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# Making of the A3243g Mutant Template Through Site Directed Mutagenesis as Positive Control in PASA-Mismatch Three Bases

**Iman P. Maksum[1]*, Athiyah Farhani [1], Saadah D. Rachman[1] and Yohanis Ngili[2]**

**[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Bandung, Indonesia**
**[2]Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Cenderawasih, Jayapura, Indonesia**

***Corres. Author: ip_maksum@unpad.ac.id**
**Telp. +62-22-2507873; Fax: +62-22-2507873**

**Abstract:** Nucleotide mutation at position 3243 that changes adenine to guanine nucleotide (A3243G) on $tRNA^{Leu(UUR)}$ gene in mtDNA is the most discovered mutation and is expressed in phenotype MIDD (*Maternally Inherited Diabetes and Deafness*) and MELAS (*Mitochondrial myopathy, Encephalopathy, Lactic Acidosis, and Stroke-like episodes*). The A3243G mutation was heteroplasmy, so it was difficult to analyze with low levels, especially in the blood. Some of methods have limited sensitivity and potentially cause mistake. In previous research has been used PASA-mismatch three bases, but it was still less validity, because it didn't have positive control of the A3243G mutant. One of the method commonly used to make mutant template is site directed mutagenesis (SDM).This research aims to make the A3243G mutant template by SDM which will be used as positive control in PASA-mismatch three bases and to determine optimization condition of SDM. Normal mtDNA was isolated from lymphocytes, then adenine base in position 3243 of $tRNA^{Leu(UUR)}$ gene was mutated into guanine base and amplified using mutant forward primer $Mt3243_F$ and reverse primer $Mt3243_R$. Determination of PCR optimum condition was done including varied annealing temperature and mutant primer concentration. Then, to optimize mutant template concentration which can be detected by PASA-mismatch three bases. The presence of the A3243G mutant template was confirmed by direct sequencing. The result of SDM showed the A3243G mutant template with size of 489 bp in the optimize of annealing temperature and ratio of mutant primer to normal primer were 74 °C and 3:1, respectively. Mutant template with 100× optimum dilution can be used as a PASA-mismatch three bases positive control in A3243G mtDNA mutation analysis.
**Keywords**: Heteroplasmy, PASA, site directed mutagenesis, A3243G, mutant template.

## Introduction

Among the diseases caused by changes in the mitochondrial genome, most studies to date has focused on A3243G mutation in $tRNA^{Leu(UUR)}$ gene[1]. Several studies on mtDNA gene mutations associated with type 2 diabetes have been conducted in various populations, such as Japan, Korea, China, France, Australia, England, Netherlands, Poland, and Indonesia. There are several mutations that have been reported, of which the most commonly found A3243G mutations in the $tRNA^{Leu(UUR)}$gene which is heteroplasmy[2-9].

The method has been used to detect the presence of mutations now have limited sensitivity and this could potentially lead to misclassification of patients especially those with a low level of heteroplasmy[10]. Previous studies have used PASA-mismatch three bases with the forward primer design $D_{M3}$ mutants were obtained from the previous researchers[11]. From these studies a problem arises, that the A3243G mutation had been detected by PASA-mismatch three bases do not have strong validity, so it is necessary to use a positive control mutant A3243G, during which only use positive samples A3243G mutation of type 2 diabetes patients are detected with another method, namely the PCR-RFLP.

Based on this background, the research will be conducted with the A3243G mutant template fabrication with site directed mutagenesis (SDM) method to be used as a positive control PASA-mismatch three bases, with the hope that with the positive control mutants could be avoid false-positive results. In this study, also carried out the determination of the optimum conditions and the determination of the optimum concentration of SDM template mutant products that can be detected by PASA-mismatch three bases.

## Materials and Methods

### *Isolation of normal mtDNA template of lymphocytes cells*

Lymphocytes cell obtained by inserting 200 µL of blood samples into 1.5 mL micro eppendorf tube, washed with 1000 µL TE buffer (tris-HCl 10 mM pH 8,0; EDTA 0,5 mM pH 8,0), centrifuged at 12,000 rpm for 1 min, at 0 ºC and the supernatant was discarded. Washing is done repeatedly until become a white pellet. White pellets added with 264 mL $ddH_2O$, 30 mL lysis buffer consisting of 50 mM Tris-HCl pH 8.5, 1 mM EDTA pH 8.0, and 0.5% Tween-20. Then, added 6 µL proteinase K 0.04 mg/mL. The reaction mixture was incubated at 56 ºC for 1 hour and incubated at 95 ºC for 5 min in the incubator. After incubation, the reaction mixture was centrifuged at 20,000 rpm for 5 min and then the supernatant was taken and transferred to another tube for SDM reaction.

### *Design primer directed mutagenesis*

Directed mutagenesis primer design for A3243G mutant template model is done using *Perlprimer* software to facilitate simulation of the determination annealing temperature ($(T_m–5)^oC$). Mutant forward primer was designed to contain the desired mutation is located in the center of the primers. Both forward and reverse primer has length of about 20-30 nucleotides, containing at least 40% GC, no self-complementary bases G/C, especially at the 3' end, and has adjacent $T_m$. Primers were designed first is mutant forward primer. Annealing temperature in PCR determined look $T_m$ of each primer and primer combinations $T_m$ could be determined using *Perlprimer* application by entering base sequence of both the forward primer and reverse primer. Several alternative design forward and reverse primers were tested with software NCBI/BLAST (*Basic Local Alignment Search Tool*) is done on-line to get non-specific attachment region information. Design primers were selected that had number of non-specific attachment of the least and the homologous percentage is less than 50%. Primer designed to be named $Mt3243_F$ for forward primer and $Mt3243_R$ for reverse primer.

### *Making A3243G mutant by directed mutagenesis*

A3243G mutant template will be created through the process of PCR using normal mtDNA isolated from lymphocytes cells as template. The reaction mixture with total volume of 25 µL consisting of 11.3 mL $ddH_2O$, 2.5 µL $MgCl_2$ 15 mM, 2.5 µL dNTP 200 µM, 2.5 mL *Pfu Turbo* 10× buffer, 0.5 mL primer $Mt3243_F$ 20 pmol/µL, 0.5 µL $Mt3243_R$ primer 20 pmol/µL, 45 ng or equivalent to 5 µL normal mtDNA template, and 0.2 mL *Pfu Turbo* DNA polymerase 5u/ µL.

PCR reaction started with denaturation at 95 ºC for 1 min. Amplification will be performed by 30 cycles of denaturation at 95 ºC for 30 sec, annealing for 1 min by optimizing the annealing temperature (55-74°C) and optimization of the ratio of mutant primer on normal primer (1:1, 2:1 and 3:1 ) and elongation at 68 ºC for 10 min. PCR reactions ended with a post-elongation at 68 ºC for 5 min (12).

PCR products were electrophoresed with 1% agarose gel (w/v) using Mini Sub™ DNA Electrophoresis Unit (Biorad). The process of electrophoresis was performed in 1×TAE buffer as a conductor medium voltage at 60 volts, the current is 100 mA, for 60 min. DNA standards or markers used was 100 bp ladder. The results of electrophoresis was visualized with UV light.

### *A3243G mutation confirmation by direct sequencing*

Direct sequencing performed for the analysis of mutant mtDNA base sequence amplification results of SDM to saw if the results of the PCR product is already contained A3243G mutant or not. To prepare templates that will read the order, use the same method for the preparation of template mutant, which directed mutagenesis via PCR amplification products 489 bp with primers $Mt3243_F$/$Mt3243_R$ (Fig. 1), SDM template results sent to Macrogene (Korea) for the direct sequencing. Readings of base sequences used reverse primer $Mt3243_R$.

Results of analysis of direct sequencing performed with DNASTAR software, with the applications EditSeq and MegAlign. EditSeq used to enter the base sequences data that are known nucleotide sequence number that read. All data will be processed by DNASTAR stored as file *seq*. Data in the form *seq file* then aligned in MegAlign application to determine the similarity between the sequence of nucleotide bases. Homology analysis is done by comparing the nucleotide sequence of the sample results of direct sequencing with the standard sequence of *Cambridge* on MegAlign program by choosing menu clustal method.

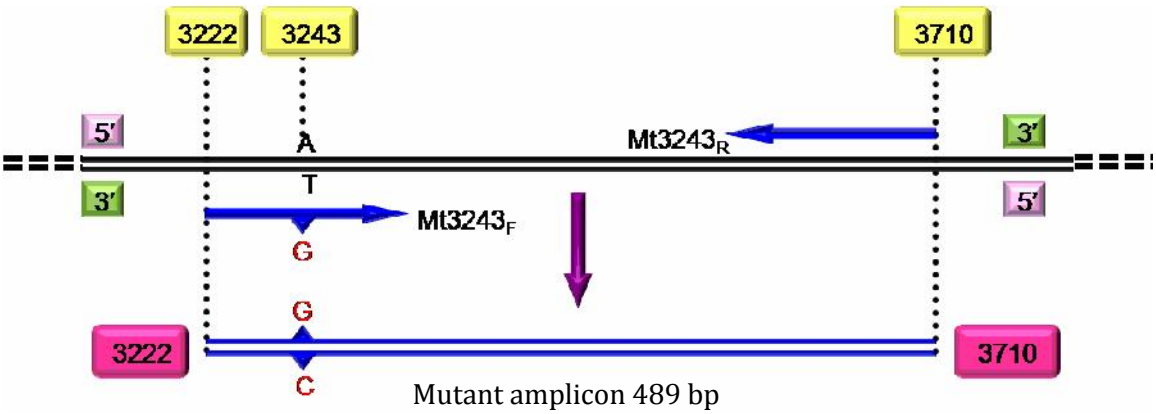


**Fig 1**. Design of site directed mutagenesis

### *Concentration optimization of A3243G mutant as template on the PASA-mismatch three bases*

A3243G mutant derived from the SDM, was used as a template for the PASA-mismatch three bases. To find out how the concentration of the mutant template, then do optimization with various dilution 1x, 10x, 100x, and 1000x. Reagent composition of PASA-mismatch three bases, namely the reaction mixture with total volume of 25 µL consisting of 11.3 µL $ddH_2O$, 2.5 µL $MgCl_2$ 15 mM, 2.5 µL dNTP 200 µM, 2.5 µL Taq polymerase buffer 10×, 10 pmol or equal to 0.5 µL forward and reverse primer 20 pmol/ µL, 45 ng or equivalent to 5 µL of mutant mtDNA template directed mutagenesis results, and 1 unit or equivalent to 0.2 µL *Taq* DNA polymerase 5u/µL[12]. A3243G mutation analysis by PASA using forward primer *mismatch* three specific base (for the mutant allele), $D_{M3}$ and normal reverse primer $D_R$. For normal template amplification, carried out using non-specific forward primers (for the normal allele), 3243tot and normal reverse primer $D_R$[11]. Conditions of PCR namely through stages denaturation beginning at 94 ºC for 5 min, then go to PCR cycles program by 30 cycles with steps of denaturation at 94 ºC for 1 min, annealing at 62 ºC for 1 min, and elongation at 72 ºC for 1 min, the post-elongation process at 72 ºC for 4 min. PCR products were characterized by electrophoresis on agarose gel 2 % (w/v), voltage 70 V, current 100 mA, for 60 min, and use the standard 100 bp DNA ladder.

## Results and Discussion

### Isolation of normal mtDNA template of lymphocytes cell

Normal mitochondrial DNA to be used as template in the reaction of human lymphocytes taken from normal human cells with consideration has no history of the disease and do not indicate any symptoms related phenotypes arising from mutations A3243G on *tRNA*$^{Leu(UUR)}$ region. The use of normal mtDNA template on directed mutagenesis reaction was to saw there is bases change from the normal template of the mutant template or not. The use of lymphocytes cell as a source of mtDNA cells because relatively easy to taken.

Confirmation of normal mtDNA lysis results was tested by PCR using *forward* universal primers $D_2$[13] and reverse primer $D_R$ and PCR fragments with length of 190 bp. PCR results then electrophoresed with 2% agarose gel (w/v) at voltage of 70 V, 100 mA for 60 min (Fig 2).

To confirm whether the template lysis results are normal mtDNA, then do PASA-mismatch three bases using primer *mismatch* three bases $D_{M3}$ that specific for A3243G mutants and $D_R$, and the results were analyzed by agarose gel electrophoresis 2% (w/v) at voltage of 70V, 100 mA, for 60 min. However, all of template lysis results were tested did not produce the bands on electropherogram (data not shown), this means that the sample did not contain the mutation A3243G.

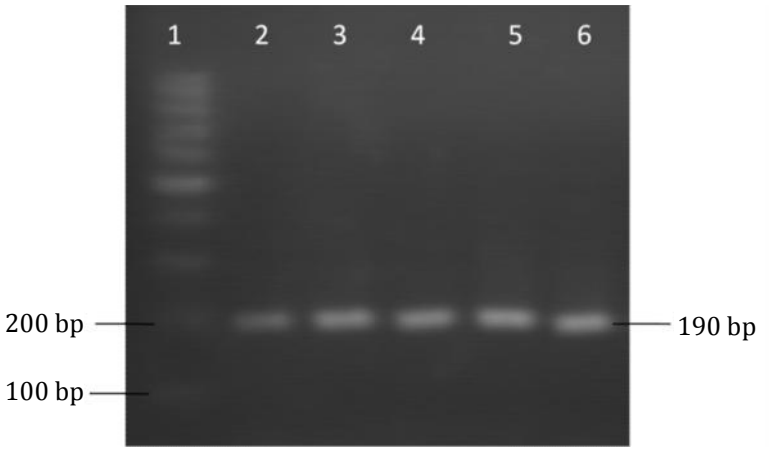


**Fig 2**. Electropherogram of the results of confirmation normal mtDNA template by PCR using primers $D_2/D_R$. Characterization of PCR results with normal mtDNA template lymphocyte cell lysis results in tubes 1-5 (lanes 2-6) with 2% agarose gel electrophoresis (w/v) at voltage of 70 V, current 100 mA for 60 min and compared with marker 100 bp ladder (lane 1).

### *Design of directed mutagenesis primer*

Design primer for directed mutagenesis to the manufactured of the model template of mutant A3243G conducted in accordance with the terms of the design of primers[14] using the *Perlprimer* application to facilitate the simulation of the determination of the annealing temperature optimal theoretical (($T_m$-5) $^oC$) to support the work processes of PCR.

This study uses only two primers, one forward mutant primer and one reverse primer. Mutant forward primer was designed to contain the desired mutation is located in the center of the primer, this is because if the primer mutation is at the end, then the amplification process directed mutagenesis could not run because there is mismatch of bases at the 3′ so that the elongation process is not could take place.

Annealing temperature in PCR work is determined by first finding each primer $T_m$ and $T_m$ primer combinations which can be determined using *Fast PCR program, oligo analyzer*, *oligo explorer,* and *Perlprimer*. In this study, determination of primer only using *Perlprimer* application by entering the bases sequence of both the forward primer and reverse primer.

Primer that first designed is forward primer, cause the determination of the order of bases more limited, which should be on region that have mutation A3243G in the middle of the primer, while the reverse primer sequence determination could be customized with forward primer with more flexibility.

After the results obtained by the forward primer selection, then analyzed by using the software NCBI/BLAST (*Basic Local Alignment Search Tool*) is on-line to get the non-specific primer (attachment of the primer on other template than the region of interest). Design of primers were selected that had at least homologous to the mtDNA template and the percent is less than 50% homologous. From the analysis of these data, mutant forward primer to be used in PCR reaction is $Mt3243_F$ which has 30 nucleotides with base sequence 5'-AGGGTTT GTTAAGATGGCAG**G**GCCCGGTA-3' (3222-3251) with $T_m$ is 74.92 ºC.

After obtained the forward primer design, then, is to design reverse primer in similar way when design of forward primer. The design should have reverse primer $T_m$ similar to the forward primer with an estimated 200-500 bp amplification results. Reverse primer that selected is $Mt3243_R1$ with size of 22 bp, because it primer has $T_m$ adjacent to the primer $Mt3243_F$, % GC has very large, and have percent homologous and the total of non-specific attachment is less.

From the results of the data processing and selection of design both forward and reverse primers, primer sequence mutant data results obtained forward mutant $Mt3243_F$: 5'-AGGGTTTG TTAAGATGG CAG**G**GCCCGGTA-3' (3222-3251) with $T_m$ 74.92 ºC and normal reverse primer $Mt3243_R$: 5'-CCGA TCAGGGGCTCGCAGTGCG-3'(3710-3689) with $T_m$ 74.74 ºC. Primers have % GC over 55% which means it is still showing good primer requirement in strong primer bond with the template during the PCR reaction.

Based on that design, the primer was then synthesized and forward primer to be used should be purified first by using FPLC (*Fast Nucleotide Liquid Chromatography*). Purification is intended to avoid significant decline in the efficiency of mutation[12].

***Making of A3243G mutant by directed mutagenesis***

Directed mutagenesis is commonly used PCR methods for mutated bases of the protein that associated with the expression of these proteins. In this study, SDM used to create mutant A3243G mtDNA template that will be used as positive control in the process of mutation analysis using the PASA-mismatch three bases.

Problems often arise in the process of PCR is the annealing temperature handling could make this method is not specific to amplified of the template, especially if the method is used to create mutations at specific point, therefore, in this research, namely optimization annealing temperature at 55 °C (12) to 74 °C (1 °C below the melting temperature).

Optimization is done by SDM reactions using mutant forward primer, $Mt3243_F$ and reverse primer, $Mt3243_R$. Product of yield optimization of annealing temperature electrophoresed using 1% agarose gel (w/v) and the obtained results with the fragment size of 489 bp, as shown in Fig 3.

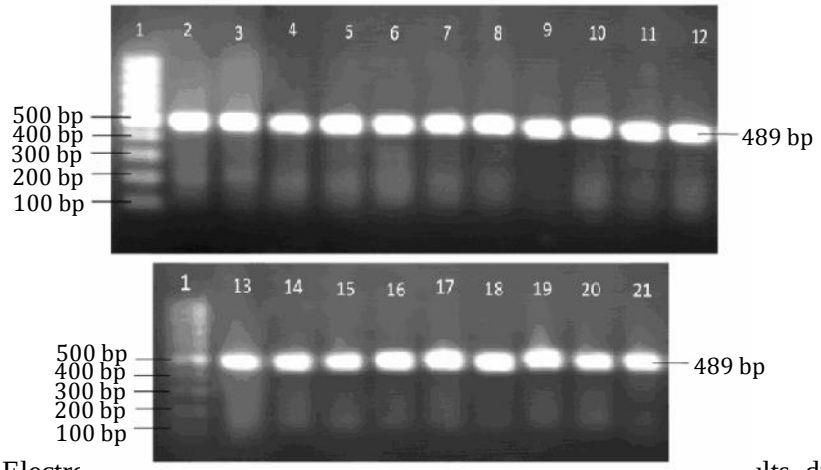


**Fig 3**. Electrophierogram of annealing temperature optimization results directed mutagenesis. Agarose gel electrophoresis using 1% (w/v) at 60 V, 100 mA, for 60 min. The first column states fragments of 100 bp ladder marker. Lanes 2-21 respectively expressed fragment directed mutagenesis results at a temperature of 55-74 °C.

From fig. 3, it can be seen that the optimization results at all temperatures produces good bands. So the result of this optimization, the temperature is used as the annealing temperature is the highest temperature, which is 74 °C, this is because the higher the annealing temperature, the resulting fragments are more specific, meaning that when the primer attached to the non-specific bonding between primer and templates in a non-specific are released again, so just stick to the primer specific base sequences only. Formation of secondary structure could also inhibit mutagenesis reaction. Increasing the annealing temperature to temperature of 74 °C could reduce the formation of secondary structure and improve the efficiency of mutagenesis[12].

Further optimization of primer concentration, ie the concentration variation ratio of the primer mutant on the normal primer ie 1:1, 2:1, 3:1. The purpose of optimization of primer concentration variation of this is to avoid the conversion of mutant template back to normal template, due to point mutation is not positioned in the middle of the mutant templates, but adjacent to the tip (Fig 1). Optimization is done by looking at the effect of the mutant primer concentration for the amount of product formed mutant. The composition of the mixture of reagents used are shown in Table 1.

**Table 1**. Directed mutagenesis reagent mixtures with various concentrations of primers.

| Reagent | Primer $Mt3243_F$:$Mt3243_R$/µl | | |
|---|---|---|---|
| | 1:1 | 2:1 | 3:1 |
| $ddH_2O$ | 11,3 | 10,8 | 10,3 |
| Buffer *pfu* DNA Polymerase 10× | 2,5 | 2,5 | 2,5 |
| $MgCl_2$/ 25 mM | 2,5 | 2,5 | 2,5 |
| dNTP/2mM | 2,5 | 2,5 | 2,5 |
| Primer $Mt3243_F$/(20 pmol/µl) | 0,5 | 1,0 | 1,5 |
| Primer $Mt3243_R$/(20 pmol/µl) | 0,5 | 0,5 | 0,5 |
| mtDNA normal template of lysis results | 5 | 5 | 5 |
| Enzyme of *pfu* DNA polymerase /(5u/µl) | 0,2 | 0,2 | 0,2 |
| Total volume | 25 | 25 | 25 |

Directed mutagenesis results with primer concentration variation then characterized by agarose gel electrophoresis and the resulting electropherogram as shown in Fig 4. All bands fragment on the electropherogram directed mutagenesis results provide results that thick/good, so that comparisons can not be

known primer mutagenesis can provide optimal results. To see the optimization of mutagenesis, directed mutagenesis results of the three primer comparison was confirmed by PASA-mismatch three base.

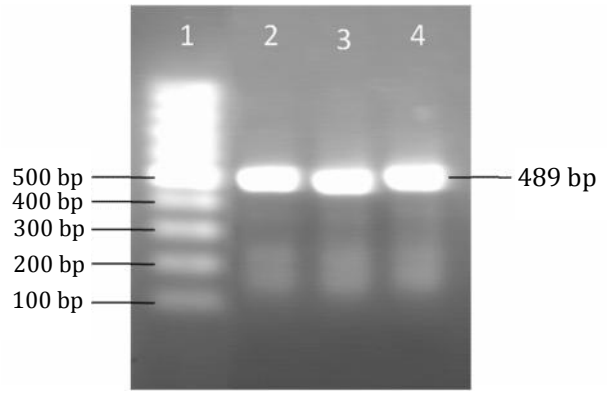


**Fig 4**. Electropherogram directed mutagenesis results with forward primer concentration variations. PCR performed on the annealing temperature 74°C. Electrophoresis performed using 1% agarose gel (w/v) at 60 V, 100 mA, for 60 min. The first lane marker 100 bp fragment expressed ladder. Lanes 2-4 respectively expressed fragment directed mutagenesis results with the concentration ratio of the forward primer : reverse 1:1, 2:1, and 3:1.

### *A3243G mutation confirmation by direct sequencing*

Existence A3243G mutant as result of SDM sequence was confirmed by direct sequencing. Preparation of mtDNA templates for direct sequencing, amplification performed using the primers used in the reaction of SDM at annealing temperature, 74°C and the ratio of mutant primer to normal primer, 3:1 and produce amplification fragments sized 489 bp, then the SDM template results were sequence analyzed by direct sequencing of reverse directions using primers $Mt3243_R$.

Analysis of the results of direct sequencing electropherogram A3243G mutant template using primers $Mt3243_R$ showed A3243G mutation (Fig 5). From the results of this analysis could be proved that the method directed mutagenesis can be used to create template A3243G mutant.

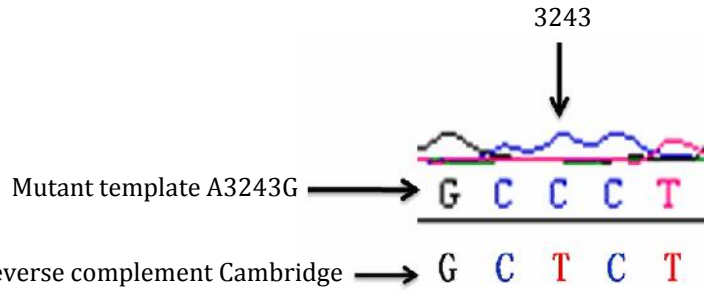


**Fig 5**. Electropherogram results of direct sequencing A3243G mutant template. Peak spectrums A3243G mutant template base sequences using the reverse primer Mt3243R compared with the reverse complement sequence of bases *Cambridge*. The existence of peaks C nucleotides on 3243 position that is complementary to the G nucleotide base sequences showing mutations A3243G.

***Optimization of A3243G mutant as template concentration on the PASA-mismatch three bases***

PASA-mismatch three bases is PCR method that used to analyze mutations. In previous study, optimization was done PASA-mismatch three bases with variations in annealing temperature and $Mg^{2+}$ concentrations obtained optimal annealing temperature at 62 °C with concentration of $Mg^{2+}$ 5.0 mM and characterization agarose gel 1%.

A3243G mutation analysis through PASA-mismatch three bases using two pairs of primers, 3243tot/$D_R$ for tube 1 (normal allele) and primer $D_{M3}$/$D_R$ for tube 2 (mutant allele) in separate tube PCR system using PASA system-specific mismatch three bases for normal or mutant allele. PASA produced fragment sizes of 97 bp in the normal allele and the mutant allele.

Optimization of the concentration of mutant A3243G SDM results, as templates for the PASA-mismatch three bases, performed at various concentration dilution of 1×, 10×, 100×, and 1,000×. These variation is amplified template concentration PASA-mismatch three bases using forward primer mismatch three bases ($D_{M3}$) and universal reverse primer ($D_R$) and the resulting fragments are 97 bp long (Fig 6).

From fig. 6, it can be seen that at concentration of templates 1× (lane 2) there is still a residual template that has not been amplified 489 bp, no formed mutant fragment 97 bp, and the formation of many non-specific fragments. In the templates by diluting 10× (lane 3) target of 97 bp fragments are formed, but there are non-specific fragment by fragment length of approximately 350 bp and 450 bp. Templates are best used as a template PASA-mismatch three bases are templates with 100× dilution (lane 3), because it shows the quality of the band is thick and the absence of non-specific fragment, whereas the template with 1,000× dilution band formed thinning, this condition means that the number of templates is not proportional to the amount of PCR reagents.

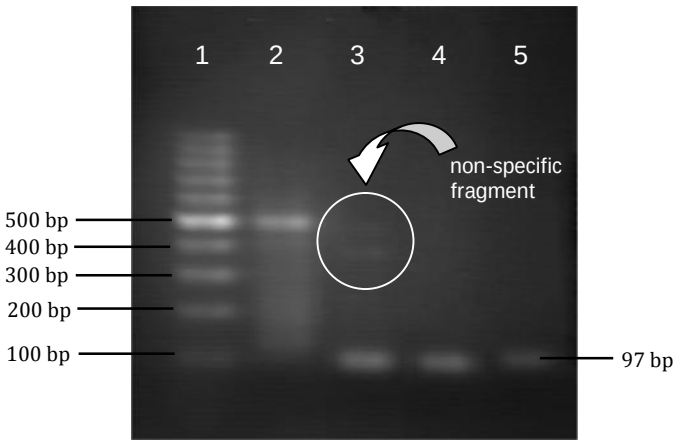


**Fig 6**. Electropherogram results PASA-mismatch three bases with A3243G mutant template concentration variation. Lane 1 indicates marker 100 bp ladder; lanes 2-5 consecutive declare the results PASA-mismatch three bases with variety of mutant template concentration 1×, 10× dilution, 100×, and 1,000×.

After the optimum concentration of template is known, mutant template of directed mutagenesis yield comparisons primer diluted 100× and the that mutations were analyzed by PASA-mismatch three bases for determining the optimum primer that optimum. PASA is done by system of two separate tubes between normal alleles and mutant alleles, respectively, and produces 97 bp fragments, as shown in Fig 7.

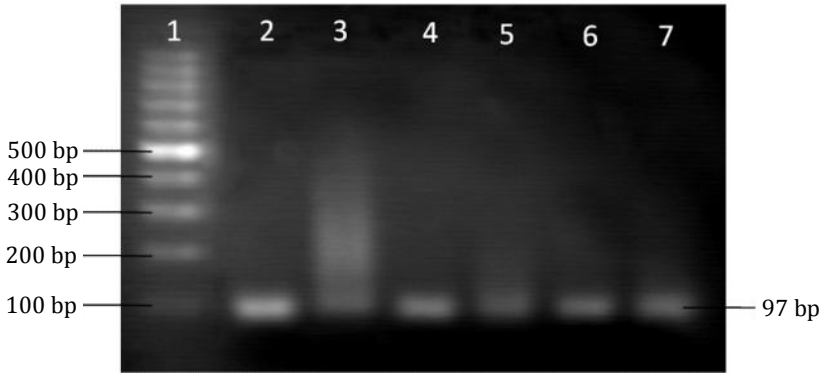


**Fig 7**. Electropherogram results PASA-mismatch three bases using template directed mutagenesis results with primer concentration ratio variation. Each template diluted 100× and then do the PASA-mismatch three bases, and the results electrophoresed with 2% agarose (w/v), the condition of 70V, 100 mA for 60 min. Lane 1 shows the marker 100 bp ladder; lane 2 and 3 respectively show the results of amplification of normal and mutant templates with mutant primer concentration ratio of 1:1, lane 4 and 5 respectively show the results of normal and mutant template amplification with primer concentration ratio of mutant 2:1, lane 6 and 7 respectively show the results of amplification of normal and mutant templates with mutant primer concentration ratio 3:1.

From the results of electropherogram (Fig 7), it could be seen that the three samples with various concentrations of the primer containing the normal allele and the mutant allele with different proportions (mutant heteroplasmy). The greater of concentration of the mutant forward primers in PCR reaction, the amount of mutant template higher. It could be seen in Fig 7 that the normal allele bands were formed (lanes 2, 4, and 6) are running low due to rising concentrations of mutant forward primer. In contrast, mutant alleles formed bands (lanes 3, 5, and 7) are clear and specific in line with the increase in the concentration of mutant forward primer. From this analysis, it could be seen that the optimal process of mutagenesis is the primer ratio, 3:1.

## Conclusion

From the results of this research that has been done, it can be concluded that the directed mutagenesis could be used to make the A3243G mutant template that can be used as positive control PASA-mismatch three bases in analyzing the A3243G mutation. In addition, the process of directed mutagenesis could run optimally at annealing temperature, 74 °C, primer concentration ratio of mutant : normal 3:1, and the product could be used as positive control PASA-mismatch three bases with concentration of 100× dilution. Further research suggested is necessary to tested for patients suspected of having the mutation A3243G and perform optimization factors other PCR to obtained better mutant results.

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