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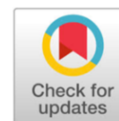
Original Research

Optimization of DCR1 and DCR2 epigenetic annealing temperatures for breast cancer biomarker using in-silico and in-vitro study



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Abstract: The epigenetics of methylated and unmethylated DCR1 and DCR2 (decoy receptors 1 and 2) are genes encoding membrane receptors that can bind to TRAIL causing TRAIL inhibition in the apoptotic pathway. Epigenetic detection of DCR1 and DCR2 was developed as a biomarker of breast cancer. One of the detection methods is using PCR. The most important step in the PCR process is the determination of the annealing temperature. This research performs Tm analysis using the insilico program from Neb, insilico, Thermofisher, and Promega and in vitro optimization. Methylated DCR1 can be amplified at annealing temperatures of 51.4°C, 52.4°C, 53.6°C, 54.7°C measuring about 600bp according to Tm analysis of insilico and promega. DCR1 could also be amplified at annealing temperatures of 50,1, 49, and 48.8 but the primers were also amplified at non-specific sites. Methylated DCR2 could be amplified at annealing temperatures of 48.8°C, 49°C and 50.1°C and a specific size of about 500 bp according to the Tm analysis of promega. Unmethylated DCR1 and DCR2 genes could not be amplified at the annealing temperature which were analyzed using Neb, insilico, promega, and thermofisher.

Keywords: Melting temperature; Annealing temperature; PCR.

INTRODUCTION

Breast cancer is the leading cause of woman death in America about 13%. The incidence of breast cancer in Indonesia ranks first, and the second highest cause of death after lung cancer [1]. Breast cancer develops from an early stage in the form of benign cancer that can divide continuously (hyperplasia and low grade dysplasia), pre-cancer (high grade dysplasia and carcinoma in situ), and cancer (invasive, and metastatic carcinoma). The incidence of breast cancer can be influenced by genetic and epigenetic factors. Epigenetic factors that can cause tumor development are DNA methylation and histone acetylation. DNA methylation aberrations are responsible for the silencing of tumor suppressor genes, leading to tumor development and cancer progression.

Research that has been carried out regarding DNA methylation modification in cancer cells occurs in the cytosine residue region at the CpG dinucleotide location on the gene promoter or the first exon where CpG islands are formed [2]. Changes in DNA methylation in the promoter gene cause the formation of suppressed chromatin structures so that it inhibits transcription factors from being able to bind to the active site, thereby causing uncontrolled DNA functions involved in tumor development, such as tumor suppressors and DNA repair, cell cycle regulators, and transcription factors [3]. One of the genes that control the process of apoptosis is DCR1 and DCR2. DNA methylation on the promoter site of DCR 1 and 2 genes causes inhibition of cell cytotoxic processes. DCR1 and 2

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are tumor necrosis factor receptor superfamily member 10C. DCR1 and DCR2 are receptors for TRAIL ligands that play a role in cytotoxic processes. DCR1 is able to prevent apoptosis by binding to TRAIL[4–6].

One of the detections is to determine the presence of DCR1 and DCR2 gene methylation by the PCR method. PCR is a method to amplify or duplicate specific genes in a short time [7,8]. The PCR process produces specific data when using the right steps. One of the PCR steps that plays an important role in determining gene specificity is annealing [9–12]. Annealing is the stage of attaching the primers to the target gene. Annealing requires the right temperature by calculating the melting temperature (Tm). Melting temperature is highly dependent on the composition of adenine, guanine, cytosine, and thymine. The more guanine and cytosine content in the primers will determine the higher temperature, so the annealing temperature of each primer will be different [13]. Before carrying out the PCR process, the steps carried out were optimizing the annealing temperature [11,12]. The annealing temperature was determined manually or using the insilico program. The annealing temperature optimization process is an important step before PCR because the conditions of the samples, reagents, and tools to be used are different for each researcher [14].

Insilico analysis simplifies trial and error annealing steps without clear references. Insilico analysis is the calculation of Tm using bioinformatics. There are several factors used to consider the annealing temperature calculation, namely the type of DNA polymerase, G and C content, and primers concentration. Insilico temperature melting analysis has many options including Neb, Thermofisher, Promega. Choosing the right insilico program is very important, especially if researchers don't have a thermocycler engine with a temperature gradient. This study aims to confirm the results of insilico annealing temperature analysis of several programs with in vitro PCR of methylated and unmethylated DCR1 and DCR2.

MATERIAL AND METHOD

The experimental study design used serum samples from breast cancer patients. The variables studied were the primers annealing temperature of methylated and unmethylated DCR1 and DCR2. Annealing temperature search analysis using the Insilico program. Promega Wizard®SV Genomic DNA Purification System kit, GoTaq® Green Master Mix 2x M7128(Promega), Primers methylated and unmethylated DCR1 and DCR2, DNA template, Nuclease free water, Agarose (GeneDireX), Nucleic Acid Gel Stain (Smobio NS 1000 FlouroVueTM), 5x DNA Loading Dye Smobio DL4000 Excel DyeTM, marker 1 kb vivantis.

Nanodrop MaestroGen, PCRmax 10570-1 version 2.41 thermocycler machine, UV transilluminator (MS Major Science), Mupid electrophoresis machine. DNA isolation using the promega Wizard®SV Genomic DNA Purification System kit. The isolated DNA was then calculated for concentration and purity using the MaestroGen nanodrop machine.

Insilico analysis of primary annealing temperature using https://tmcalculator.neb.com/, http://insilico.ehu.es/tm, https://worldwide.promega.com, https://www.thermofisher.com. The type of material used for the gene amplification process is a consideration for the annealing temperature data to be obtained. DNA polymerase using GoTaq® Green Master Mix 2x, M7128 Promega. Primers concentration was 100 pmol and template DNA was 3 ng/µl.

Specific gene amplification using the PCRmax 10570-1 version 2.41 thermocycler machine. 3 µl of 1 ng/µl template DNA was pipetted and put into a PCR microtube. Primers DCR 1 unmethylated F5'-GAATTTTTTTATGTGTATGAATTTAGTTAAT-3' and R5'-CCATCAAACAACCAAAACA-3' were added 2µl each reaction. GoTaq® Green

Master Mix 2x M7128 (Promega) added 12.5µl. Nuclease free water was added as much as 5.5µl. The reaction was homogenized using a vortex and spindown. The total volume of the reaction was 25µl. The PCR steps include predenaturation at 95°C for 15 minutes, denaturation at 95°C for 30 seconds, annealing at 48.8°C, 49°C, 50.1°C, 51.4°C, 52.4°C, 53.6°C, 54.7°C, 55.9°C, 57°C, 58.2°C, 59.1°C, 59.3°C for 1 minute, elongation temperature 72°C for 1 minute, extra extension at 72°C for 10 minutes, and cooling down at 4°C for 10 minutes. The same steps and PCR components were used to amplify the DCR1 methylated F5'-TTACGCGTACGAATTTAGTTAAC-3' and R5'-ATCAACGACCGACCGAAACG-3' genes, unmethylated DCR2 genes F5'-TTGGGGATAAAGTGTTTTGATT-3' and R5'-AAACCAACAACAAAACCA-3', and methylated DCR2 genes F5'-GGGATAAAGCGTTTCGATC-3' and R5'-CGACAACAAAACCGCG-3' (Tserga, 2011). Two (2)g of agarose (GeneDireX) was weighed, then the agarose was dissolved with 100 ml of TAE 1X. The solution is heated in a microwave until all the agarose powder is dissolved and the liquid is clear. Nucleic Acid Gel Stain (Smobio NS 1000 FlouroVueTM) as much as 4 µl was added to the agarose solution. The warm agarose solution is poured into the mold and the comb is installed. The hardened agarose gel is used for the electrophoresis process. 2 µl of loading dye (5x DNA Loading Dye Smobio DL4000 Excel DyeTM) was mixed with 8 µl of PCR product, then resuspended and put into agarose wells. 1 kb marker was used to determine the size of the PCR product. The sample running process was carried out using a voltage of 50V for 1 hour. The visualization process uses a UV transilluminator (MS Major Science).

The gene bands of DCR1 methylated at about 600 bp and DCR2 methylated at about 550 bp appeared at certain annealing temperatures and matched the suggested insilico data

RESULTS AND DISCUSSION

Insilico analysis using the programs https://tmcalculator.neb.com/, http://insilico.ehu.es/tm,https://worldwide.promega.com,https://www.thermofisher.com produces annealing temperatures that different (Table 1.). The Neb program produces one annealing temperature for a pair of primers. The information obtained from the NEB program is the annealing temperature, the number of nucleotides from a pair of primers, and the G+C content. The data entered in the Neb program are the type of polymerase kit, forward and reverse primers nucleotide sequences and the concentration of primers.

The insilico program from http://insilico.ehu.es/tm produces different annealing temperatures between the forward and reverse primers, so the annealing temperature of both primers must be confirmed simultaneously (Table 1). The information obtained was primers nucleotide length, G+C content, molecular weight, and annealing temperature. The data that is processed in the http://insilico.ehu.es/tm program is the primers nucleotide sequence, and the determination uses basic Tm or base-stacking Tm. In the base-stacking Tm data can be entered primary concentration, salt concentration, and concentration of Mg^{2+}.

The insilico program from https://worldwide.promega.com resulted in different Tm calculations between the forward and reverse primers (Table 1). The data needed in the Promega program are the primers nucleotide sequence, the concentration of the primers, and the type of buffer and polymerase used.

The insilico program from https://www.thermofisher.com generated Tm calculations for both primers (Table 1). In addition, the information provided is the annealing temperature of each primers, molecular weight, extinction coefficient, and annealing temperature.

Table 1. Annealing temperature based on insilico and in vitro analysis

Gen	Neb	Insilico	Promega	Thermo	Invitro
DCR1 unmet	31 °C	F-49.8 °C R-41.2 °C	F-53 °C R-47 °C	51,7 °C	
DCR1 met	39 °C	F-49.9 °C R-53.8 °C	F-52 °C R-57 °C	55,7 °C	52.4 °C 53.6 °C 54.7 °C 51.4 °C 50.1 °C 49 °C 48.8 °C
DCR2 unmet	38 °C	F-47.4 °C R-45.6 °C	F-51 °C R-50 °C	53,7 °C	
DCR2 met	37 °C	F-48.9 °C R-45.9 °C	F-50 °C R-49 °C	53,8 °C	48.8 °C 49 °C 50.1 °C

Based on the annealing temperature of several insilico programs, in vitro tests have been carried out using the PCR method. The annealing temperature range used is 50°C to 60°C with 12 different temperature gradients. The genes that were successfully amplified were methylated DCR1 and DCR2 (Table 1).

3.2 Invitro test optimization annealing temperature insilico program

The methylated DCR1 PCR product of about 600 bp was successfully amplified at an annealing temperature of 52.4°C (Figure 1). Thin amplified DNA bands indicate low amplicon. Low amplicon can be affected due to the low concentration of template DNA. The thick DNA band indicates a high concentration of template DNA [13,15]. The annealing temperature of DCR 1 methylated in vitro is almost the same as the insilico program from http://insilico.ehu.es/tm.

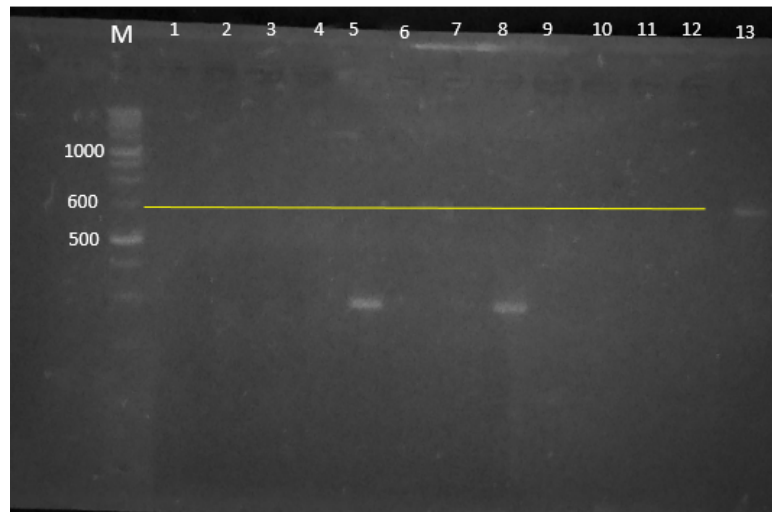

Figure 1. Optimization of the annealing temperature of the methylated DCR 2 gene (1) 50.1°C; (2) 49°C; (3) 48.8°C, LRP1 gene (5) 59.1°C ; (6) 58.2°C ; (7) 57°C ; (8) 55.9°C , unmethylated DCR2 gene (9) 57°C ; (10) 55.9°C , methylated DCR1 gene (11) 54.7°C ; (12) 53.6°C ; (13) 52.4°C, (M) marker

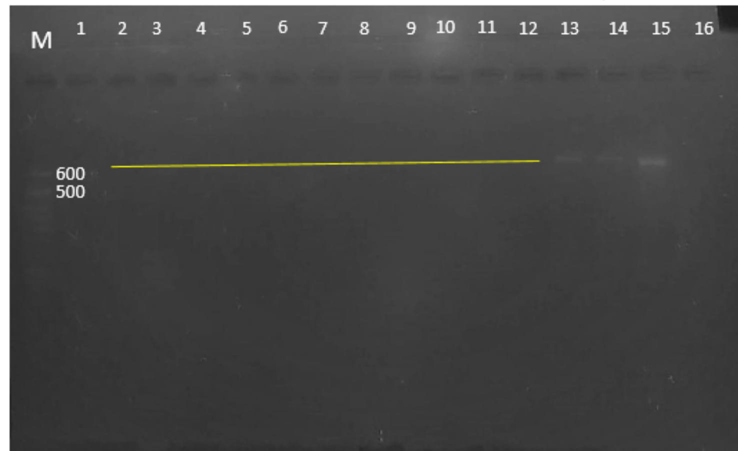

Figure 2. Optimization of annealing temperature of unmethylated DCR 2 gene (1) 53.6°C; (2) 52.4°C; (3) 51.4°C ; (5) 50.1°C ; (6) 49°C ; (7) 48.8°C ; methylated DCR 1 gene (8) 59.3°C ; (9) 59.1 °C ; (10) 58.2°C ; (11) 57 °C ; (12) 55.9°C ; (13) 54.7°C ; (14) 53.6°C ; (15) 52.4 °C , methylated DCR2 gene (16) 51.4 °C, (M) marker

Methylated DCR1 was also successfully amplified at annealing temperatures of 54.7°C, 53.6°C, 52.4°C (Figure 2). The methylated DCR1 gene produced the most amplicons at 52.4°C (Figure 2). The difference in the intensity and thickness of the thin or faint bands was due to the total DNA concentration being too small. However, if the DNA concentration is too high, dilution is also required [7]. The methylated DCR1 gene has almost the same annealing temperature as the insilico program http://insilico.ehu.es/tm.

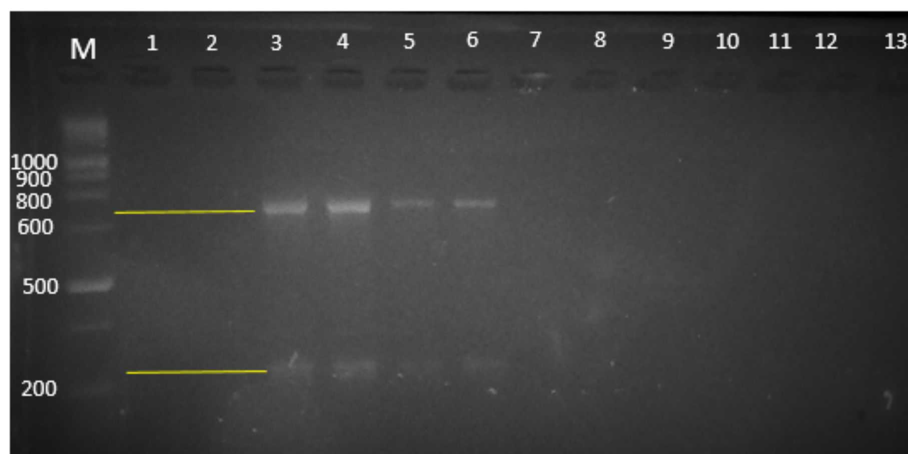

Figure 3. Optimization of the annealing temperature of the methylated DCR 2 gene (1) 53.6 °C; (2) 52.4 °C, methylated DCR 1 gene (3) 51.4 °C; (4) 50.1°C; (5) 49°C; (6) 48.8°C, unmethylated DCR 2 gene (7) 59.1°C; (8) 58.2°C ; (9) 57°C ; (10) 55.9°C ; (11) 54.7°C ; (12) 53.6°C ; (13) 52.4°C , (M) marker

The methylated DCR1 gene was amplified at 51.4°C, 50.1°C, 49°C, 48.8°C. The amplification temperature is too low, causing the PCR product to be non-specific. This is indicated by the appearance of two bands around 600 bp and 200 bp (Figure 3). The basis of the success of the PCR process lies in the suitability of the primers and the efficiency and optimization of the PCR process. The annealing stage requires a very specific and optimum temperature. Annealing temperature that is not specific can cause misspriming, namely primers amplify areas that are not the target or even do not amplify the target DNA [12]. Tm that is too high causes the release of the primer that has been attached to the DNA template so that the PCR product will not be formed, on the contrary if the tm is too low, the primer will stick to the non-specific side[14,16,17].

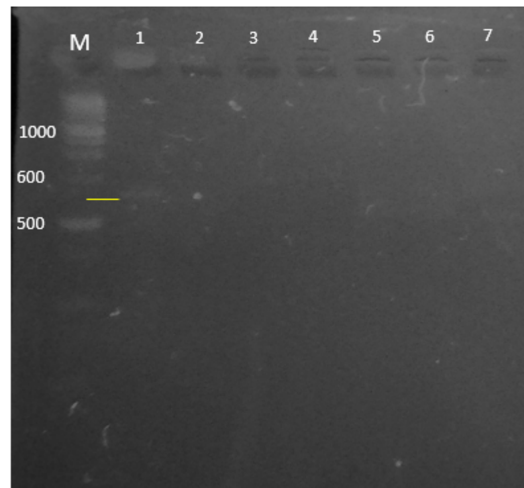

Figure 4. Optimization of annealing temperature of methylated DCR 2 gene (1) 48.8 °C ;(2) 59.3 °C ;(3)59.1 °C ; (4) 58.2 °C ;(5) 57 °C ;(6)55.9 °C ;(7) 54.7 °C, (M) marker

The methylated DCR2 gene was amplified at 48.8°C, 49°C, 50.1°C (Figures 4 and 5). The DNA band is about 500 bp in size and is single indicating a specific PCR product. The DNA band of the DCR2 gene is thin showing the slightly formed amplicons. The annealing temperature of the DCR2 gene in vitro is almost the same as the insilico annealing temperature of https://worldwide.promega.com.

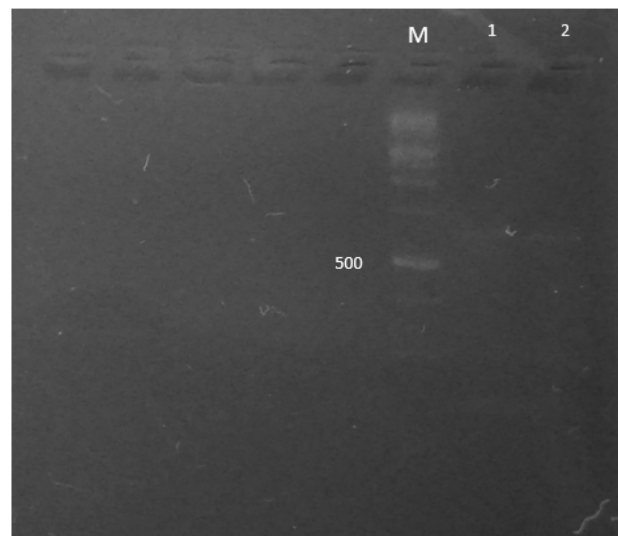

Figure 5. Optimization of the annealing temperature of the methylated DCR 2 gene (1) 50.1 °C ;(2) 49 °C (M) marker

The unmethylated DCR1 and DCR2 genes were not amplified in the annealing temperature range of 50-60 °C according to Tm analysis of various insilico programs. The basis of the success of the PCR process based on in the suitability of the temperature and time at each stage of the PCR, especially annealing. the annealing stage requires a very specific and optimum temperature[18]. The non-specific annealing temperature can cause the target DNA not to be amplified[19,20].

CONCLUSION

The methylated DCR1 gene had an annealing temperature of 51.4°C, 52.4°C, 53.6°C, 54.7°C almost the same as the Tm analysis from http://insilico.ehu.es/tm. The methylated DCR2 gene had an annealing temperature of 48.8°C, 49°C and 50.1°C almost the same as the Tm analysis program https://worldwide.promega.com. The unmethylated DCR1 and DCR2 genes could not be amplified at Tm as suggested by the insilico program.

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DATA AVAILABILITY STATEMENT

The utilized data to contribute to this investigation are available from the corresponding author on reasonable request.

DISCLOSURE STATEMENT

The views and opinions expressed in this article are those of the authors and do not necessarily reflect the official policy or position of any affiliated agency of the authors. The data is the result of the author's research and has never been published in other journals.

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