD, particularly the common mutation Viangchan (rs137852327 - a G>A) in Vietnamese with the sensitivity and specificity of it reaching 100%. Comparing with the traditional methods in general, and ARMS-PCR in specific, HRM was much faster as well as significantly dominant in accuracy. These promising results will be an essential precondition to apply the HRM method for other types of G6PD mutations or continue developing the HRM-Multiplex reactions.

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TỐI ƯU HÓA PHƯƠNG PHÁP HRM CHO VIỆC CHUẨN ĐOÁN BỆNH THIẾU MEN G6PD Ở DÂN TỘC KINH THÔNG QUA ĐỘT BIẾN VIANGCHAN

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TÓM TẮT

Thiếu men glucose-6-photphase (G6PD) là một trong những bệnh di truyền về enzyme phổ biến nhất ở người ngày nay. Đã có một vài phương pháp phân tử được áp dụng để chẩn đoán bệnh. Tuy nhiên, những phương pháp hiện nay rất tốn thời gian, độ nhạy kém và chi phí cao. Gần đây, High Resolution Melting (HRM) đã được nghiên cứu và chứng minh là hiệu quả trong việc phát hiện kiểu gen và kiểu đột biến. Theo đó, phương pháp HRM đã được chọn để chẩn đoán bệnh thiếu men G6PD thông qua việc phát hiện đột biến Viangchan. Trong bài nghiên cứu này, tổng số 56 mẫu máu khô (sáu mẫu đối chứng và 50 mẫu chưa biết kiểu gene) được thu thập và trích DNA bằng bộ Kit QIAamp DNA Blood Mini. Các cặp mồi dùng trong phân tích HRM được thiết kế bằng phần mềm Umelt. Sau đó, phản ứng HRM đã được tối ưu hóa với nồng độ $MgCl_2$ là 2.5µM và nhiệt độ bắt cặp của mồi là 62^{o}C được áp dụng cho 50 mẫu và được so sánh với kết quả từ phương pháp ARMS-PCR để thực hiện bước đánh giá. Những mẫu có kết quả không khớp giữa 2 phương pháp sẽ được giải trình tự gene. Thật đáng ngạc nhiên khi mà cả độ nhạy và độ đặc hiệu của HRM đều đạt mức 100%. Kết quả chính xác đó cho thấy nghiên cứu này đã thành công trong việc tối ưu hóa các điều kiện của HRM và là điều kiện thiết yếu cho việc áp dụng phương pháp này cho các loại đột biến gây bệnh G6PD khác, cũng như tiếp tục phát triển phản ứng Multiplex HRM để chẩn đoán bệnh.

Từ khóa: *G6PD Viangchan, HRM, Phân tích đường cong nóng chảy, Phần mềm Umelt, ARMS-PCR.*