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Novel detection of IVS I-1 G>T mutation on β-globin gene

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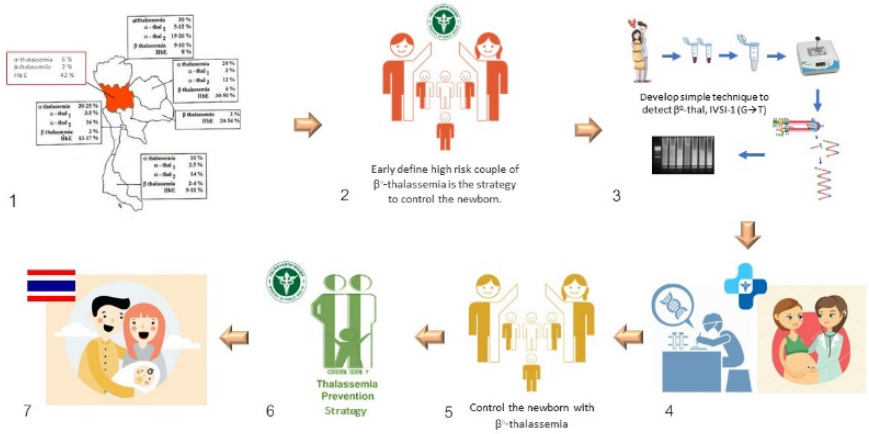
Abstract: Mutation at IVS I-1, G>T on β-globin gene is one of the β^0-thalassemia, caused severe clinical symptoms found in Thailand. Detection of this β^0-globin gene mutation in the carriers to define the risk couple is a strategy to control newborn with β^0-thalassemia. We developed the reliable Loop-mediated isothermal amplification (LAMP) assay to detect this mutation in one-tube isothermal amplification. Firstly, we designed 4 sets of LAMP primers specific to β^0-globin gene, IVS I-1, G>T mutation, then optimized the amplification condition and the DNA amount; and finally applied on wildtype and heterozygote for IVS I-1, G>T mutation. The results showed that LAMP primer set 4 was successfully differentiated between normal allele and IVS I-1, G>T mutation allele with no cross amplification. This assay could detect mutation allele using 5-10 ng DNA and completed reaction in a single temperature within 50 min. The result was in accordance with the conventional technique, multiplex Amplification Refractory Mutation System (multiplex ARMS) technique. Moreover, we found that heating block, an inexpensive equipment could replace a thermo cycler machine, to incubate LAMP reaction with one temperature. This simple, rapid and cost-effectiveness LAMP assay could be useful to identify IVS I-1, G>T mutation and other β^0-globin gene mutations in primary hospitals. Further studies are needed to develop the LAMP assay to identify other β^0-globin gene mutations mostly found in Thailand. This assay could support the national program to control the incidence of β^0-thalassemia in Thailand.

Graphical abstract:



Keywords: β^0-thalassemia, IVS I-1 detection, LAMP

1. Introduction

β-thalassemia is a common genetic disorder and a major public health concern. There are 20 types of β^0-globin gene mutations, caused severe clinical symptoms found in Thailand [1]. IVS I-1, G>T mutation is one of the six common β-thalassemia with 7.7%

prevalence in northern Thailand [1]. There is an autosomal recessive inheritance, with the very severe type is homozygous β^0-thalassemia and compound heterozygous β^0-thalassemia with hemoglobin E (β^0-thalassemia/ Hb E) has severe clinical symptoms such as jaundice caused by anemia, dark skin due to high iron deposits in the body big belly due to an enlarged liver and spleen. And in very pale patients, regular blood transfusions are required. Medical treatment cost of these severe β-thalassemia patients impacts health economics of the country. And it also affects the patient and family because the patient has low immunity, so it is prone to various complications. It is difficult for families care to the patients. In general, the gene-level diagnosis for risk couples cannot be detected at the primary hospital level. There are numerous gene amplification techniques based on the polymerase chain reaction (PCR) with expensive thermocycling equipment [2-8]. Loop-mediated isothermal amplification (LAMP) is an established nucleic acid amplification method for molecular diagnostics in the biomedical field [9-20]. This method offers sensitivity and high specificity, rapidity, accuracy, and cost-effectiveness [9]. Moreover, no thermal cycler is required for this method. The LAMP method has been used to detect several pathogens [11-19]. The result of the LAMP reaction is interpreted by DNA staining [9], turbidity [10,11], fluorescent dye [12], SYBR green [13] or pH indicator dye [14-20]. Recently the LAMP assay was introduced to genotype human genetic disease, α-thalassemia 1 [20,25] and β-thalassemia [21]. This study aimed to develop LAMP assay for identification of IVS I-1, G>T mutation on β-globin gene.

2. Materials and Methods

The study was approved by the Naresuan University Institutional Review Board, Phitsanulok Province, Thailand. Thirty blood samples including normal β-globin gene, heterozygote for β^0-globin genes, IVS I-1 G>T; cd 17, A>T; cd 41/42, -TCTT; cd 71/72, +A and abnormal hemoglobin, Hb E, cd 26 G>A were collected from Thalassemia Research Unit, Naresuan University Hospital. The normal and mutation of β^0-globin gene were detected and confirmed with standard amplification-refractory mutation system (ARMS)-PCR technique [22]. The genomic DNA was extracted from peripheral blood applying Masterpure™ (Epicenter, USA).

2.1. Primer Designing for detection of IVS I-1 G>T mutations

Primer explorer V5 software (http://primerexplorer.jp/e/) was used to design 4 sets of LAMP primers specific to β0-globin gene, IVS I-1 G>T mutation with some modifications (Table 1), and U_01317.1 reference sequence from GenBank was considered as a template. Figure 1 showed the β-globin gene location of each LAMP primer. All primers were synthesized by 1st Base Pte. Ltd. Co., Gemini Singapore Science Park II, Singapore.

Table 1. The sequences of LAMP primers for detection of IVS I-1 G>T mutation.

LAMP Primers[1]	Sequences (5′>3′)
Forward Outer (F3)	CAA CCT CAA ACA GAC ACC ATG
Backward Outer (B3)	AGA CCA CCA GCA GCC TAA G
Backward Inner (BIP)	AAC TGG GCA TGT GGA GAC AGA GAA TAG GCA GAG AGA GTC AGT
Forward Inner (FIP 1)	ACT GCC CAG GGC CTC ACC AGG AGA AGT CTG CCG TTA C
Forward Inner (FIP 2)	AGA GCC CAG GGC CTC ACC AGG AGA AGT CTG CCG TTA C
Forward Inner (FIP 3)	AAC TGC CCA GGG CCT CAC CAG GAG AAG TCT GCC GTT AC
Forward Inner (FIP 4)	AAG AGC CCA GGG CCT CAC CAG GAG AAG TCT GCC GTT AC

[1]All LAMP primer sets consist of 4 primers; BIP, FIP, F3 and B3. Primers BIP, F3 and B3 were the same sequences except FIP1-FIP4 primers with difference base sequences at 3′ end in each set.

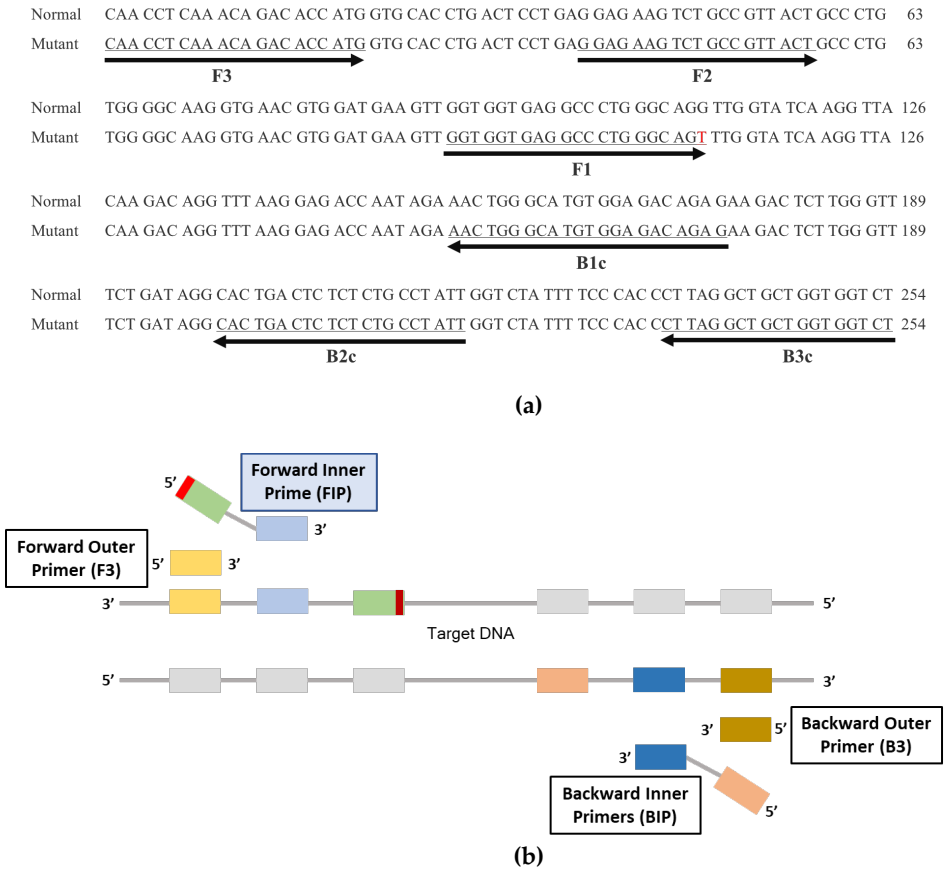


Figure 1. Sequences of LAMP primers for of IVS I-1 G>T mutation detection and their locations on β-globin gene. (a) Nucleotide sequence alignment of the target region β-globin gene in wildtype and mutant at IVS I-1 G>T. The sequences used for LAMP primers are indicated by thick arrow lines and IVS I-1 G>T mutation is in red letters. (b) Schematic representation of LAMP primers composition and location. Construction of the inner primers FIP, BIP and outer primers F3, B3 are shown. Abbreviations: FIP, Forward inner primer; BIP, Backward inner primer; F3, Forward outer primer; B3, Backward outer primer.

2.2. LAMP amplification reaction

The LAMP reaction was carried out in a total volume of 25 ◎L containing 1x Thermo-Pol buffer [20 mM Tris-HCl, 10 mM (NH4)2SO4, 10 mM KCl, 2 mM MgSO4, 0.1% Triton®-X-100], 6mM MgSO4, 1.4 mM dNTPs, 1.6 ◎mol of each forward inner primer (FIP) and backward inner primer (BIP), 0.2 ◎mol of each F3 and B3 primers, 0.32 U of Bst DNA Polymerase (New England Biolabs Ltd., UK), and 50 ng of template DNA. The LAMP reaction was incubated in thermo cycler (Kyratec, Australia) at 65 °C for 60 min. The LAMP products were analysed in agarose gel electrophoresis. The positive LAMP result appears in a ladder-like pattern on agarose gel electrophoresis.

2.3. Optimization of LAMP reaction

One of 4 LAMP primer sets that gave the best LAMP reaction resulted from previous reaction was optimized in 5 various temperatures: 62, 63, 64, 65, and 66 °C for 60 min. Then, the temperature with the best result was further performed AMP reaction with varied incubation times: 20, 30, 40, 50, and 60 min.

2.4. Sensitivity and specificity of LAMP reaction

To assess the sensitivity of LAMP technique, genomic DNA was determined at the concentrations of 1, 5, 10, 25 and 50 ng. For specificity, an optimized LAMP protocol was

performed to differentiate the IVS I-1 G>T mutation from a normal β-globin gene, other β0-globin gene mutations (cd 17, A>T; cd 41/42, -TCTT; cd 71/72, +A) and abnormal hemoglobin, (Hb E, cd 26 G>A). Visual detection was compared to 2% agarose gel electrophoresis under a UV-transilluminator.

2.5. Confirmation with multiplex ARMS technique

ARMS reaction was set up as described previously [22].

2.6. Application of LAMP reaction with heating block

The LAMP reaction was performed as described above, then incubated at 64o C for 50 min with inexpensive equipment, heating block (Major Science, Taiwan) and thermo cycler machine. Results were analysed in agarose gel electrophoresis.

3. Results

The goal of this study was to develop the rapid assay to diagnose IVS I-1, G>T, β^0-globin gene mutation.

3.1. LAMP amplification reaction

The LAMP reaction was carried out with same condition and compared to 4 sets of LAMP primer specific to IVS I-1, G>T mutation on β^0-globin gene. LAMP primer set number 1 and 3 were unable to differentiate the normal allele from mutant allele while the prime set number 2 and 4 could perform. The result is shown in Figure 2.

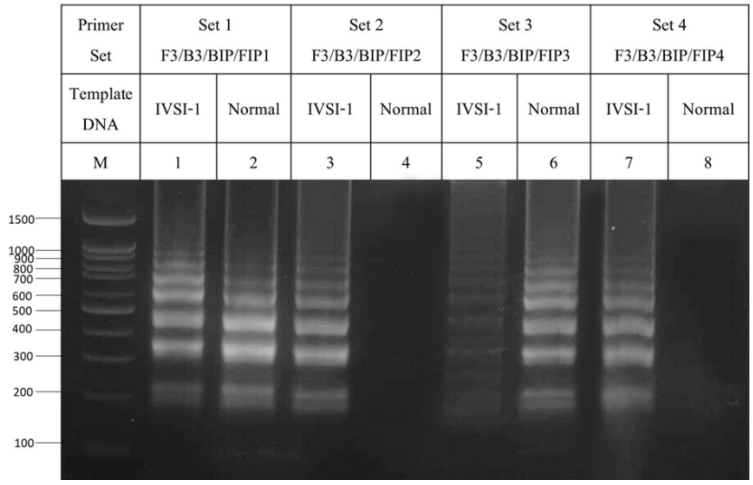
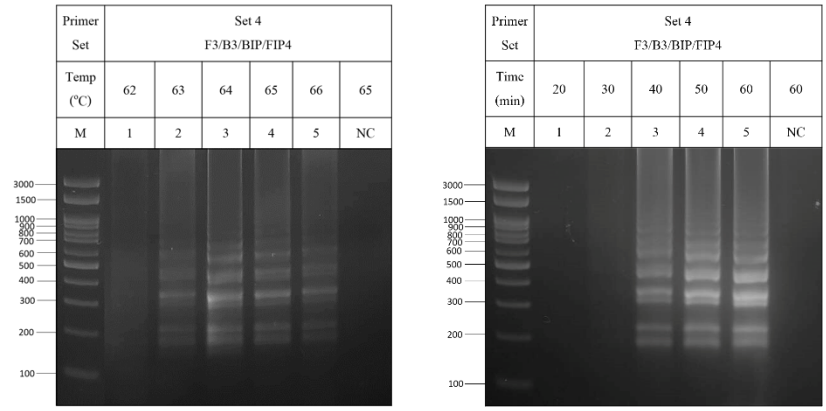


Figure 2. The ladder-like pattern results of LAMP reaction with 4 sets of LAMP primers in IVS I-1, G>T mutation and normal β-globin gene. M = 100 bp DNA Ladder (GeneDireX, USA), Lanes 1, 3, 5, and 7 were mutant DNA carried IVS I-1 G>T mutation. Lanes 2, 4, 6 and 8 were normal DNA of β -globin gene.

3.2. Optimization of LAMP reaction

The LAMP primer sets number 2 and 4 were optimized for the temperature and incubation time. The temperature of LAMP reaction was determined at the various temperatures: 62, 63, 64, 65 and 66°C. The optimum temperature of LAMP reaction was 64°C and only LAMP primer set 4 gave the best positive result as shown in Figure 3(a). LAMP primer set 4 was further used at 64°C in a various incubation times: 20, 30, 40, 50, and 60 min. The incubation time to perform LAMP reaction was 40, 50 and 60 min as shown in Figure 3(b). We summerized that the optimum LAMP condition to diagnose IVS I-1, G>T mutation on β0-globin gene was at 64°C for 50 min.



(a) **(b)**

Figure 3. (a) LAMP assay using primer set 4 for IVS I-1, G>T detection (a) Reaction temperature. Lanes 1, 2, 3, 4 and 5 were genomic DNA with mutant allele at 62, 63, 64, 65 and 66° C, respectively. Lane 6 was a negative control (NC). (b) Reaction time. Lanes 1, 2, 3, 4 and 5 were genomic DNA with mutant allele at 20, 30, 40, 50 and 60 min, respectively. Lane 6 was a negative control (NC). M = 100 bp DNA ladder.

3.3. Sensitivity and specificity of LAMP reaction

The sensitivity of LAMP reaction with LAMP primer sets 2 and set 4 were determined in genomic DNA at the concentrations of 1, 5, 10, 25 and 50 ng as shown in Figure 4(a). LAMP primer set 2 could identify IVS I-1, G>T mutation at 25-50 ng of genomic DNA while set 4 could identify at 5-50 ng. We chose LAMP primer set 4 performed at 64°C for 50 min to diagnose IVS I-1, G>T mutation on β0-globin gene. The specificity of LAMP primer set 4 was performed to differentiate IVS I-1, G>T mutation from a normal β-globin gene and other β*0*-globin gene mutations *(*cd 17, A>T; cd 41/42, -TCTT; and cd 71/72, +A*)*. The result showed that cross detection was not found (Figure 4b).

3.4. Confirmation with multiplex ARMS technique

Multiplex ARMS reaction was performed to comfirm the LAMP method. The positive ARMS of IVSI-1, G>T mutation showed band at 281 bp and internal control at 861 bp in Lane 1, while Lanes 2-5 showed only 861 bp DNA band of internal control. the 281 bp of IVSI-1, G>T mutation DNA band were absent. Lane 6, a negative control confirmed that there was no contamination in this reaction. The result was shown in Figure 5(a) and 5(b).

3.5. Application of LAMP reaction with heating block

This LAMP method was applied to incubate the reaction with a heating block with the same condition. The LAMP result was in accordance with the incubating reaction with an expensive thermocycler equipment as shown in Figure 6.

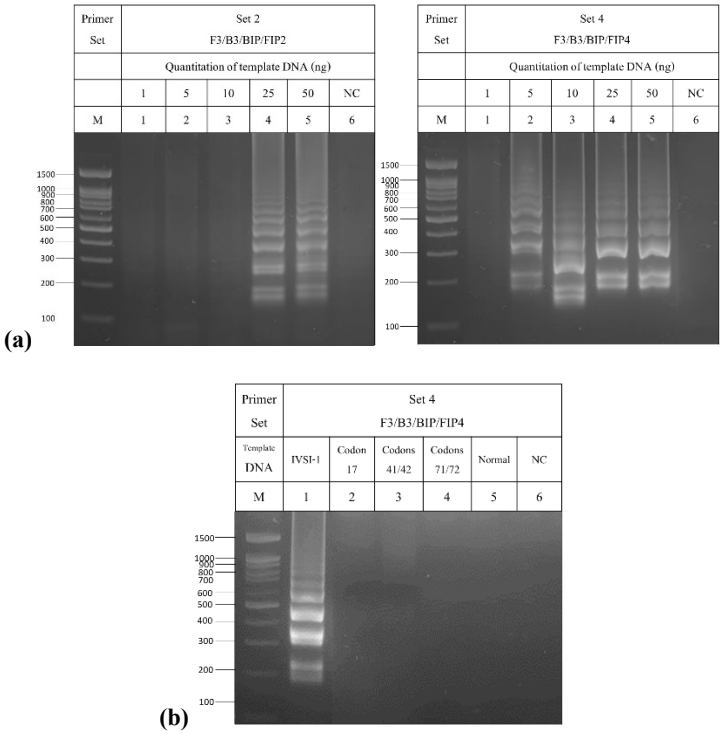


Figure 4. Sensitivity and specificity of LAMP for detection of IVS I-1, G>T detection (a) Sensitivity of LAMP assay using primer sets 2 and 4. Lanes 1, 2, 3, 4 and 5 were the genomic DNA with mutant allele at 1, 5, 10, 25 and 50 ng, respectively. Lane 6 was a negative control (NC). (b) Specificity of LAMP assay with primer set 4. Lane 1 was the mutant allele IVS I-1, G>T mutation, Lane 2 was cd 17, A>T mutation, Lane 3 was cd 41/42, -TCTT mutation, Lane 4 was cd 71/72, +A mutation, Lane 5 was a normal allele of β-globin gene, Lane 6 was a negative control (NC). M = 100 bp DNA ladder.

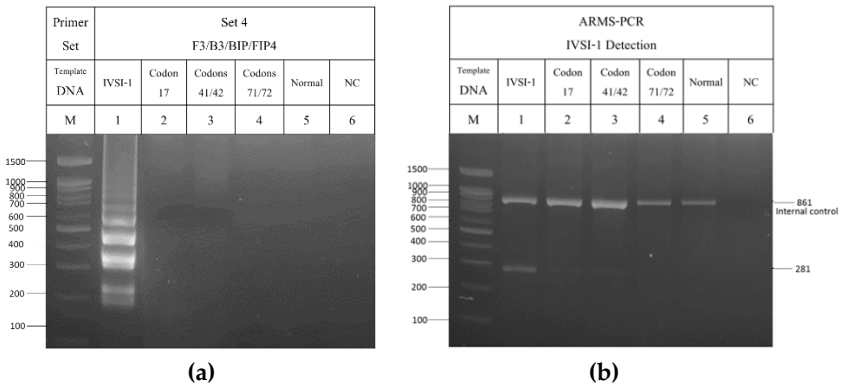


Figure 5. Confirmation of β0-thal mutant genotyping, IVS I-1, G>T mutation by LAMP reaction with standard technique, multipex ARMS. (a) LAMP assay using primer set 4 for IVS I-1, G>T detection. Lane 1 was the mutant allele of IVS I-1, G>T mutation in ladder-like pattern, Lane 2 was cd 17, A>T mutation, Lane 3 was cd 41/42, -TCTT mutation, Lane 4 was cd 71/72, +A mutation, Lane 5 was normal allele to β-globin gene, Lane 6 was a negative control (NC). (b) ARMS assay. Lane 1 was the mutant allele with IVS I-1, G>T, Lane 2 was cd 17, A>T mutation, Lane 3 was cd 41/42, -TCTT mutation, Lane 4 was cd 71/72, +A mutation, Lane 5 was normal allele to β-globin gene, Lane 6 was a negative control (NC). M = 100 bp DNA ladder.

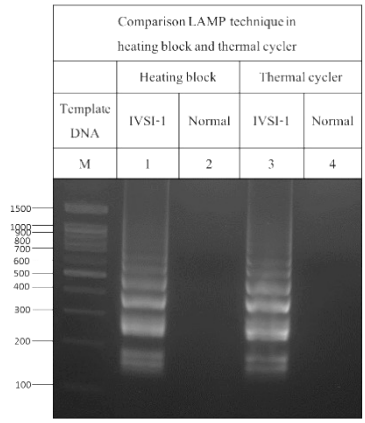


Figure 6. Comparison of LAMP reaction performed in heating block and thermoclycler. Lanes 1 and 3 were the IVS I-1, G>T mutant allele, Lanes 2 and 4 were normal β-globin allele. M = 100 bp DNA ladder.

4. Discussion

Recently, LAMP assay was firstly applied to genotype mutation of genetic disease; β-thalassemia [21] and α-thalassemia mutation [20, 25]. Gill *et al*, developed ARMS-LAMP method to detect IVS II-1, G>A mutation, the highest frequency in the north of Iran [21]. Unfortunately, their method was unable to differentiate between a normal allele and IVS II-1, G>A mutation allele. We designed 4 sets of LAMP primers specific to IVS I-1, G>T mutation on β-globin gene with some modifications. The results showed that primer set 4 with base modified at 3′ end of FIP2 and FIP3 primers (Table 1 and Figure 2) were more specific to IVS I-1, G>T mutation. The optimal condition of the LAMP method to detect IVS I-1, G>T, β^0-globin gene mutation was at 64° C for 50 min. This LAMP assay could detect using 5-10 ng of DNA that was 25 times less than that of PCR reaction. The diagnostic ability of LAMP in the present study was validated (Figure 4) and compared to the conventional ARMS method. As shown in Figure 5, the diagnostic power of LAMP was identical to the ARMS method. Moreover, we found that heating block, an inexpensive equipment could replace an expensive thermo cycler machine, to incubate LAMP reaction with one temperature as shown in Figure 6.

5. Conclusions

This study was successfully to develop LAMP assay to differentiate between a normal allele and IVS I-1, G>T mutation on β^0-globin gene. The novel LAMP assay can be completed within 50 min using a thermocycler machine or a heating block. Further studies are needed to develop the LAMP assay to identify other β^0-globin gene mutations mostly found in Thailand. This simple, rapid and cost-effectiveness LAMP assay could be useful to identify IVS I-1, G>T mutation and other β^0-globin gene mutations in primary care units or rural hospitals without access to thermocycler machines. This assay could support the national program to control the incidence of β^0-thalassemia in Thailand.

Author Contributions: Conceptualization, S.M; methodology, S.M., S.S. and P.H.; validation, S.S. and P.H.; formal analysis, S.S. and P.H.; investigation, P.H.; resources, S.M.; data curation, P.H.; writing—original draft preparation, S.S.; writing—review and editing, S.M.; visualization, S.M.; supervision, S.M.; project administration, S.M.; funding acquisition, S.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by Board Naresuan University Institutional Review Board (COA no.174/2019, IRB no.0037/62 and date of approval; 22 April, 2019 and extended to 22 April 2022).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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