

IN SILICO PRIMER DESIGN AND ANNEALING TEMPERATURE OPTIMIZATION TO AMPLIFY THE FRAGMENT OF *gyrB* GENE *Mycobacterium tuberculosis* ISOLATE P010 USING POLYMERASE CHAIN REACTION

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Abstract

One of the factors causing XDR-TB is due to mutations in the *Mycobacterium tuberculosis* gene, one of them is in the *gyrB* gene. Amplification of *gyrB* gene fragments from *Mycobacterium tuberculosis* DNA using Polymerase Chain Reaction (PCR) method. The amplification process by the PCR method requires a pair of primers (forward and reverse) to limit the area to be amplified. The current study aims to obtain the best primer pair generated by in silico design using Clone Manager Suite 6 program while simultaneously optimizing the annealing temperature to amplify the fragment of *gyrB* *Mycobacterium tuberculosis*. The template used in designing the primer is the sequence of *gyrB* *Mycobacterium tuberculosis* H37Rv isolate obtained from NCBI database of genbank code AL123456.3.

The current study obtained a pair of primer which respectively had 19 oligonucleotide length and the best annealing temperature of 56°C. The primer is able to do in silico amplification of the fragment of *gyrB* *Mycobacterium tuberculosis* gene isolate P010 in the nucleotide area range from 1271-1755 bp with 485 bp fragment length.

Key words: primer design, annealing temperature, *gyrB* gene, PCR.

I. INTRODUCTION

Indonesia is included in 30 countries with a high burden of MDR-TB which reaches 32,000 new cases of MDR-TB from 480,000 global data on new cases of MDR-TB [8]. According to WHO data (2017) MDR-TB cases have grown by 6.2% to Extensively Drug Resistant Tuberculosis (XDR-TB). XDR-TB is a TB caused by bacteria resistant to INH and RIF (MDR-TB) moreover it is also resistant to at least Fluorokuinolone (FQ) and one of the second-line tuberculosis drugs in the form of injection [8]. In an tuberculosis drugs resistance treatment study, an examination has been performed on some second-line tuberculosis drugs. It is found out that Fluorokuinolone (FQ) is the most important drug of

the core MDR-TB regimen, this is due to the effectiveness of the therapy given and the low toxicity of the use of FQ compared to other second-line OAT.

The mechanism of development of FQ resistance in *M. tuberculosis* is the presence of mutations in the Quinolone Resistance Determining Region (QRDR) *gyrB* gene [5]. QRDR *gyrB* gene is defined as the region at codons 500 to 540 [4].

The identification of mutations in *gyrB* gene fragments can be done by PCR technique. In the process of amplification of gene fragments required a pair of primers (forward and reverse) is required. Primer serves as a limiting fragment of DNA to be amplified. The primer used must meet the criteria of a

good primer to amplify *gyrB* fragments specifically. These criteria include: primary length, % GC, T_m, dimer and hairpins, primary stability, repeats, runs and false priming. Therefore the current study aims to design a pair of primer used to amplify *gyrB* gene fragment as well as to find out the optimum annealing temperature to amplify *gyrB* fragment by conducting annealing temperature optimization.

II. METHODS

2.1 Primer Design

Primer used to amplify *gyrB* gene fragment in the current study was designed to utilize Clone Manager Suite 6 program. The first stage to be done was inputting the sequence nucleotide data of *gyrB* *M. tuberculosis* data (Gen Bank AL123456.3), which was provided in the database at URL://www.ncbi.nlm.nih.gov, to Clone Manager Suite 6 program. A pair of primer was designed by selecting menu bar primer design. The length of primer base produced was input and target area to amplified based on the mutation data literature was determined. Primers produced in the rank line was analyzed based on the primer criteria desired for *gyrB* gene area.

2.2 Annealing Temperature Optimization and Amplification of *gyrB* *M. tuberculosis* Gene Fragment using PCR

Determination of primer optimum temperature to be used to amplify *gyrB* fragment on isolate H37Rv was done by conducting an experiment at 56°C, 58°C, 60°C

DNA template used for amplification was obtained from the isolation result of isolate P010. PCR was conducted using a pair of primer specially designed on the current study used Clone Manager Suite 6 where the amplification process started with predenaturation at 95°C for 15 minutes followed by 45 amplification cycle (denaturation at 94°C for 1 minute, annealing at optimization temperature at 56°C, 58°C, 60°C for 1 minute 20 seconds, extension at 72°C for 2 minutes and final extension at 72°C for 10 minutes).

2.3 PCR Product Detection

PCR product was detected using electrophoresis agarose gel 1,5% b/v. The result of electrophoresis was then conducted visualization using UV Transilluminator.

III. RESULT AND DISCUSSION

3.1 Primer Design

The primer design performed using Clone Manager Suite 6 obtains the result of a pair of primer which are forward primer with a nucleotide base

sequence:5'-GAGAGTTGGTGC GGCGTAA-3' and reverse primer with a nucleotide base sequence:5'GCGGTCGGAGTATGCGAAT-3'.

The length of the forward and reverse primer of *gyrB* genes is 19 bases. Primers that have a length of less than 18 bases will have lower specificity and will easily cause mispriming [2].

Percent (%) of GC of forward and reverse primer of *gyrB* gene show the same amount that is 57%. The GC percentage has met the primer general requirements of 40% to 60% [7]. If a primer has a low GC level, the primer will not be able to attach effectively to the target so that there will be a decrease in the efficiency of the PCR process. In the other hand, the high level of GC will complicate the separation of double helix chains on primers and templates [2].

Forward and reverse primers of the *gyrB* gene have the same T_m (melting temperature) of 64 °C. T_m is the temperature at which 50% of the DNA's double helix has been separated. T_m in a pair of primer of *gyrB* gene has met the criteria ranging from 50-65 °C and do not have a gap of T_m between one primer with the others [2]. A pair of primer should not have a high gap of T_m. The gap recommended is not more than 5°C, if it is higher, it will cause a decrease in the amplification process or even the amplification process can be performed. It also aims to facilitate the determination of annealing temperatures in the multiplex-PCR process [7].

The pair of primer generated from the design has a dimer at the tip of 3' as much as 2 pb on each forward and reverse primer, while dimer-any presence as much as 2 pb on forward primer and 3 pb on reverse primer. It shows that primer generated from the design still meets the criteria of Clone Manager suite 6 program which has a tolerance limits of dimer as much as <3 pb at the tip of 3' and <7pb other than 3' area.

The designed primer has a stability of 2.5 kcal and 2.3 kcal for forward and reverse primers respectively. So the stability of the two primer design has met the criteria of Clone Manager Suite 6 that is more than or equal to 1.2 kcal. This figure indicates the stability of 5 bases (pentamer) of the tip of 5' is greater than at the tip of 3'.

Runs and repeats that are allowed in the Clone Manager Suite 6 program are less than 3. In the primer generated from the design, both primers meet the allowed runs and repeats criteria. Repetition of both nucleotide and dinucleotide bases that is more than 3 will increase the possibility of false priming [6].

Hairpins condition was not found in the primer design so it has met the criteria of a good primer based on Clone Manager Suite 6. Hairpins is a

condition which the tip of primers complement each other [3].

The last important criterion is false priming. When the presence possibility of false priming is not found in the pair of primer generated from the design, the primer design has met the Clone Manager Suite 6 criteria. False priming is not allowed to occur on primers. The existence of false priming will result in an amplification error beyond the annealing temperature so that the desired PCR product is not formed [1].

Based on literature and criteria reference of Clone Manager Suite 6, it can be concluded that a pair of primer design for *gyrB* gene amplification has met the criteria of a good primer. Therefore, the primer can be used to amplify the *gyrB* gene sequence so that it is able to produce the desired PCR product.

Table 1. The result of Primer Design of *gyrB* gene based on the reference in *Clone Manager Suite 6*

Criteria	Primer For-ward	Primer Reverse	References to Clone Manager Suite 6
Long primer (basa)	19	19	18-22
Percent (%) GC	57	57	50-60
Tm (°C)	64	64	55-80
Dimer on tip 3'	2	2	<3
Dimer besides the tip 3'	2	3	<7
Stability (kcal)	2.5	2.3	≥1,2
Runs (nukleotida)	2	2	<3
Repeats (dinukleotida)	2	-	<3
Hairpins False priming	-	-	-

3.2 Optimization of Annealing Temperature and Amplification of the Fragment of *gyrB* M. Tuberculosis Gene Isolate P010 Using PCR Technique

Annealing temperature is temperature required for the primer to attach to the target DNA. The low

annealing temperature of the optimum temperature will result in false priming and if the annealing temperature used is higher than the optimum temperature, it will cause the primer to be unable to attach to the target DNA so that the expected PCR product will not be formed, in other words the PCR process is not successful [2]. Therefore, it is necessary to process annealing temperature optimization so that the process of PCR will work properly and produce the product which is appropriate to the target.

Optimization of PCR process on the annealing temperature in this study used 56°C, 58 °C dan 60 °C. Temperature variations are performed with the aim of obtaining the most optimum temperature for the PCR process so that the amplification process will also work as it is planned. PCR in this study was conditioned at 95 ° C for 15 minutes for predenaturation, followed by 45 cycles of amplification with denaturation at 94 °C for 1 minute, annealing temperature variation 56 °C, 58 °C dan 60 °C for 1 minute 20 seconds, elongation at 72 °C for 2 minutes and final elongation process at 72 °C for 10 minutes. From annealing temperature optimization, it is obtained amplicon visualized by the process of electrophoresis agarose 1.5% w / v.

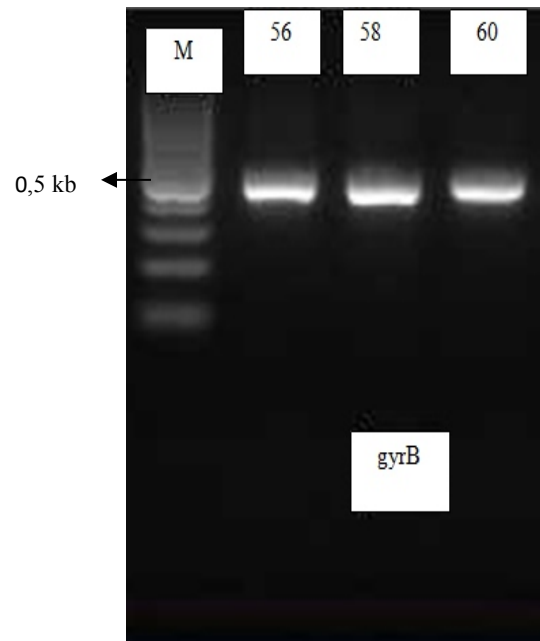


Figure 1. Electroforegram amplification *gyrB* gene fragment isolate P010 on UV- transilluminator; *gyrB*= product *gyrB* gen; M= Marker DNA ladder 100 bp (0,1 kb)

3 bands with different optimization temperatures show the same result, just as bright and bold. The number of products produced is also

suitably seen on marker ± 485 bp. If the resulting product is as good at two different temperatures, in this case there is no secondary hybridization and the DNA bands obtained are sufficiently thick, then a lower annealing temperature will be selected [1]. This consideration is caused by high annealing temperatures that can inhibit primary hybridization with DNA templates resulting in fewer PCR products so that temperatures 56°C can be used for *gyrB* gene amplification with primer design results. So that the PCR process is conducted at 56°C annealing temperature according to the Clone Manager Suite 6 annealing temperature criteria is 56°C .

After optimizing the annealing temperature and amplification using the PCR method on the P010 isolate using a pair of primer design with Clone Manager Suite 6 that is a forward primer with a nucleotide base sequence:

5'-GAGAGTTGGTGC GGCGTAA-3' and reverse primer with nucleotide base sequence:

5'-GCGGTCGGAGTATGCGAAT-3' proved to be able to amplify the *gyrB* gene in the P010 isolate used as a template.

It can be seen by detecting the PCR products performed using electrophoresis method on 1.5% agarose gel seen on UV Transilluminator. The electrophoresis results showed that PCR products formed correctly were products of ± 485 bp in accordance with the size of the *gyrB* gene.

IV. CONCLUSION

In silico primer design produced primers which had good criteria as it had met the criteria such as primer length, %GC, T_m , *dimer*, *hairpins*, primer stability and *false priming*. A pair of primer obtained were primer forward:

5'-GAGAGTTGGTGC GGCGTAA-3' and primer reverse:

5'-GAGAGTTGGTGC GGCGTAA-3'. It was proved that the pair of primer produced by in vitro were able to amplify *gyrB* gene fragment and produced amplicon with the size of ± 485 bp after detecting using electrophoresis method and was seen using UV Transilluminator with temperature optimization results of 56°C .

ACKNOWLEDGEMENT

I would like to thank everyone who has helped me either directly or indirectly in order to conduct this study well.

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