Optimization of Annealing Temperature of HIF-1 A and 18s rRNA in Blood of Swimming Athletes Using RT-PCR

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ABSTRACT
Gene expression is the process of reading from DNA into a functional product. Regulation of gene expression through complex mechanisms. HIF1-1α is one of the regulatory genes that regulates the return of homeostasis when there is a lack of oxygen (hypoxia). Hypoxia is often experienced by athletes during training, especially during swimming training. Detection of gene expression can use qualitative and quantitative Polymerase chain reaction (PCR) methods. Each has advantages and disadvantages. Research on the expression of the HIF-1α gene in both humans and mice has been widely reported. However, the primary annealing temperature used varies, even with the same primer sequence. Therefore, it is necessary to optimize the primer annealing temperature before measuring gene expression. Annealing temperature optimization using the in-silico program showed annealing temperature at 52°C for the HIF-1alpa and 18s rRNA genes. Because the previous research used annealing temperature of 59ºC, the range of optimization temperature gradient was at 52ºC - 59ºC. Agarose gel electrophoresis results showed the HIF-1α gene product at 157bp and 18S rRNA gene 155bp. Optimum annealing temperature can be seen from the band thickness in the path of 53.6°C and 18S rRNA gene 55.8°C. Ct values for the HIF-1alpha gene are on average Ct to 25 and 18S rRNA genes at 10 ct. Based on good primer criteria, optimization of annealing temperature in silico, annealing temperature from the primer reference used, and the results of optimization of both genes in this study, the optimum annealing temperature recommended for further testing of the qPCR of all samples in this study series was 55.8 ° C.
Keywords: *in silico, optimization, HIF-1, 18s rRNA, annealing temperature*

1. INTRODUCTION

Gene expression is the process of reading from DNA into a functional product. Functional products can be RNA or protein. Regulation of gene expression through complex mechanisms. Regulations can occur simultaneously at various levels and factors work together to stimulate and inhibit the expression of a gene. These factors are called regulatory genes [1].

HIF1-1α is one of the regulatory genes that regulates the return of homeostasis when there is a lack of oxygen (hypoxia). HIF-1α was discovered by Semenza et al in 2000. Since then many studies have reported that HIF helps regulate the expression of a number of genes [2] [3]. One gene that is in the regulation of HIF-1α reported by Farma (2014) is CA9 and ACE1 in the lung tissue of hypoxic Rats [4].

Hypoxia is often experienced by athletes during training, especially during swimming training. The impact of excessive physical exercise is an imbalance between the physical training and recovery time [5]. The intensity and pattern of improper training can trigger the risk of athlete injury.

Detection of gene expression can use the method of Polymerase chain reaction (PCR). PCR is a technique used to amplify specific DNA fragments from certain genes. The principle of PCR and Real time PCR (quantitative PCR) in amplifying DNA is basically the same. The difference is the number of amplified copies amplified on conventional PCR is only known in the final phase (plateau phase) of the amplification process and is interpreted in the form of a visualization of DNA bands in agarose gels, whereas in real time PCR, the number of amplified copies can be known during the amplification cycle ("In real time"), starting from the optimum amplification condition (exponential phase) - the linear phase when the amplification reaction slows down - until the plateau phase is when the amplification reaction starts to stop. Therefore, the measurement of the number of copies of an amplicon on a PCR is said to be qualitative, whereas in real time PCR is quantitative because the curves formed in the exponential phase can be interpreted in numerical form [6].

The expression level of a gene can be determined by using mRNA. mRNA is a product of DNA transcription that can be detected simply, sensitive, and strongly. Identification of mRNA can be used to measure differences in

expression after mRNA is converted to from cDNA using the reverse transcriptase enzyme. This process is known as Reverse transcription (RT) PCR.

Detection of mRNA expression can be done using one step or two step quantitative methods Real Time Polymerase Chain Reaction (qRT-PCR). Real-time RT-PCR (or kinetic RTPCR) is widely and increasingly used because of its high sensitivity, good reproducibility, and wide dynamic quantification range [7].

The one step qRT-PCR method uses RNA samples with direct cDNA synthesis in the qPCR machine. This method reduces the risk of contamination of DNA. The two-step qRT-PCR method uses RNA samples with indirect cDNA synthesis in the qPCR machine. The cDNA synthesis is performed first in an ordinary PCR machine. Then cDNA is stored at -20 and can be used for the next qPCR. However, in principle both qRT-PCR methods have the advantage of high sensitivity and specificity of high dynamic range, suitable for quantification and user and lab friendly actions [8].

qPCR must be carried out in sterile conditions, and requires an appropriate annealing temperature so that the quantification process can run well without the result of expression bias or double peak. Annealing temperature is the temperature at which the primer will stick to the DNA template, its size the temperature can be calculated based on the melting value temperature (Tm) of each primer. Search for optimal conditions of annealing temperature very important, because it deals with specificity and sensitivity of PCR products [9].

Research on the expression of the HIF-1α gene in both humans and mice has been widely reported [4] [10]. However, the primer annealing temperature used varies. Hardiany et al (2013) used the primer annealing temperature of HIF-1α at 59 ° C. The same temperature is also used for housekeeping gene amplification 18s rRNA.

The primer sequences used by Hardiany et al (2013) for the HIF-1 gene are tgatgaccagcaacttgagg (Forward) and ttgattgagtgcagggtcag (Reverse) with an amplicon product of 157 bp and a primer sequence of 18s rRNAs are aa cggctaccacatccaag (Forward) and cctccaatggatcctcgtta (Reverse) with 155 bp amplification products [10].

Korbie & Mattick (2008) states that the annealing temperature is influenced by the presence of GC-content contained in the primary or target DNA sequence, RNA concentration, and PCR reagent mix [11]. Other sources also mention that the use of different machines sometimes also affects the optimum annealing temperature for a PCR reaction even with the same primer. Therefore, this study aims to optimize the primer annealing temperature.

2. MATERIALS AND METHODS

This research is a descriptive study with the aim of obtaining the optimum temperature to pass the measurement of HIF-1α gene expression. Materials used were blood samples from subjects (swimming athletes after hypoxic swimming exercises), RNA isolation kits (Quick-RNA ™ MiniPrep Plus, Zymo Research), SensiFAST SYBR No-ROX One-Step Kit (Bioline), nuclease free water (Biotechnology) 1 st Base), forward primers and reverse genes for HIF-1α and 18s rRNA genes (IDT-DNA Technologies). The instrument used is quantitative Real Time RT-PCR (Rrotor Gene Q, Qiagen), nanofotometer, and centrifugator.

2.1. Total RNA Isolation

Add 200µl DNA/RNA sheild™ (2X concentrate) directly to each 200µl of fresh/frozen blood sample and mix thoroughly. For every 400µl of reagen/blood mixture add 8µl Proteinase K and mix thoroughly. Incubate at room temperature (20-30ºC) for 30 minutes. Add an equal volume of isopropanol and mix by vortex. Transfer the mixture to a Zymo-Spin™ IIICG Column1 (green) in a Collection.

Tube and centrifuge2. Discard the flow-through. Add 400 µl RNA Prep Buffer to the column and centrifuge. Discard the flow through. Add 700 µl RNA Wash Buffer to the column and centrifuge. Discard the flowthrough. Add 400 µl RNA Wash Buffer and centrifuge the column for 2 minutes to ensure complete removal of the wash buffer. Transfer the column carefully into an RNase-free tube. Add 50 µl DNase/RNase-Free Water directly to the column matrix and centrifuge. The eluted RNA can be used immediately or stored at ≤-70ºC.

2.2. RNA Concentration and Ppurity

The concentration and purity of isolated RNA were analyzed using a nanofotometer. The absorbance of the sample was measured at wavelengths of 260 nm and 280 nm. Measurements were made in duplicates for each sample RNA.

2.3. Annealing Temperature Optimization

The annealing temperature optimization using the in-silico program http://tmcalculator.neb.com/. The next process is the temperature obtained from in-silico then becomes the basis for choosing the temperature gradient range in the thermocyler PCR machine.

The stages are followed by making cDNA separately first to save on templates and reagent kits. cDNA synthesis used reverse transcriptase from the SensiFAST SYBR No-ROX Two-Step Kit (Bioline). The protocol for making cDNA is the activation of the reverse transcriptase enzyme at 42 ° C for 5 minutes. Inactivation of the reverse transcriptase enzyme at 95 ° C for 5 minutes.

cDNA is used as a template for annealing temperature optimization. CDNA amplification using the SensiFAST SYBR No-ROX One-Step Kit (Bioline). The optimization

protocol qPCR is activation of polymerase at 95 ° C for 2 minutes, denaturation at a temperature of 95 ° C for 5 seconds. Primary annealing at a temperature gradient of 52-59 ° C for the HIF-1α and 18s rRNA genes, extension of the DNA chain at 72 ° C for 5 seconds. Repetition of reactions from stages 3 to 5 to 40 cycles. The PCR product was then confirmed to be sized using 1% agarose gel electrophoresis

3. RESULT AND DISCUSSION

The average RNA concentration was 23.27 ng / µl with an average purity index of 1.85. According to the SensiFAST SYBR No-ROX One-Step Kit (Bioline) mix reaction protocol, the RNA level is in the range of 1pg to 1g, the level used is 78ng.

The results of primer annealing temperature analysis in silico showed HIF-1α and 18s rRNA at 52ºC. This value then becomes the basis for choosing the gradient temperature during optimization. Because Hardiany et al (2013) used annealing temperature of 59ºC [10], the range of gradient temperatures was at 52ºC - 59ºC. The results of the HIF-1α and 18s rRNA gene melting curve are shown in Figure 1.

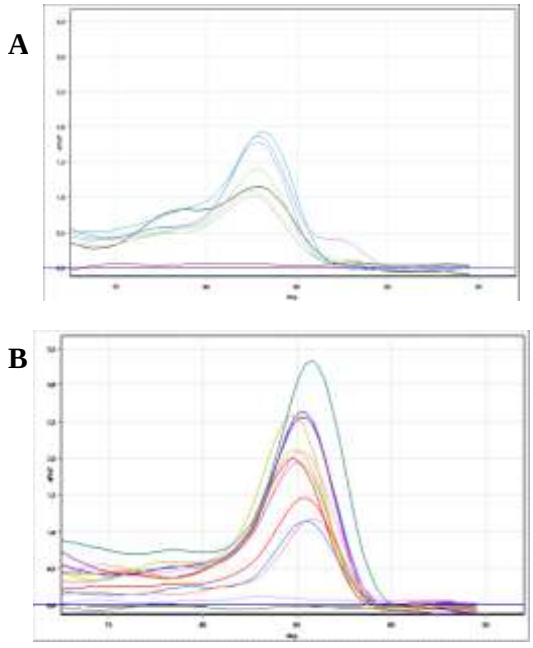

Fig 1. Melting Curve gen HIF-1 α (A) and gen 18S rRNA (B)

The agarose gel electrophoresis results shown in Figure 2 show the HIF-1α gene product at 157bp and 18S rRNA gene 155bp. Optimum annealing temperature seen from the thickness of the tape. The HIF-1α gene is seen in the band 53.6°C and 18S rRNA gene 55.8°C.

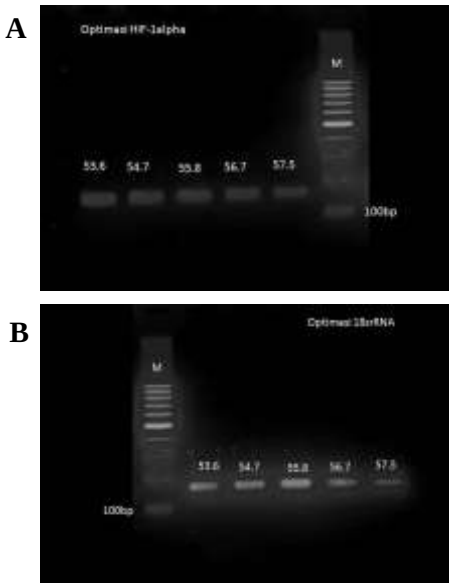

Fig 2. Results of agarose electrophoresis in PCR products of the HIF-1 α (A) gene and 18S rRNA (B) gene

Changes in mRNA expression are markers of changes in cell activity [12]. The RT-PCR real time technique is very powerful for quantifying the expression of a gene [13]. This is to ensure the repeatability and accuracy of the measurement there are standard rules that need to be followed as stated by Udvardi et al (2008). Some of the standard rules include the use of RNA isolation procedures that produce high quality RNA, DNA purification using DNAse to eliminate genomic DNA contamination, use gene specific primers that are in accordance with the standard (Tm = 55-60 ± 1 ° C, 18-25nt, content GC 40-60%, product 60-150 bp), use good reference genes, and use gene expression calculation methods that take into account the efficiency of PCR [14].

At real time RT-PCR data generated in the form of crossings thresholds (Ct) representing the number of PCR cycles needed to achieve an intensity The size of the Ct value is determined by the initial number of target genes in the sample. The difference in the value of Ct can be determined by ΔCt. In the calculation of normalized gene expression with the reference gene, the perhitunganCt calculation is performed for both the target gene and the reference gene. Amplification of sample genes and reference genes is best when done together to minimize variation. However, in cases where the annealing temperature of the two genes is different as reported by Farma (2016) between the CA9 and β-actin genes, amplification can be done separately.

The reference gene or also called housekeeping genes selected is the gene that is expressed by cells consistently so that it can accurately represent the total mRNA that is present in each the sample. Categories for housekeeping

genes are genes involved in general regulation and the basis of cellular functions needed for cell resistance. Some reference genes that are often used are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, β2-microglobulin, cyclooxygenase 1, hypoxanthine phosphoribosyl transferase 1, glucose-6-phosphate dehydrogenase, cyclophilin A, tubulin, transferrin receptor and ribs 18 RNA [15]. The selection of reference genes is very important because variations that arise will directly affect the final results of the calculation of target gene expression [13].

In figure 1 there is only one peak on dan gene analysis of the melting curve for the HIF-1alpha 18S rRNA gene. According to Hardianty et al (2013) this proves that primers have been specifically designed for HIF-1alpha and 18s rRNA. Ct values for the HIF-1alpha gene are on average Ct to 25 and 18S rRNA genes at ct to 10.

Figure 2 shows the results of the qPCR product electrophoresis showing only one band for the HIF-1alpha gen gene (157 bp) and the 18s rRNA gene (155 bp) also support that the primer has been specifically designed for that gene. The thickest band picture is seen in the annealing temperature lane 53.6 ° C for HIF-1alpha and 55.8 ° C 18s rRNA gene.

These results indicate that the annealing temperature is very different from that reported by Hardianty et al (2019), where the optimum annealing temperature for both genes is 59 ° C. It is also known that Hardianty et al (2019) used the qRT-PCR meisn (CFX96, BioRad Laboratories-USA) and iScript One Step RT-PCR Kit with SYBR Green (BioRad). This further strengthens the basis that the use of different qPCR machines can cause different optimum annealing temperatures. Therefore, it is necessary to optimize the annealing temperature first, before analyzing gene expression. difference in results can be caused by several factors, one of them is the use of solvents to extract active compounds which are not appropriate. In this research, the solvent used was ethanol, which is polar. According to (11), the type of solvent was a factor that influences the concentration and type of compound to be extracted. The polarity of the solvent is an important thing that influences the antimicrobial activity. Therefore, further experiments need to be carried out using non-polar solvents.

4. CONCLUSION

Based on the best primer criteria, optimization of annealing temperature in silico, annealing temperature from the primer reference used, as well as the results of optimization of both genes in this study, the optimum annealing temperature recommended for qPCR further testing of all samples in this study series was 55.8 ° C..

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