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OPTIMIZATION OF IN HOUSE POLYMERASE CHAIN REACTION FOR HIGH RISK HUMAN PAPILLOMAVIRUS (HPV 18) E1 GENE-BASED DETECTION

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Abstract

Human Papillomavirus is a virus that can infect human and a causative agent for cervical cancer. This disease mostly caused by HPV type 16 and 18. Polymerase Chain Reaction (PCR) is one of the molecular biology method can be used to detect HPV in sample. Gen E1 in HPV genome has a role in virus replication and transcription, and relatively conserved. This gene can be used in HPV genotyping and detection. This research aim is to find the PCR optimal reaction for HPV type 18 detection. The E1 gene data from the National Center for Biotechnology Information (NCBI) was used in designing primer with Primer-BLAST NCBI. Selection of the primers was done by in silico analyzes using DINAmelt and Mfold. Optimization of annealing temperature and primer concentration was done for selected primers. The result showed that optimal annealing temperature is 59^{o}C with optimal primer concentration is 600 nM.

Keywords: *Human Papillomavirus, Polymerase Chain Reaction, E1 gene*

Abstrak

Human Papillomavirus merupakan virus yang dapat menginfeksi manusia dan dapat menyebabkan kanker serviks. Umumnya, kanker serviks disebabkan oleh HPV tipe 16 dan 18. Polymerase Chain Reaction (PCR) merupakan salah satu metode biologi molekuler yang digunakan untuk deteksi HPV pada sampel. Gen E1 pada genom HPV berperan dalam proses replikasi dan transkripsi virus dan umumnya lestari untuk setiap genotipe. Gen E1 juga dapat digunakan dalam penentuan genotipe HPV dan untuk deteksi infeksi HPV. Penelitian ini bertujuan untuk mencari reaksi PCR yang optimal dalam deteksi HPV tipe 18. Desain primer dibuat berdasarkan data dari National Center for Biotechnology Information (NCBI) dan dibuat dengan Primer-BLAST NCBI menggunakan gen E1. Primer diseleksi dengan analisis in silico menggunakan DINAmelt dan Mfold. Optimasi terhadap suhu annealing dan konsentrasi primer dilakukan menggunakan primer yang telah diseleksi. Diperoleh suhu annealing yang paling optimal adalah 59^{o}C dengan konsentrasi primer 600 nM.

Kata Kunci: *Human Papillomavirus, Polymerase Chain Reaction, gen E1*

1. Introduction

Human Papillomavirus (HPV) is a DNA virus that can infect humans and consists of 200 types. Human Papillomavirus infection can infect anyone regardless of age. Transmission can also infect through sexual contact (vaginal, anal, and oral), or direct contact and exposure with sufferers. There are two types of HPV based on the level of risk, high

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risk HPV (causing cancer, mostly cervical cancer) and low risk HPV (low risk and almost doesn't cause disease, only cause warts on the surface of the skin).

There are 70% of cervical cancer occurrences in women around the world due to HPV types 16 and 18, and 20% are also at high risk and are caused by other types which also include the high risk HPV such as types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 (Prodia Occupational Health Institute, 2014). Other types that also have the possibility to cause cancer are HPV types 26, 53, 66, 67, 70, 73, and 82. Based on data obtained from the HPV Information Centre in 2019, 569.847 women are diagnosed with cervical cancer in the world each year, and 311.365 women died of cervical cancer. Indonesia annually has cervical cancer cases in 32.479 women, and 18.279 die each year which is also explained by the HPV Information Centre with an age range of 15-44 years. The dominant types of HPV occured in Indonesia are HPV types 16, 18, 45, and 52. There are other types of cancer caused by HPV infections but in a small percentage, such as anal, vulva, vaginal, penile, and oropharyngeal cancer.

Currently detection of HPV infection in patients can be done using tools and techniques that are already commercially based on antibodies, such as Hybrid Capture (Digene, USA), INNO-LiPA (Fujirebio, Japan), and Cobas Amplicor (Cobas, USA). Polymerase Chain Reaction (PCR) is another method based on molecular biology that can also be used to detect HPV. This technique has high sensitivity and specificity and can be used in laboratories that don't yet have facilities and access to commercial HPV detection devices.

E1 gene is a gene that has a role in the process of virus replication and has a sequence base that is higly conserved for each HPV genotype. The specialty of this gene is its tendency to remain intact in the process of integrating the viral genome into the human genome. This gene can be used in the process of detecting HPV infections in patients.

This research aims to find the optimal PCR reaction for HPV type 18 detection based on the E1 gene.

2. Methods

2.1 Research Sample

Patients recruitment was carried out based on Kaji Etik No 0688/UN2.F1/ETIK/2018 with the object being sampled Poli Kebidanan patients of the RSUPN Dr. Cipto Mangunkusumo with inclusion criteria: (1) Female, (2) Diagnosed with cervical epithelial cells abnormalities, (3) Age 15-57 years, and (4) Not suffering from ovarian cancer.

Patients with inclusion criteria were taken biopsy of tumor tissue in their cervix which was stored in a 50 ml tube with 10% formalin. Samples are stored in an icebox and taken to a laboratory for DNA isolation.

2.2 Primer Design

Primer design begins with searching for HPV 18 E1 gene nucleotide using data at the National Center for Biotechnology Information (NCBI). The data obtained is stored in one file and align them using GeneStudio Pro ver 2.2.0.0 software. The results of the alignment are formed by consensus sequence for primer using the Primary-BLAST software in NCBI. There are 8 pairs of HPV 18 primers corresponding to the E1 gene. The eight primer pairs were analyzed according to good primary criteria: (1) Self 3' complimentarity < 3, (2) Self complimentarity < 4, (3) Difference Tm between primary forward and reverse primer < 5 ºC , (4) GC % range from 40% to 60%, (5) Primary lengths are 16-28 bases, and (6) Product length < 2000. Primers that comply the criteria are primer pairs 2, 4, 5, and 6. Primers were analyzed in silico.

2.3 DINAmelt and Mfold Analysis

In silico analysis was carried out using Mfold software (http://unafold.rna.albany.edu/) to see whether there was a secondary structure in the primary binding region. DINAmelt (http://unafold.rna.albany.edu/) is used to see the bond between primary pairs.

2.4 Hela DNA Isolation

DNA isolation was carried out using Hela cells that had been cultured before. Isolation was carried out in the Biosafety Cabinet (BSC) at Biosafety Level 2 (BSL-2) using the Quick-DNA TM Miniprep Plus Kit (ZYMO, USA). Isolated Hela DNA sample concentration was measured using spectrophotometer (Infinite 200 PRO NanoQuant Microplate Reader TECAN, Switzerland).

2.5 DNA Isolation for Patient Biopsy

DNA isolation on patient's cervical biopsy was carried out in the Biosafety Cabinet (BSC) at Biosafety Level 2 (BSL-2) using the Quick-DNA TM Miniprep Plus Kit (ZYMO, USA). Biopsy samples were divided into three treatments volume elution buffers, 30 µl, 60 µl, and 100 µl. Isolated DNA sample concentration was measured using a spectrophotometer (Infinite 200 PRO NanoQuant Microplate Reader TECAN, Switzerland).

2.6 Annealing Temperature and Primer Concentration Optimization

Annealing temperature used in optimization is 55 ºC - 65 ºC . There are two primer concentrations used, 300 nM and 600 nM for each primer.

The PCR mix components used are: (1) 5x Mytaq Reaction Buffer (Bioline, USA), (2) HPV 18 Primer Forward & Reverse, (3) ddH_2O, (4) Mytaq HS-DNA Polymerase (Bioline, USA) (5) DNA template. Total PCR mix in one reaction is 25 µl.

The PCR cycle consisted of early denaturation for 95 ºC for 1 minute, denaturation with 95 ºC for 15 seconds, annealing temperature gradient (55 ºC - 65 ºC) for 15 seconds, and elongation with 72 ºC for 15 seconds. The PCR cycle is done with repetitions up to 30x.

2.7 Visualization of PCR Results

Visualization of PCR results was performed using electrophoresis and observation with UV Transilluminator (UVITEC Fire Reader V10, UK). The agarose concentration used was 2% with 100 V voltage for 25 minutes. Staining was carried out with Florosafe DNA (1st Base, Singapore) using the pre-casting method.

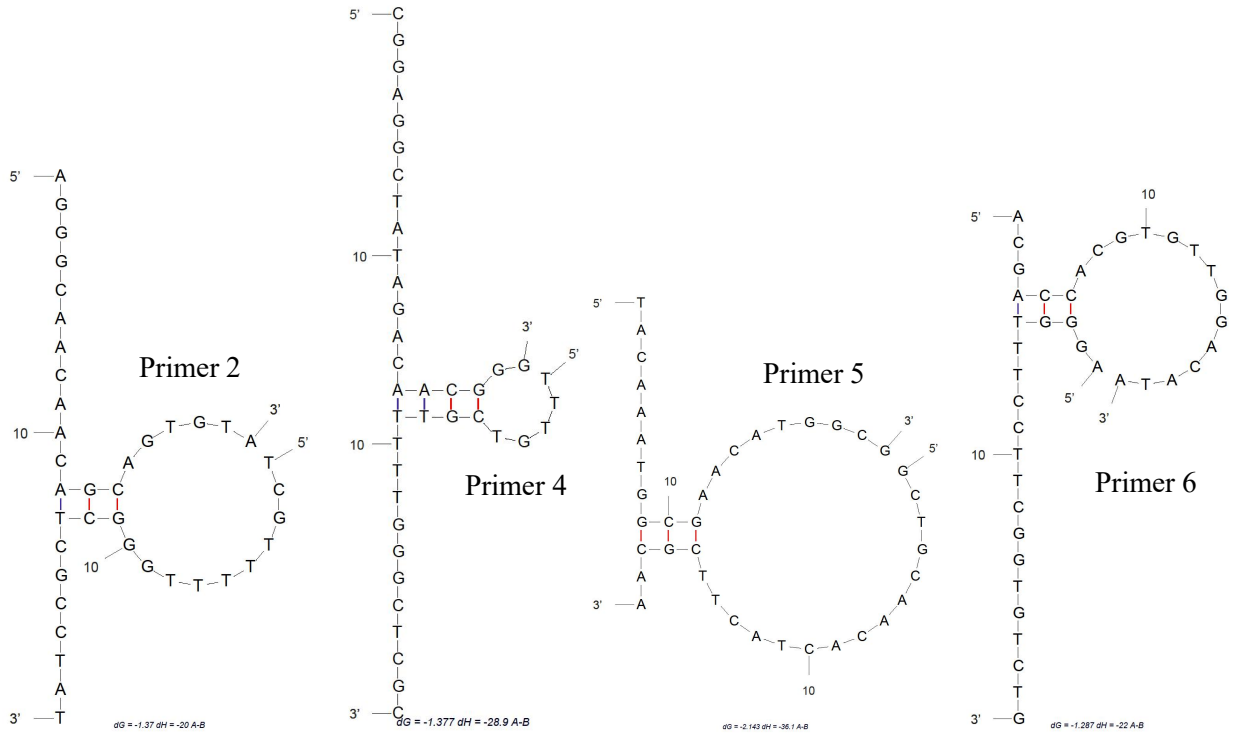
2.8 Validation with Patient Samples

The results of PCR reaction optimization were tested using samples of patients with the same reaction conditions.

3. Research Result

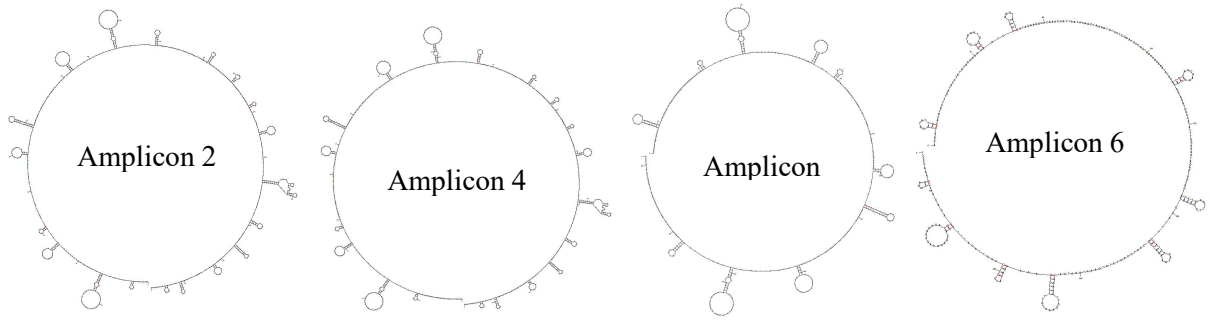
The primer design was carried out based on data obtained from searching for HPV 18 nucleotides using NCBI with access numbers KU298886.1, KC456644.1 and X04773.1. The selected nucleotide data are complete genomes and have E1 protein. Alignment was performed using GeneStudio Pro on the data obtained. The results of the alignment are made consensus and formed into primers using Primer-BLAST. There are several primers of Primer-BLAST results.

Based on research conducted by Dinda Eling K. Sasmito (2014), good primer characteristics for PCR include: (1) Self 3 'complimentarity < 3, (2) Self complimentarity < 4, (3) Difference in Tm between forward primers and reverse primers < 5 ºC , (4) GC % range from 40% to 60%, (5) Primary lengths are 16-28 bases, and (6) Product length < 2000. Based on primers produced using Primer-BLAST, four pairs of primer candidates are found good primer criteria (primers 2, 4, 5, and 6). Primer candidates are analyzed in silico using the DINAmelt and Mfold software to see the secondary structure in the primary binding region and the potential for fellow primers (dimers primer) of the primer candidates to be used (Picture 1 and Picture 2).



Picture 1. DINAmelt Analysis Results.

The four primer candidates have different results. The G-C bond at primer 2 tends to be close to the 3' end, the G-C bond at primer 4 is far from both ends 3' and 5', the G-C bond at primer 5 is more than two and close to the 3' end, the G-C bond at primer 6 is close to the end 5'.



Picture 2. Mfold Analysis Results.

The four amplicons of primer candidates have different secondary structures. Amplicon 2 has a secondary structure that is close to the ends 3 'and 5', amplicon 4 has a secondary structure that is far from the ends 3' and 5', amplicon 5 has a secondary structure that is far from the ends 3' and 5', and amplicon 6 has a secondary structure that is close to the 5' end.

The best primer obtained is the fourth primer pair, because the G-C bond is far from the 3' and 5' ends as the primer attachment area so that the chances of forming dimers primer are getting smaller, and the secondary structure is also not close to the primer attachment area. Based on the results of in silico analysis, the good primer is primer 4 with a length of 20 bases and are at 786 bp.

Besides HPV primers, HPV consensus primers are also used, namely CPI and CPIIG primers which are used as indicators for HPV detection in samples based on the results of Frank Karlsen's research on HPV detection (1996). The list of primers used appears in Table 1.

In house PCR optimization for HPV 18 detection was carried out using DNA from HeLa cells, because HeLa cell DNA positive had HPV 18. Optimization of annealing temperature was carried out by PCR temperature gradient using HPV 18 primers (786 bp) primer concentrations of 600 nM at 55 ºC temperature gradation up to 65 ºC. The results of the visualization were carried out using agarose electrophoresis. The results are shown in Picture 3.

Table 1. List of Primers Used

No.	Primer	Sequence
1.	HPV18f	CGG AGG CTA TAG ACA ACG GG
2.	HPV18r	TTT GTC GTT TTT GGG CTC GC
3.	CPI	TTA TCW TAT GCC CAY TGT ACC AT
4.	CPIIG	ATG TTA ATW SAG CCW CCA AAA TT

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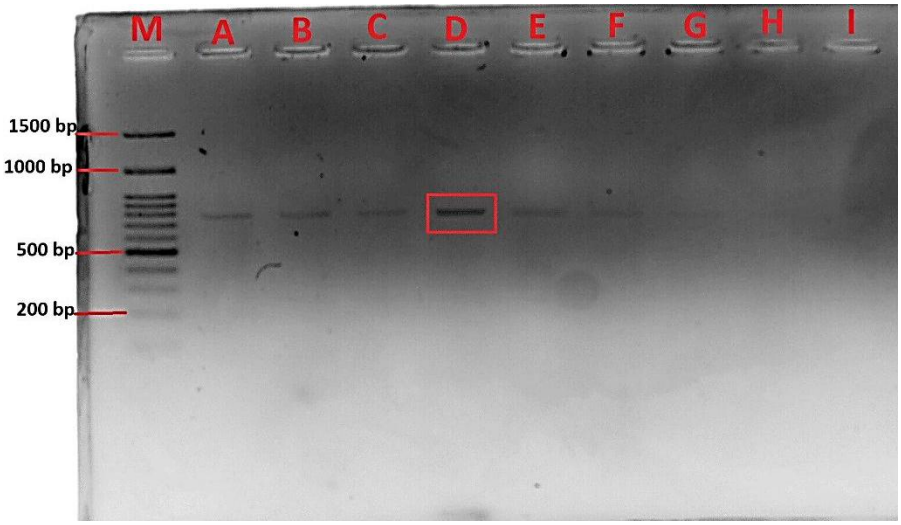
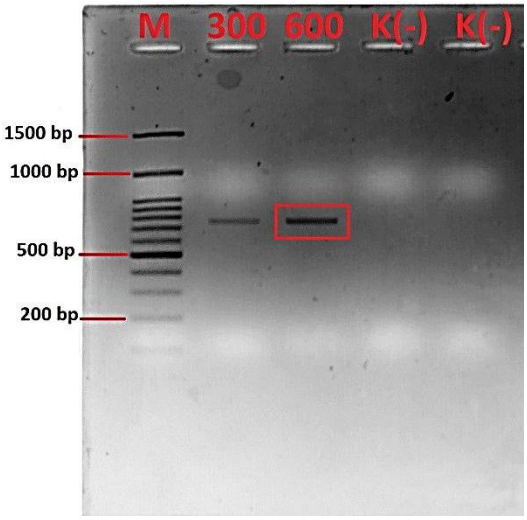


Picture 3. Annealing Temperature Optimization Results (M = marker, A = HPV 18 primer with temperature 55 ºC, B = HPV 18 primer with temperature 55.5 ºC, C = HPV 18 primer with temperature 56.9 ºC, D = HPV 18 primer with temperature 58.9 ºC, E = HPV 18 primer with temperature 61.1 ºC, F = HPV 18 primer with temperature 63.1 ºC, G = HPV 18 primer with temperature 64.5 ºC, H = HPV 18 primer with temperature 65 ºC, I = negative control).

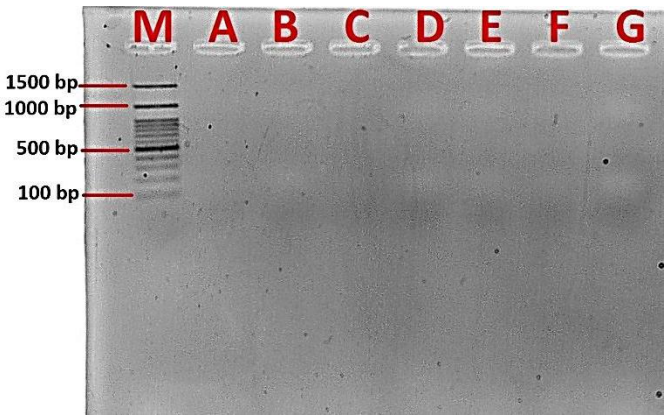
The brightest band was produced in well D (Picture 3), with 58.9 ºC annealing temperature. At this temperature, replication the DNA band produces the highest concentration. At this annealing temperature the most optimal PCR reaction results in a DNA replication of about 786 bp in length. This temperature value is then rounded to 59 ºC. The next optimization is optimization of the primer concentration.

The primer concentrations used were 30 nM and 600 nM.



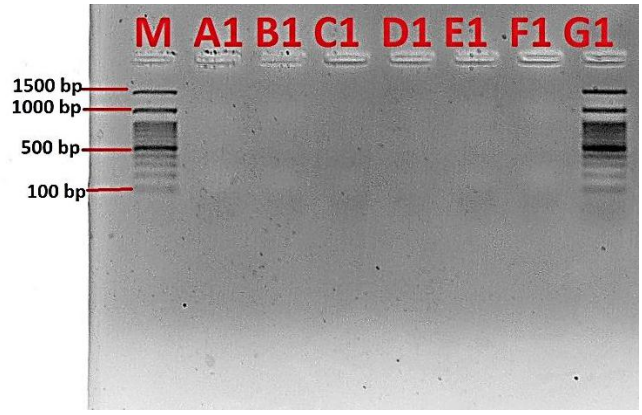
Picture 4. Primer Concentration Optimization Results (M = marker, 300 = 300 nM primer concentration, 600 = 600 nM primer concentration, K (-) = negative control).

The primer concentration obtained which produced the brighter band was the 600 nM primer compared to the 300 nM primer (Picture 4). So it was concluded that the primers with concentrations of 600 nM produce optimal DNA replication. Observations in the next stage were validated by the optimization results using DNA samples from patient biopsies.



Picture 5. Primer Validation of HPV 18 with Sample Patient 5 and Patient 7 (M = marker, A = patient 5 with elution buffer 30 µl, B = patient 5 with elution buffer 60 µl, C = patient 5 with elution buffer 100 µl, D = patient 7 with elution buffer 30 µl, E = patient 7 with elution buffer 60 µl, F = patient 7 with elution buffer 100 µl, G = negative control.

Validation was carried out using samples of patients 5 and 7 with elution buffer concentrations of 30 µl, 60 µl, and 100 µl in each sample. Validation using annealing temperature and primer concentration that has been obtained from the optimization results (annealing temperature of 59 ^{o}C and 600 nM primer concentratio). The results of validating this patient sample didn't produce a target DNA band (Picture 5).



Picture 6. Primer Consensus Validation with Sample Patient 5 and Patient 7 (M = marker, A1 = patient 5 with elution buffer 30 µl, B = patient 5 with elution buffer 60 µl, C = patient 5 with elution buffer 100 µl, D = patient 7 with elution buffer 30 µl , E = patient 7 with elution buffer 60 µl, F = patient 7 with elution buffer 100 µl, G = marker).

Validation was also performed using consensus primers with the same patient sample. The consensus primers (CPI and CPIIG primers) were used as indicators of the detection of the presence of HPV DNA in the samples used. The results of validating patient samples in this trial also couldn't produce the 188 bp target DNA band (Figure 6).

4. Discussion

The results of the primer design produced several primer candidates. According to the research results of Dinda Eling K. Sasmito (2014), good primer characteristics for PCR include: (1) Self 3' complimentarity < 3, (2) Self complimentarity < 4, (3) Difference in Tm between forward primers and reverse primers < 5 ^{o}C, (4) GC % range of 40% to 60%, (5) Primer lengths are 16-28 bases, and (6) Product length < 2000. Self complimentarity with numbers that are too large can cause primers to form dimer structures, especially at the ends 3' which must be avoided. Primer pairs with a Tm difference of more than 5 ^{o}C can cause a decrease in the performance of the amplification process, so that the amplification does not run optimally. GC percentage that exceeds 60% can interfere with the primer attachment process with the target DNA. The lengths of the primers are used in each study differ, but generally range from 18-30 bases. Primers with a length of more than 30 can reduce specific primary performance because of the lower specificity. The distance between the two ends of the 5' primer (product length) is no more than 800 bp, the smaller the easier the amplification process because it requires fewer enzyme concentrations. The good primer candidates based on criteria are primer 2, 4, 5, and 6. Primer candidates were further analyzed using Mfold and DINAmelt software.

In silico analysis was carried out using DINAmelt and Mfold to avoid the risk of primer attachment failure with the target gene in the sample used. DINAmelt is used to see the presence of G-C bonding structures at the 3' or 5' ends. The closer the structure of the G-C bonds at the 3' and 5' ends, the more likely the primers bond and recognize each other and form dimer primer. The formation of a dimer primer makes the primer unable to recognize and bind to the target gene. The existence of a secondary structure of the amplicon in the form of a hairpin loop is seen using Mfold analysis. The secondary structure must not be near the end of 3' because it's the primer attachment area. The closer the secondary structure to the 3' end, the more difficult the primer can attach to the target gene.

DINAmelt analysis on primer pair 2 produces G-C bonds which are slightly at the end 3'. In primer pair 4 the G-C bonds are formed far from the ends of 3' and 5'. The primer pair 5 has more than two pairs of G-C bond and close to the end of 3', the possibility of primer dimer formed is even greater. Primer pair 6 has a G-C bond that is at the end of 5', this primer pair has possibility forming a primary dimer (Picture 1).

Mfold analysis of the amplicon in primer 2 results secondary structure at the end of 3'. In primer pairs 4 and 5 the secondary structure isn't closed to the 3' or 5' ends, whereas in the primer 6 the secondary structure is very close to the 5' end which can interfere with the primer attachment process with the target DNA (Picture 2).

The four primer candidates that have been analyzed produce that the best primer is primer 4 compared to the other three primers, because it fulfills good primary criteria and the results of in silico analysis that don't form dimer primer and secondary structures don't interfere with the primary attachment process.

Primers that have been analyzed in silico are used for optimization of the PCR reaction. Optimization is done using DNA from HeLa cells that have been isolated before, because it has a genome with integration of HPV DNA type 18. Optimization is carried out using the gradient of annealing temperature and primer concentration. The annealing temperature used is between 55 ºC to 65 ºC because it is the optimal temperature range in the PCR process. The brightest and clearest band is the band in the sample with a temperature of 58.9 ºC which is in well D (Picture 3). The higher the temperature the more vague the band is formed, which indicates that the reaction isn't running optimally in the amplification process with too high temperature. In this reaction the best band is specific to around 786 bp.

The next optimization is primer concentration, with primary concentrations used 300 nM and 600 nM. It's seen that the concentration of primers of 600 nM produces clearer bands compared to primers of 300 nM. The greater primer concentration, the more optimal primer attachment process with target gene that causes the reaction to run better and produces a better band during visualization. Good results are also obtained because the primers fulfill the optimal criteria, so the process of attaching primers with the target DNA when amplification can work well.

The results of this optimization are then used for validation of the patient sample. The patient sample used was patient sample 5 and 7, because the sample had the highest DNA concentration compared to the other four samples. In the validation also used consensus primers which are indicators to detect the presence of HPV DNA in patient samples. Validation using patient samples with the reaction according to the results of optimization gives negative results, this is because the patient sample has a low DNA concentration so that the amount of DNA in the sample is very small or even non-existent. Based on the instructions for use from the PCR reagent manufacturer, minimum DNA sample concentration required for PCR process is 10 ng, while the DNA in the patient biopsy sample has a concentration of less than 10 ng (not written on this paper), so that the PCR reaction does not run optimally. Another thing that can cause negative results is the possibility in a patient's cervical biopsy sample that there are more blood clots than tumor tissue, so the results of HPV DNA isolation are very small.

Conclusion

Based on the results of in silico analysis using DINAmelt and Mfold, the most effective primers can be determined for use in PCR when optimization. Optimization performed obtained an optimal reaction with annealing temperature (59 ºC) and a primer concentration (600 nM). Visualization of the results showed that the PCR reaction produced a target DNA band of 786 bp.

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