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Optimization of Pyrosequencing Method to Detect IVS1-NT5 β-Globin Gene Mutation in β-Thalassemia

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Abstract : β-thalassemia incidence in Indonesia is reaching 2.500 cases per year, so that it is still a threat for Indonesian's society due to the high therapeutic cost. Prenatal diagnosis for detecting β-globin gene mutation is important to be developed in order to decrease β-thalassemia incidence. Pyrosequencing, a new DNA sequencing method, owns the ability to detect DNA mutation quickly for prenatal diagnosis importance. A specific β-globin gene mutation for Indonesian population is needed to make the mutation detection process become more effective, which is the mutation in the intervening sequence 1 nucleotide 5 (IVS1-NT5) region. Hence, the objective of this study is to know the optimal pyrosequencing condition to detect the IVS1-NT5 β-globin gene mutation in β-thalassemia. The sample used was the stored DNA material which the mutation had been detected using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for pyrosequencing optimization. There were two steps of optimization, both in the amplification step using polymerase chain reaction (PCR), and pyrosequencing step. The obtained result in amplification step was 55°C for primer annealing temperature. In pyrosequencing step, the enzyme and substrate storage condition, cartridge's needle performance, and quality of sequencing primer were observed as factors which influence peak formation in the pyrograms, Nevertheless, due to the absence of the proper peaks on the pyrograms of this study, the pyrosequencing method to detect IVS1-NT5 is not yet optimized.
Keywords : β-globin, β-thalassemia, IVS1-NT5, Pyrosequencing.

Introduction

Thalassemia is a syndrome caused by mutation in the globin gene, which decrease the globin chain synthesis. Thalassemia is classified into α-thalassemiadue to deficiency of α-globin chain synthesis, and β-thalassemia due to deficiency of β-globin chain synthesis[1,2]. The number of β-thalassemia carriers in Indonesia reach 3-10%. According to the data and the number of Indonesian inhabitant and Indonesian birth rate, it is

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estimated that each year, 2.500 children will born with β-thalassemia[3]. This thing shows that thalassemia is still a threat for Indonesian's society, because the therapeutic cost is reaching 200 until 300 million rupiah each year for every patient, and it has to be done by the patients throughout their lifetime. Therefore, thalassemia prevention strategy, such as β-thalassemia prenatal diagnosis, must be developed in order to decrease the β-thalassemia incidence.

The molecular defect on β-globin gene mutation can be different and specific in every population and particular ethnic[4–6].Hence, β-thalassemia prenatal diagnosis in Indonesian population can be more effective if it is specified for the most common β-globin gene mutation type in Indonesia, where 54% happens in the intervening sequence 1 nucleotide 5 (IVS1-NT5) region (G→C)[4,7].The DNA sequencing method becomes the primary method in the detection of point mutation for β-thalassemia diagnosis, due to its ability to quickly know the exact sequence of the studied DNA[8]. Nowadays, pyrosequencing appears as a new promising sequencing method, which gains a big potency to be used in the routine practice of β-thalassemia prenatal diagnosis. Pyrosequencing can perform faster than Sanger sequencing, the most common sequencing method. Pyrosequencing can give the sequencing signal immediately after reaching the downstream of the primer and analyze 96 samples at the same time[9,10].

There are cascade of steps which have to be done before the pyrosequencing process starts, named as pyrosequencing workflow, which only take 3-4 hours to be completed. The cascades consist of the assay design for 5 minutes, DNA amplification using Polymerase Chain Reaction (PCR) for 2 hours, sample preparation for 15 minutes, then pyrosequencing for approximately 10 until 60 minutes[11].Nevertheless, an optimal pyrosequencing condition must be achieved in order to make this process fast and correct. Therefore, this research is important to be conducted to know the optimal pyrosequencing condition for detecting the IVS1-NT5 β-globin gene mutation in β-thalassemia.

Experimental

This study used descriptive design with one variable, the optimal condition of pyrosequencing method. The optimal condition was observed both in the amplification and pyrosequencing steps. In the amplification step, the primer annealing temperature was observed; while in the pyrosequencing step, factors which influence the peak formation in the pyrograms were observed. This study was conducted in Molecular Genetics Laboratory, Faculty of Medicine, Universitas Padjadjaran from August until November 2016.

DNA Samples

The subject of this study was the stored DNA material which the mutation type had been detected using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method. It was isolated from the blood of children with β-thalassemia in 2008, and was stored in Molecular Genetic Laboratory, Faculty of Medicine, Universitas Padjadjaran. The inclusion criterion was the DNA which hadβ-globin gene mutation in IVS1-NT5 region, whilethe exclusion criterion was DNA with low concentration (less than 25ng/μL). The instruments used to measure the DNA concentration were NanoDrop 2000 spectrophotometer (Thermo Scientific Inc., MA, USA) and NanoDrop 2000 software(Thermo Scientific Inc., MA, USA). There were randomly selected 13 DNA samples which met the requirements and included in this study.

Polymerase Chain Reaction (PCR)

The PCR primers used in this study were synthesized by Integrated DNA Technologies, Inc. (Iowa, USA). The forward primer was IVS1nt5-F (5'-AAG GTG AAC GTG GAG ATA GTT-3'), while the biotinylated reverse primer was IVS1nt5-R (5'-biotin-TEG/CTC CAC ATA CCC AAT TTC TAT TAA TCT-3'). Reactions were carried out using 1000™ Thermal Cycler (Bio-Rad Laboratories, USA). Each PCR tubes contained 25μL of mixtures which consist of: 12.5μL PCR master mix 2X, 2.5μL coralloid concentrate 10X, 5μL Q solution 5X, 1μL primer forward 20μM, 1μL primer reverse 20μM, 2μL nuclease free water, and 1μL DNA. All PCR reagents used in this study were using Pyromark PCR Kit (Qiagen N.V., Hilden, Germany).

PCR Condition and Optimization

The PCR condition for this reaction was started with 1 cycle of initial denaturation phase at 95^{o}C for 15 minutes, followed by 40 cycles of denaturation phase at 94^{o}C for 30 seconds, 40 cycles of annealing for 30

seconds, 40 cycles of extension phase at 72^{o} for 30 seconds, and terminated by the final extension phase at 72^{o}C for 10 minutes. The annealing temperature was obtained from the optimization process at 55^{o}C, 57^{o}C, 59^{o}C, and 61^{o}C.

Electrophoresis

The amplification products which obtained from PCR must be evaluated firstly before utilized in pyrosequencing by using electrophoresis in order to know the quality of the PCR products. Electrophoresis process was done by resolving 5µL of PCR products into the 1.5% agarose gel. The agarose gel was made using 0.45g agarose powder (1st BASE, Singapore), mixed with 30mL TAE buffer 1X, and stained with 0.5µL gel red (Biotium, Inc., CA, USA). Before the electrophoresis ran, 2µL KAPA Universal DNA ladder (Kapa Biosystems, MA, USA) was put in the first well to determine the size and quantity of the PCR products. The electrophoresis process ran at 90V, 78mA, and 7W for 40 minutes using EPS 3501 XL Electrophoresis Power Supply (Amersham Pharmacia Biotech Inc., NJ, USA). The agarose gel was then visualized with ultraviolet transilluminator G-Box Range Fluorescence Image Capture (Syngene Bioimaging Private Ltd, India) and read using GeneSnap from SynGene software (Syngene Bioimaging Private Ltd, India).

Pyrosequencing Sample Preparation

All pyrosequencing materials, steps and instruments used in this study were referred to the pyrosequencing protocol from QIAGEN N.V., Hilden, Germany. The first step of sample preparation in pyrosequencing was preparation of pyrosequencing working station by filling the four plates with sterile aquabides, denaturation buffer, washing buffer, and ethyl alcohol (EtOH) 70%. This step was followed with the DNA immobilization to streptavidin beads. The DNA samples from PCR products must be prepared in pyrosequencing mix consisting of streptavidin beads, PyroMark binding buffer, nuclease free water, and the PCR products themselves, with total volume of 80µL for one sample (Table 1).

Table 1: Master Mix Components for DNA Immobilization.

Master mix for DNA Immobilization	1 sample	n samples (n= …)
Streptavidin beads	3 µL	-
PyroMark Binding Buffer	37 µL	-
PCR Product	15 µL	-
Nuclease Free Water	25 µL	-
Total Volume	**80 µL**	-

The mix was then placed in the PCR plate, sealed, and agitated for 10 minutes with 1,400 rpm. While the samples were agitating, the PyroMark annealing buffer and sequencing primer must be prepared and put in Q96 plate. The sequencing primer used in this study was IVS1nt5 S (5'-TGA GGC CCT GGG CAG-3') (Integrated DNA Technologies, Inc., Iowa, USA). The next step was vacuum processes in pyrosequencing working station to prepare a single-stranded pyrosequencing template DNA. The vacuum processes started with lowered the filter probes into PCR plates for 15 seconds, transferred to EtOH 70% for 5 seconds, transferred to deaturation solution for 5 seconds, transferred to wash buffer for 5 seconds, and raised the tool up and back to back to 90^{o} vertically for 5 seconds to drain. After that, the samples needed to be heating by incubating them at 80^{o}C for 2 minutes, and then cooled at room temperature to let the sequencing primer to anneal.

Pyrosequencing reaction

PyroMark Q96 ID System and PyroMark software (Qiagen N.V., Hilden, Germany) were used to perform the pyrosequencing. First, the assay was designed using the PyroMark software prior the running of pyrosequencing; there were the sequence to analyze (GTTG/CGTATCAAGGTTACAAGACAGGTTTA), forward primer, and reverse primer. The software then showed the determined amount of pyrosequencing reagents to be load in the cartridge. The reagents were the enzyme; substrate; and nucleotide A, C, G, and T. The pyrosequencing then ran and the result, the pyrograms, were read and interpreted on PyroMark software.

Data Analysis

The obtained data during the optimization processes were qualitatively described using the images captured from the SynGene software (Syngene Bioimaging Private Ltd, India) and PyroMark software (Qiagen N.V., Hilden, Germany). The analysis was constructed based on the PyroMark Q96 ID user manual and pyrosequencing troubleshooting criteria from nature protocol[12–14].

Ethical Approval

Ethical approval of this study was obtained from the ethical committee of Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia, on 15thJuly 2016. The project identification code was 630/UN6.C1.3.2/KEPK/PN/2016.

Results

PCR Optimization

The optimized PCR was known from the primer annealing temperature, which the result was visualized using electrophoresis. In electrophoresis, the PCR product should give one strong band with minimal excess of primers[12]. In this study, the optimal temperature for the primer to anneal was 55^{o}C (Figure 1). This anneling temperature was then used to did the PCR for all 13 samples, and there were no unspecific bands found (Figure 2).

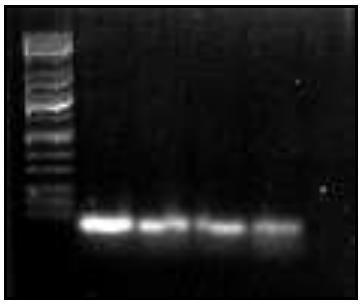

Fig. 1: Electrophoresis visualization of primer annealing temperature optimization at 55^{o}, 57^{o}, 59^{o}, and 61^{o} C (left to right).

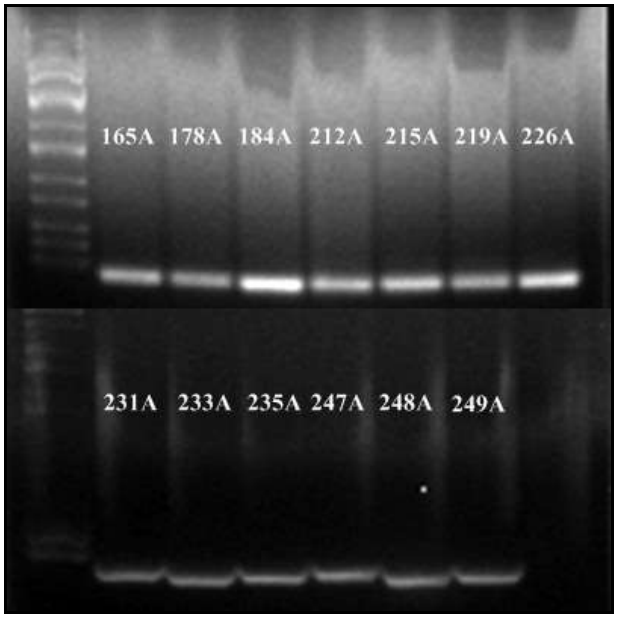

Fig. 2: The visualization of all samples used in this study on agarose gel. There were no unspecific bands found.

Pyrosequencing Optimization

The first pyrosequencing optimization was conducted based on the protocol on one sample, and the result was as follows (Figure 3). After the pyrograms was analyzed, it is found that there was no peak detected, which probably because of the damage of the reagents used due to improper storage[12]. Reagents should be frozen at -20^{o}C for long term storage, until expiry date on kit (less than one year)[15]. In this study, the utilized reagents, the enzyme and the substrate, were stored from July 2015. It meant that the storage was more than one year. Because of that, the second optimization was done using new enzyme and substrate (Figure 4). The undetected peak on nucleotides sequence which found during the second optimization was possibly caused by the blocked of the cartridge's needle, which made the substrate and/or enzyme had not been dispensed. This consideration was based on the presence of peak on the substrate dispensation, so that it could be assumed that the enzyme and substrate worked well[14]. The third optimization was then performed by changing the old cartridge with the new one (Figure 5). Nevertheless, there were still no peaks detected after the third optimization. This result might indicate a blocked in the needle of the new cartridge, but there were also other possibilities which caused undetected peak such as wrong sequencing primer or sequence to analyze, unheated plate, biotin degradation, and contaminated samples[13–15].

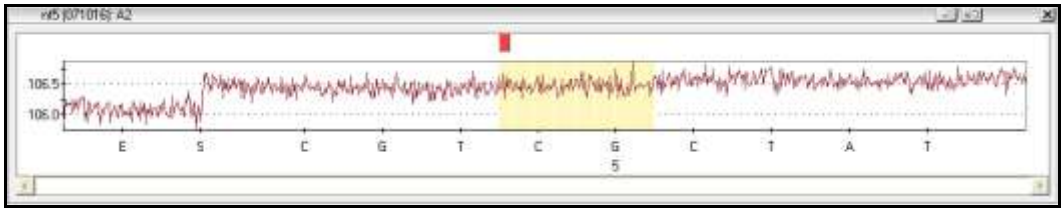

Fig. 3:The pyrograms of the first optimization with no peaks detected.

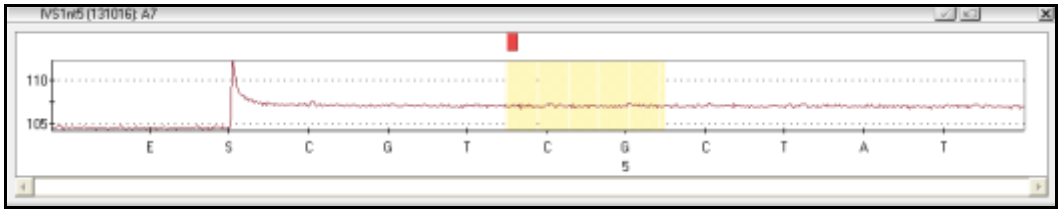

Fig. 4:The pyrograms after the second optimization.

There was a peak detected on substrate dispensation, but no peaks detected on nucleotides sequence.

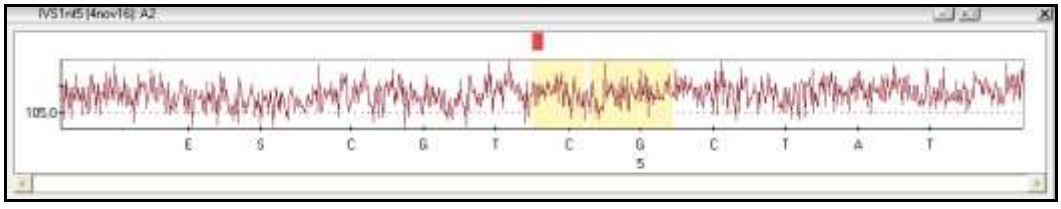

Fig. 5: The pyrograms of the third optimization with no peak detected.

Discussion

The result of pyrosequencing is determined using the peaks generated in the pyrogram, therefore the peak quality reflects the quality of pyrosequencing method. The peaks appear as a result from the light formation in pyrosequencing reaction, which is detected by a charge coupled device (CCD) camera in pyrosequencing instrument[15]. Nevertheless, the peaks are not always detected during the pyrosequencing reaction. There are several factors contributing the peaks formation which can be found in each step of pyrosequencing cascade; the assay design, amplification, sample preparation, and pyrosequencing steps. In the assay design step, attention must be given to the sequence to analyze, a certain target sequence which contain the mutation of interest[13]. A faulty of the peak formation in nucleotide sequence can be caused by the wrong sequence to analyze which entered to the pyromark software[12].

During the amplification step, the PCR step, there are three things to be considered. First consideration is the quality of the biotin-labeled primer. Biotin is important to create the single strand DNA as template for pyrosequencing reaction, therefore biotin degradation will lead to the failure of the reaction and cause undetected peak[12,13]. In this study, the biotin-labeled primer was ordered from Integrated DNA Technologies, Inc., Iowa, USA; so that it can be assumed that the quality of the biotin was good and the only way to test this problem is reorder new biotin. Second consideration is the primer annealing temperature. The primer annealing temperature is different for different gene, therefore it needs to be optimized in order to achieve an optimized PCR condition. PCR must be optimized to increase the quality of the PCR product which later used as pyrosequencing sample, so that it will highly influence the peak formation and the height of the peak[12]. In a study about CYP2D6 allele identification using pyrosequencing, it is proven that the highest peak is formed at the optimal primer annealing temperature[16]. However, the height of the peak in this study cannot be analyzed because there is no peak detected, so that the observation about the influence of the primer annealing temperature to the peak height is limited. Third consideration is the contaminant that can be found during the PCR preparation, such as the addition of the third primer which will generates unspecific bands or a detectable smear on agarose gel during electrophoresis[13]. In this study, there were no unspecific bands formed on electrophoresis result, as has been discussed previously.

In the sample preparation step, there are also three things which must be concerned; the plate incubation, sequencing primer, and reagent storage condition[12,14]. The pyrosequencing plate must be heated to 80^{o}C so that the sequencing primer can anneal into the DNA template. In this study, the plate has been heated to 80^{o}C for 2 minutes by referring into pyrosequencing protocol from QIAGEN N. V., Hilden, Germany[12]. Hence, this possibility does not become the primary priority. The sequencing primer which is added into the sample should be verified that it is in accordance with the sequence to analyze[14]. In this study, the sequencing primer is ordered from Integrated DNA Technologies, Inc., Iowa, USA, so that the possibility of the presence of mistake is low. Nevertheless, redesigning the sequencing primer must be considered if non-specific peaks appear. The pyrosequencing reaction relies on the enzymatic reaction. Therefore, pyrosequencing reagents particularly the enzyme and substrate need to be stored correctly by freeze them at-20^{o}C for long term storage, in order to maintain their quality[15]. The wrong reagent storage condition will cause undetected peak as in the first optimization of this study.

Attention of cartridge condition must be considered in the last step, the pyrosequencing step, if there is no significant peak detected[12]. The reagents and nucleotides should be filled correctly in the cartridge's well. An obstruction or damage of the cartridge's needle should be checked to avoid blockage in the cartridge. A blocked cartridge will cause a failure in enzyme and substrate dispensation which cause no detected peak[14]. The result of the second and third optimization in this study is possibly caused by the blockage of the cartridge's needle.

Based on the findings found above, the optimal condition of pyrosequencing is determined by several factors. In the amplification step, the factor is primer annealing temperature. In pyrosequencing step, the factors are enzyme and substrate storage condition and the cartridge's needle performance. All of the factors above are proven in influencing the peak formation in the pyrograms. Nevertheless, there is a limitation of this study, which is the lack of time available to finish the study, so that there is still no peak detected on the pyrograms. Therefore, the pyrosequencing method to detect IVS1-NT5 β-globin gene mutation is not yet optimized. This research may be continued by referring into pyrosequencing troubleshooting criteria from PyroMark Q96 ID user manual, which focus on primer labeling with biotin in the following research.

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References

1. V. Kumar, A.K. Abbas, N. Fausto and JC. Aster, Robbins and Cotran Pathologic Basis of Disease. Saunders, Elsevier Inc., Philadelphia, Edn. 8, pp. 648-652 (2010).
2. A.J. Marengo-Rowe, *Proc. Bayl. Univ. Med. Cent.*, **20**, 27 (2007); doi:10.1080/08998280.2007.11928230.
3. Health Technology Assessment Indonesia, Pencegahan Thalassemia. Dirjen Bina Pelayanan Medik Kementrian Kesehatan Republik Indonesia, Jakarta, pp. 6–15 (2009).
4. S. Fucharoen and P. Winichagoon, *Southeast Asian J. Trop. Med. Public Health*, **23**, 647 (1992).
5. S.H. Ansari, T.S. Shamsi and M. Ashraf, *Int. J. Mol. Epidemiol. Genet.*, **2**, 403 (2011).
6. W. Wang, E.S.K. Ma, A.Y.Y. Chan, D.H.K. Chui and S.S. Chong, *Clin. Chem.*, **49**, 800 (2003); doi:10.1373/49.5.800.
7. M. Tamam, S. Hadisaputro, S. Sutaryo, I. Setianingsih, R. Astuti and A. Soemantri, *J. Kedokteran Brawijaya*, **26**, 48 (2010); doi:10.21776/ub.jkb.2010.026.01.9.
8. J. Old, C.L. Harteveld and J. Traeger-Synodinos, Molecular Diagnosis, Thalassaemia International Federation, Cyprus, 2 (2012).
9. B. Gharizadeh, M. Ghaderi and P. Nyren, *Seibutsu Butsuri.*, **47**, 129 (2007); doi:10.2142/biophys.47.129.
10. A. Chowdhury, S.B.I.N. Mannan and R.M. Mazumdar, *Int. J. Life Sci. Pharma Res.*, **2**, 65 (2012).
11. N. Hochstein, D. Honsel and C. Kappmeier, Pyrosequencing ® and its applications Sample & Assay Technologies, Genetic Testing (2010).
12. QIAGEN, PyroMark ® Q96 ID User Manual Sample & Assay Technologies, Vol. 5, pp. 78-84 (2016).
13. J.L. Royo, M. Hidalgo and A. Ruiz, *Nature Publishing Group*, **2**, 1734 (2007).
14. J. Tost and I.G. Gut, *Nature Publishing Group*, **2**, 2265 (2007).
15. AJ. McKnight, Pyrosequencing Protocol, Pyrosequencing AB, Sweden, Edn. 1, pp. 1–16 (2004).
16. E. So, Determination of CYP2D6 Gene Copy Number by Pyrosequencing, Vol. 51, pp. 522–31 (2005).

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