

# THE USE OF THE AMPLIFICATION REFRACTORY MUTATION SYSTEM (ARMS) IN THE DETECTION OF RARE $\beta$ -THALASSEMIA MUTATIONS IN THE MALAYS AND CHINESE IN MALAYSIA

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**Abstract.** Molecular characterization and prenatal diagnosis for  $\beta$ -thalassemia can be carried out using the Amplification Refractory Mutation System (ARMS). The ARMS is a rapid and direct molecular technique in which  $\beta$ -thalassemia mutations are visualized immediately after DNA amplification by gel electrophoresis. In the University of Malaya Medical Center, molecular characterization and prenatal diagnosis for  $\beta$ -thalassemia is carried out using ARMS for about 96% of the Chinese and 84.6% of the Malay patients. The remaining 4% and 15.4% of the uncharacterized mutations in the Chinese and Malay patients respectively are detected using DNA sequencing. DNA sequencing is an accurate technique but it is more time-consuming and expensive compared with the ARMS. The ARMS for the rare Chinese  $\beta$ -mutations at position -29 (A→G) and the ATG→AGG base substitution at the initiator codon for translation in the  $\beta$ -gene was developed. In the Malays, ARMS was optimized for the  $\beta$ -mutations at codon 8/9 (+G), Cap (+1) (A→C) and the AATAAA→AATAGA base substitution in the polyadenylation region of the  $\beta$ -gene. The ARMS protocols were developed by optimization of the parameters for DNA amplification to ensure sensitivity, specificity and reproducibility. ARMS primers (sequences and concentration), magnesium chloride concentration, *Taq* DNA polymerase and PCR cycling parameters were optimized for the specific amplification of each rare  $\beta$ -thalassemia mutation. The newly-developed ARMS for the 5 rare  $\beta$ -thalassemia mutations in the Chinese and Malays in Malaysia will allow for more rapid and cost-effective molecular characterization and prenatal diagnosis for  $\beta$ -thalassemia in Malaysia.

## INTRODUCTION

Beta-thalassemia is the most common autosomal recessive gene disorder and poses a major health problem in Southeast Asia, India, Pakistan, Mediterranean, North Africa, Middle East and South China (Weatherall, 1983; Laosombat *et al*, 1992; Verma *et al*, 1997; Zahed *et al*, 1997).  $\beta$ -thalassemia has been most often observed in four different populations: namely the Mediterranean, the Asian Indian, the

Chinese and the Black African. In each of these populations, a small number of common  $\beta$ -mutations are responsible for more than 90% of the disease while the rest of the  $\beta$ -thalassemia is due to a handful of rarer  $\beta$ -mutations (Winichagoon *et al*, 1992; Thein, 1993).

In Malaysia, seven  $\beta$ -thalassemia mutations are responsible for the majority of  $\beta$ -thalassemia in the Malay and Chinese ethnic groups. These include the  $\beta$ -mutations at -28 TATA Box, codon 17, codon 41-42 and IVSII #654 in the Malaysian Chinese (George *et al*, 1990). In the Malaysian Malays, the common mutations responsible for the majority of the  $\beta$ -thalassemia are codon 26 (Hb E), IVSI #1 and IVSI #5 (George *et al*, 1992). Various molecular techniques have been developed to

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characterize these mutations and the choice of the detection protocols used in the molecular characterization and prenatal diagnosis of  $\beta$ -thalassemia in each region depends on the diversity of the ethnic groups and types of  $\beta$ -mutations present in the region (Old and Ludlam, 1991; Sutcharithchan *et al*, 1995).

In the University of Malaya Medical Center (UMMC), molecular characterization and prenatal diagnosis of  $\beta$ -thalassemia is carried out using the Amplification Refractory Mutation System (ARMS) (Tan *et al*, 1998). These mutations include 14 common and rare  $\beta$ -mutations along the  $\beta$ -gene: -29 (A→G), -28 (A→G), Cap (+1) (A→C), cd 8/9 (+G), cd 15 (G→A), cd 17 (A→T), cd 19 (A→G), cd 26 (HbE, G→A), IVSI #1 (G→T), IVSI #5 (G→C), cd 41-42 (-TCTT), cd 71-72 (+A), IVSII #654 (C→T) and the 619 bp deletion at the 3'-end of the  $\beta$ -gene. The basis of the ARMS technique is that the terminal 3'-nucleotide of a PCR primer must be allele-specific for DNA amplification to occur (Newton *et al*, 1989). This specificity allows ARMS to directly detect  $\beta$ -mutations by gel electrophoresis after DNA amplification without any further need of restriction enzyme digestion or oligonucleotide probing (Old and Ludlam, 1991; Venkatesan *et al*, 1992; George *et al*, 1993).

The identification of rare  $\beta$ -mutations can be made accurately using DNA sequencing. While genomic sequencing is a sensitive and specific technique, it requires specialized equipment and trained staff. DNA sequencing is also more expensive and time-consuming compared with the ARMS because the sequencing reactions use special fluorescence dyes available only in kits and because it requires highly purified PCR products. Our objective was to develop and optimize the current rapid and inexpensive ARMS protocols to include five  $\beta$ -mutations which are rare but nevertheless present in our Malay and Chinese patients.

The -29 (A→G) mutation was originally reported in the American Black and Chinese population and is a transcriptional mutation generally giving rise to  $\beta^+$ -thalassemia (Huang *et al*, 1986; Kazazian, 1990). The ATG→AGG

base substitution at the initiator codon for translation in the  $\beta$ -gene was reported in the Chinese, Japanese and Korean populations (Kazazian, 1990) and is a translation mutation. This T→G base substitution causes  $\beta^0$ -thalassemia as it affects the translation of RNA into globin. Cd 8/9 (+G) detected in Asian Indian is a frameshift mutation and results in  $\beta^0$ -thalassemia (Kazazian *et al*, 1984). CAP (+1) was also originally found in the Asian Indian and is a RNA modification mutant but, unlike cd 8/9, Cap (+1) causes  $\beta^+$ -thalassemia (Wong *et al*, 1987). The AATAAA→AATAGA base substitution at the polyadenylation region of the  $\beta$ -gene results in abnormal unstable elongated RNA transcripts and causes  $\beta^+$ -thalassemia (Orkin *et al*, 1985; Higgs *et al*, 1989). All five  $\beta$ -mutations have since been identified at low frequencies in our Malaysian Malay and Chinese patients (George, 1998; Tan *et al*, 1998).

## MATERIALS AND METHODS

### Patient samples

Blood samples (10 ml) from  $\beta$ -thalassemia patients and their families and from normal individuals (controls) were collected in sodium-EDTA tubes and stored at -70°C until ready for DNA extraction. The five rare  $\beta$ -mutations at -29 (A→G), ATG→AGG base substitution at the initiator codon, cd 8/9 (+G), Cap (+1) (A→C) and the AATAAA→AATAGA base substitution were originally confirmed in our patients using DNA sequencing.

### DNA extraction

DNA extraction was carried out using sodium-dodecyl-sulphate and proteinase K digestion followed by purification by phenol-chloroform extractions. DNA was solubilized in water and stored at -70°C as stocks. Aliquots of DNA were also stored at 4°C and used for optimization of the ARMS.

### Amplification refractory mutation system (ARMS)

The primers for DNA amplification were

synthesized by Gibco BRL Life Technologies, USA. Primer sequences to detect the rare  $\beta$ -mutations are shown in Table 1. These primers were paired with common primers B, C or D to obtain ARMS products of specific molecular weights. An internal control to amplify an 861 bp fragment along the  $\beta$ -gene was simultaneously amplified in every PCR reaction. Primers A and B were used for amplification of the internal control.

### ARMS protocol

DNA amplification using the ARMS was carried out in 25  $\mu$ l reactions containing 200  $\mu$ M dinucleotide triphosphates (Perkin Elmer, Applied Biosystems Division, USA), 10X PCR buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl), 10-20 pmol ARMS primers, 0.5 Units *Taq* DNA polymerase and 1.0-1.5 mM magnesium chloride ( $MgCl_2$ ) (Gibco BRL Life Technologies, USA). Amplification was performed at 95°C for 5 minutes, followed by 30 cycles at 93°C for 1 minute, 65°C for 1 minute and 72°C for 1.5 minute. A final extension of 3 minutes at 72°C was allowed and the

reaction was maintained at 4°C until analysis by gel electrophoresis.

### Optimization of the ARMS

Optimization for specific amplification of the  $\beta$ -mutations was carried out by manipulation of different  $MgCl_2$  concentrations, primer concentration and annealing temperatures. The amplified products were electrophoresed in 1.5% agarose gel at 100V for 1-1½ hours. DNA bands were visualized under ultra-violet light illumination.

## RESULTS

Each ARMS protocol was optimized initially using  $MgCl_2$  concentrations between 1.0-2.5 mM and primer concentrations of 20 pmol. The ARMS reaction with the  $MgCl_2$  concentration that did not produce any non-specific bands was then used to optimize other parameters, namely annealing temperature, primer and *Taq* polymerase concentrations, for increased specificity.

Table 1  
Primer sequences used for the amplification of the rare  $\beta$ -thalassemia mutations by ARMS.

$\beta$ -thalassemia mutation	Primer sequence	Common primer <sup>a</sup>	Size (bp)
-29 (A→G)	CAG GGA GGG CAG GAG CCA GGG CTG GGT ATG	D	624
Translation mutation (ATG→AGG)	TGT TCA CTA GCA ACC TCA AAC AGA CAT CAG	D	544
Cd 8/9 (+G)	CCT TGC CCC ACA GGG CAG TAA CGG CAC ACC	C	214
Cap (+1) (A→C)	ATA AGT CAG GGC AGA GCC ATC TAT TGG TTC	D	594
Polyadenylation region (A→G)	GGC CTT GAG CAT CTG GAT TCT GCC TAT TAG	B	392

<sup>a</sup>The above primers are coupled as indicated with the common primers B: GAG TCA AGG CTG AGA AGA TGC AGG; C: ACC TCA CCC TGT GGA GCC AC or D: CCC CTT CCT ATG ACA TGA ACT TAA.

<sup>b</sup>The 861 bp internal control fragment was amplified using primer A: CAA TGT ATC ATG CCT CTT TGC ACC and B.

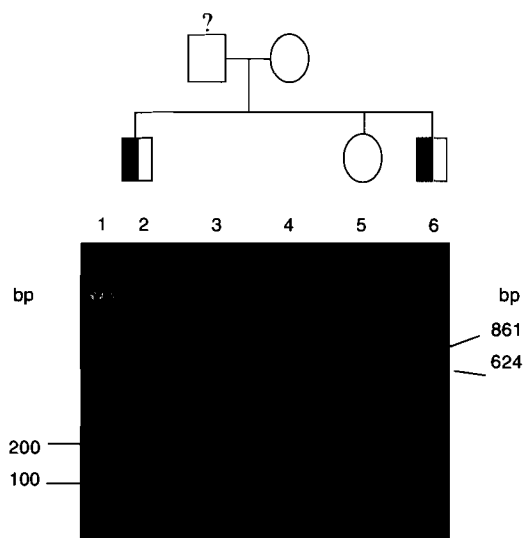


Fig 1—Electrophoresis of PCR products after amplification using the ARMS for the  $\beta$ -thalassaemia mutation at -29 (A→G). Lane 1: 100 bp molecular weight marker; lane 2:  $\beta$ -carrier son, 861 bp internal control and -29 mutant band; lane 3: father, DNA not available; lane 4: mother, 861 bp internal control and -29 mutant band; lane 5: normal daughter: 861 bp internal control band; lane 6:  $\beta$ -carrier son, 861 bp internal control and -29 mutant band.

### -29 (A→G) transcriptional mutation

One Chinese  $\beta$ -thalassaemia carrier and her two sons were confirmed as having the transcriptional  $\beta$ -mutation at position -29 by DNA sequencing. DNA from the family was amplified using  $MgCl_2$  concentrations at 1.0 mM, 1.5 mM and 2.0 mM and optimal amplification was achieved with 1.5 mM of  $MgCl_2$ . Fig 1 shows the 624 bp -29-specific mutant and 861 bp internal control bands after amplification of DNA from the mother and her two  $\beta$ -carrier sons (lanes 2, 4 and 6). Her daughter did not possess the -29  $\beta$ -mutation as only the 861 bp internal control fragment was amplified (lane 5). DNA from the father in this family was not available for molecular analysis. In the optimization reactions,  $MgCl_2$  concentration at 1.0 mM resulted in poor amplifications of the 624 bp -29 mutant and 861 bp internal control

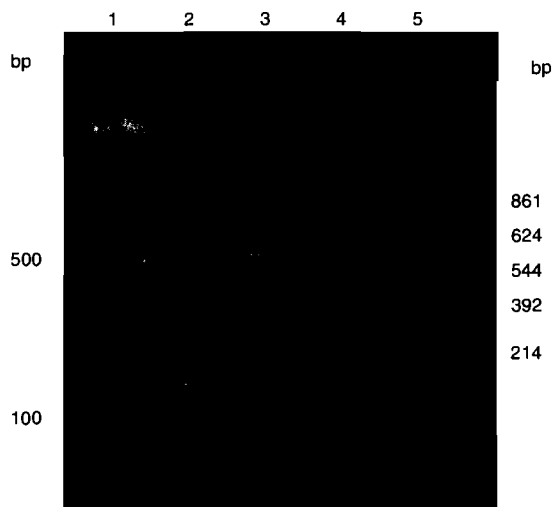


Fig 2—Electrophoresis of PCR products after amplification using the ARMS for the  $\beta$ -thalassaemia mutations at -29 (A→G), ATG→AGG base substitution at the initiator codon of translation, AATAAA→AATAGA polyadenylation mutation and cd 8/9 (+G). Lane 1: 100 bp molecular weight marker; lane 2: 624 bp -29 mutant band; lane 3: 544 bp ATG→AGG translation mutant; lane 4: 392 bp AATAAA→AATAGA polyadenylation mutant band; lane 5: 214 bp cd 8/9 mutant band. All patient DNA have also amplified the 861 bp internal control band as they were heterozygous for  $\beta$ -thalassaemia.

fragments, indicating that the condition was too stringent. Specificity studies for the -29 ARMS protocol were carried out using 20 normal DNA samples and no amplification of the 624 bp -29 mutant band was observed.

### ATG→AGG base substitution at the initiator codon of translation of the $\beta$ -gene

The ATG→AGG base substitution at the initiation codon of translation of the  $\beta$ -gene was confirmed in one Chinese  $\beta$ -thalassaemia carrier by DNA sequencing. Using the ARMS, optimal amplification was obtained using 1.5 mM  $MgCl_2$  with an annealing temperature of 65°C. When amplification was carried out using 20 pmol of the ARMS primer for the ATG→AGG translation mutation, a faint amplification of the 544 bp ATG→AGG translation mutant band was observed with DNA

from normal individuals. The primer concentration was reduced to 10 pmol and specificity was achieved with the amplification of only the 544 bp mutant band and the 861 bp internal control (Fig 2, lane 3).

### Cap (+1) (A→C) mutation

Amplification of the Cap (+1) (A→C) mutation was found to require more stringent conditions to avoid the amplification of non-specific sequences. MgCl<sub>2</sub> concentrations at 1.0, 1.5 and 2.0 mM were evaluated using DNA from a normal control and a β-carrier with a confirmed mutation at the Cap (+1) site (Fig 3). As the MgCl<sub>2</sub> concentrations were increased to 1.5 and 2.0 mM, a lower molecular weight non-specific band was amplified together with the 594 bp Cap (+1) fragment (Fig 3, lanes 4-7). Using a relaxed condition of MgCl<sub>2</sub> at 2.0 mM, normal control DNA amplified a faint 594 bp Cap (+1)-specific fragment (lane 6). The optimal MgCl<sub>2</sub> concentration was achieved at 1.0 mM at which only the 861 bp internal control fragment was amplified with normal control DNA (lane 1) and both the 861 bp and 594 bp Cap (+1) fragment were amplified as distinct bands in patient DNA (lane 2). Different annealing temperatures were also evaluated for the Cap (+1) (A→C) mutation. When amplification was carried out at 60°C, normal (Fig 4, lanes 1, 2) and patient DNA (lane 3) amplified a distinct lower molecular weight non-specific band. When the annealing temperature was increased to 65°C, control DNA amplified only the 861 bp internal control (lanes 5 and 6) and patient DNA amplified both the internal control and the 594 bp Cap (+1)-specific fragments (lane 7).

### cd 8/9 (+G) mutation

ARMS conditions for the cd 8/9 (+G) mutation was evaluated at different MgCl<sub>2</sub> and primer concentrations. MgCl<sub>2</sub> concentration at 1.5 mM produced results with distinct 861 bp and 214 bp cd 8/9-specific bands. However, faint amplification of the 214 bp cd 8/9-specific band was sometimes observed during

specificity tests with normal DNA. The concentration of the primers for amplification of the cd 8/9 β-mutation was then reduced from 20 pmol to 10 pmol. Using the new primer concentration at 10 pmol, non-specific amplification was abolished and only the 861 bp internal control and 214 bp cd 8/9-specific bands were amplified with patient DNA (Fig 2, lane 5).

### AATAAA→AATAGA base substitution in the polyadenylation region of the β-gene

The polyadenylation mutant was detected in a Malay β-thalassemia carrier by DNA sequencing. DNA amplification was carried out using 1.5 mM MgCl<sub>2</sub>. The common primer for amplification of the AATAAA→AATAGA base substitution is primer B. Since primer B is also used in the amplification of the 861 bp internal control fragment, the concentration of primer B in this ARMS protocol was increased two-fold from 20 pmol to 40 pmol. The concentration of the other primers remained at 20 pmol. Using these conditions the ARMS protocol for the A→G base substitution in the polyadenylation region of the β-gene produced a distinct 392 bp-specific fragment (Fig 2, lane 4).

## DISCUSSION

Beta-thalassemia major patients have chronic anemia and require regular blood transfusions. Molecular characterization and prenatal diagnosis programs will lead to a dramatic reduction in β-thalassemia major cases. In Malaysia, molecular analysis and prenatal diagnosis of β-thalassemia can be carried out using the ARMS technique. The ARMS was initially developed to characterize the common β-mutations in the Malays and Chinese in Malaysia.

Molecular characterization of the rare β-mutations is carried out using genomic DNA sequencing. The ARMS has a number of advantages over DNA sequencing for analysis of rare β-mutations. The ARMS protocol only requires amplification followed by direct analysis

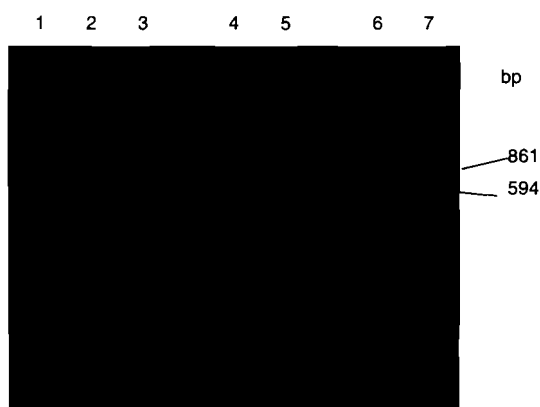


Fig 3—Effects of different  $MgCl_2$  concentrations on the specificity of the ARMS protocol for CAP (+1) (A→C) mutation. Using 1.0 mM  $MgCl_2$  - lane 1: Normal DNA amplified only the 861 bp internal control band; lane 2: DNA from  $\beta$ -carrier with Cap (+1) mutation amplified only the 861 bp internal control and 594 bp Cap (+1) mutant bands; lane 3: PCR control where no DNA was added to reaction. Using 1.5 mM  $MgCl_2$  - lane 4: Normal DNA amplified 861 bp internal control and non-specific bands; lane 5: DNA from  $\beta$ -carrier with Cap (+1) mutation amplified 861 bp internal control, 594 bp Cap (+1) mutant and non-specific bands. Using 2.0 mM  $MgCl_2$  - lane 6: Normal DNA amplified 861 bp internal control, 594 bp Cap (+1) mutant and non-specific bands; lane 7: DNA from  $\beta$ -carrier with Cap (+1) mutation amplified 861 bp control, 594 bp Cap (+1) mutant and non-specific bands.

by agarose gel electrophoresis: this versatility allows rapid and cost-effective diagnostic testing and the implementation of the ARMS for the prenatal diagnosis of  $\beta$ -thalassemia (Old and Ludlum 1991; Tan *et al*, 1994; Saxena *et al*, 1998).

The primers for the development of the ARMS for the rare  $\beta$ -mutations were carefully selected so that they were complementary to the target DNA sequences only. The prevention of non-specific annealing to other complementary sequences reduces the competition for PCR reagents and also prevents the amplification of undesirable non-specific products. Primer concentrations at 20 pmol were used for initial development of all ARMS protocols.

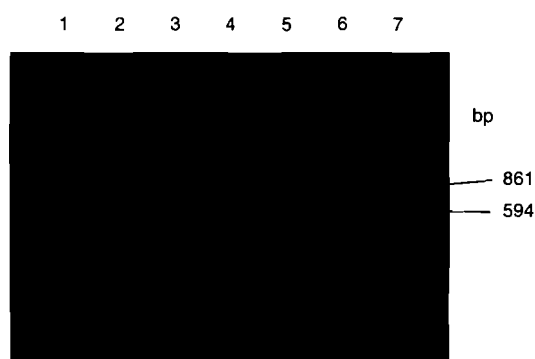


Fig 4—Effects of different annealing temperatures on the specificity of the ARMS protocol for CAP (+1) (A→C) mutation. Using 60°C annealing temperature - lanes 1 and 2: Normal DNA amplified 861 bp internal control and non-specific bands; lane 3: DNA from  $\beta$ -carrier with Cap (+1) mutation amplified 861 bp internal control, 594 bp Cap (+1) mutant and non-specific bands; lane 4: PCR control where no DNA was added to reaction.

Using 65°C annealing temperatures-lanes 5 and 6: Normal DNA amplified only the 861 bp internal control band; lane 7: DNA from  $\beta$ -carrier with Cap (+1) mutation amplified only the 861 bp internal control and 594 bp Cap (+1) mutant bands.

This concentration worked well for the -29 and Cap (+1) mutations in which the ARMS produced distinct mutant bands. However, 20 pmol of primers for cd 8/9 and the  $ATG \rightarrow AGG$  translation mutants produced faint non-specific bands with DNA from normal individuals. A reduction of the primer concentration to 10 pmol produced a more specific ARMS protocol resulting in the amplification of only the 861 bp internal control and the  $\beta$ -mutant bands.

In the development of our ARMS for the detection of rare  $\beta$ -mutations, we found that although  $MgCl_2$  concentration at 1.5 mM was optimal for -29,  $ATG \rightarrow AGG$  translation mutation and cd 8/9  $\beta$ -mutations, it produced non-specific bands with the ARMS for the Cap (+1) mutation. The optimal magnesium concentration for the Cap (+1) mutation was established at 1.0 mM (Fig 3). The use of different  $MgCl_2$  concentrations for amplifica-

tion of different gene sequences is not unusual as different primers display different magnesium optima, with ranges between 1.0 and 2.5 mM (Harris and Jones 1997).

One of the main objectives for development of the ARMS for rare  $\beta$ -mutations is to produce cost-effective protocols that can be passed on as cheaper tests to patients. We obtained specific, sensitive and reproducible results with *Taq* DNA polymerase at a concentration of 0.5 Unit per reaction. The concentration of *Taq* DNA polymerase was kept at a minimum to ensure cost-effectiveness. The majority of the investigators working on ARMS use 3% Nusieve-Seakem agarose for visualization of ARMS products (Varawalla *et al.*, 1991). Nusieve is a fairly expensive agar (Nusieve/Agarose (3:1) is RM1,100/125 g; Sea Kem is RM 625/125 g). Our laboratory uses ordinary analytical grade agarose (RM 328/100 g) for electrophoresis of the amplified ARMS products.

The development of the ARMS was accompanied by stringent tests for specificity. Using the ARMS, DNA from 20 normal individuals was amplified for each of the five rare  $\beta$ -mutations evaluated to check for false-positive results. In each of the normal DNA samples, only the 861 bp internal control was amplified after ARMS and no  $\beta$ -mutant bands were observed. A future extension of this ARMS study will be the development of Combine-ARMS for the rare  $\beta$ -mutations. Combine-ARMS detects two to three  $\beta$ -mutations per PCR reaction, providing even more rapid and inexpensive tests.

In conclusion, the ARMS is a sensitive and reproducible protocol for the detection of rare  $\beta$ -thalassaemia mutations. The specificity for all ARMS protocols should be carefully evaluated to prevent the production of false-positive results as this may lead to the termination of an otherwise normal or  $\beta$ -carrier fetus. Specificity of the ARMS can be achieved by increasing the stringency of the PCR reactions by using optimal concentrations of  $MgCl_2$  and primers and annealing temperature. The ARMS serves as a more quicker and cheaper

alternative to DNA sequencing in the molecular characterization and prenatal diagnosis of rare  $\beta$ -thalassaemia mutations in our Malaysian population.

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