International Journal of Psychosocial Rehabilitation, Vol.24, Issue 01, 2020
ISSN: 1475-7192

Optimization of In-house PCR Assay for Human BCL-2 Gene

[1] Henny Saraswati, [2] Anita Nauli Pulungan ,[3]Aroem Naroeni, [4] Esa Fauzi

Abstract: *BCL-2 gene is present on human chromosome 18 and contributes in regulating the mechanism of apoptotic cells. Mutations and overexpression of the BCL-2 gene are known to be associated with several cancers. Comprehensive research on the role of BCL-2 in the incidence of cancer is needed to understand course of the diseases and treatment. Polymerase Chain Reaction (PCR) method for copying the BCL-2 gene can be used in this research. Optimization of the in-house PCR reaction was carried out to determine the optimal conditions of the PCR reaction on the doubling of the BCL-2 gene. In-silico analysis of the primer design produces three pairs of primers used in the optimization process. The parameters used in this optimization are the annealing temperature and primer concentration used. Validation of the results of optimization of the PCR reaction was carried out by biopsy samples of tumor tissue of patients with cervical tissue abnormalities. The result showed that the best primer pairs of the reaction is primer C with optimal annealing temperature is 57^{0}C and 800 nM concentration.*

Keywords *: Apoptotic mechanism, BCL-2 gene, Polymerase Chain Reaction*

Introduction

The BCL-2 gene can be found on human chromosome 18. This gene is responsible for regulating apoptosis in a cell[1]. The mechanism of apoptosis has an important role in biological processes. It can be influenced by the cell itself or the surrounding tissue that signals the occurrence of cell apoptosis. Imperfect regulation of cell apoptosis can cause uncontrolled cell division and the emergence of several diseases such as autoimmune diseases, cancer, and neurodegenerative diseases[2].

Cervical cancer caused by Human Papillomavirus (HPV) infection, especially type 16 and type 18. Currently, the death rate from cervical cancer is the third-largest globally and ranks second after breast cancer in Indonesia.[3]

Polymerase Chain Reaction (PCR) is a technique of amplification of certain genes (DNA) in vitro DNA by repeating 25-30 cycles. PCR technique is a technique often used in molecular biology laboratories for the identification of genetic diseases, viral infections, for diagnosing a disease, identification in the field of forensics, application of biodiversity, and detection of gene mutations in cells or tissues by quantifying gene expression. The strength of this PCR technique is its high level of sensitivity and specificity.

Optimization of PCR techniques needs to be done to get the PCR reaction conditions with the results of high concentration, sensitive, and specific DNA amplification. In this study optimization of the in-house PCR reaction for optimal copying of the BCL-2 gene was carried out. It is hoped that the results of this study can be used for further research on the role of the BCL-2 gene in the incidence of diseases, especially cervical cancer.

[1]*Universitas Esa Unggul hennysaraswati@esaunggul.ac.id Corresponding author: hennysaraswati@esaunggul.ac.id*
[2] *Universitas Esa Unggul anita.nauli@esaunggul.ac.id*
[3]*Universitas Esa Unggu, Widyatama University aroem@esaunggul.ac.id*
[4] *Widyatama University. Corresponding author: hennysaraswati@esaunggul.ac.id*

1. Methods

2.1 Research Sample

Research samples are DNA from the saliva of healthy people and from tumor biopsy in patients with cervical abnormalities who attend the Obstetrics Clinic of RSUPN Dr. Cipto Mangunkusumo. A sampling of patients has passed the ethical review from the Health Research Ethics Committee of the Faculty of Medicine, the University of Indonesia with the ethical review number 0688 / UN2.F1 / ETIK / 2018.

2.2 Primer Design

The primer design was carried out using BCL-2 gene data obtained from the NCBI (National Center of Biotechnology Information) website, (https://www.ncbi.nlm.nih.gov) with accession number NM_000633.2. The sequence that has been selected is then entered into the primary design software that is freely available, namely Quest Primer from IDT (Integrated DNA Technologies) (https://sg.idtdna.com/Primerquest) Primary specificity is tested with BLAST (Basic Search Alignment Search Tool) from NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3 In silico Analysis of Primers

In silico analysis was carried out using DINAmelt http://unafold.rna.albany.edu/?q=DINAMelt) which was built by N.M Markham and Zucker[4], to see the possibility of bonding between primary pairs (primary dimers). In silico analysis was also carried out to predict secondary structures in the primary binding region.[5]

2.4 DNA Isolation from Saliva Samples

The DNA isolation from healthy subject's saliva was carried out with the kit protocol (Quick DNA Miniprep Plus KIT, Zymo Research, USA)[6]. Saliva samples were put into a 1.5 ml tube. As much as 1 ml of saliva was added with BioFluid Cell Buffer and Proteinase K, then incubated for 10 minutes at 55°C. The Genomic Binding Buffer was then added with a volume of 1: 1. DNA was washed with gDNA Wash Buffer with 2 times washing. The elution buffer is added later to shed the DNA from the column. DNA isolation result is then stored at -20°C.

2.5 RNA Isolation from Biopsy samples

Tissue samples were incubated using the Quick-RNA Miniprep Kits kit (Zymo Research, USA) in a microtube and heated for 3 hours inside. After preparation, the sample is ready for the RNA isolation process. RNA isolation was carried out according to the device protocol used by the Quick-RNA Miniprep Kits (Zymo Research, USA). Initially, the RNA Lysis Buffer is added to the sample, transferred to the Spin-Away Filter, and centrifuged at a speed of 12,000 x g for 30 seconds, and the resulting flow-through is stored. Absolute ethanol (95-100%) is added in a ratio of 1: 1 and mixed by pipetting. The solution formed is then transferred to the Zymo-Spin IIICG Column and centrifuged. Washing RNA is done with Wash Buffer RNA. Washing is done 2 times to get pure RNA. The final stage of this isolation process is to add DNase / RNase-Free Water to the column and centrifuged at 12,000 x g for 30 seconds. The RNA results are stored at -80 ° C until further analysis.

2.6 Optimization of Annealing Temperature and Primer Concentration in PCR Reactions

Optimization of annealing temperature and primary concentration on PCR using the MyTaq ™ HS DNA Polymerase PCR kit (Bioline), USA.

The reaction mixture for PCR is in following the manufacturer's instructions MyTaq HS Polymerase and Mixes (Bioline, USA). The mix components of the PCR reaction mixture are as follows: (1) 5x Mytaq Reaction Buffer (5 µl); (2) Primary A, B, and C Forward & Reverse (each as much as 1 µl for a concentration of 400 nm and 2 µl for a concentration of 800 nm); (3) ddH2O (16.5 and 14.5 µl); (4) Mytaq HS-DNA Polymerase (0.5 µl); (5) DNA template (1 µl);The total volume of the PCR reaction mixture in one reaction is 25 µl. The stages in the PCR reaction are (1) Initial denaturation of 95℃ for 1 minute, (2) Denaturation of 95℃ for 15 seconds, (3) Gradual Annealing temperature from 55 to 65℃ for 15 seconds and (4) Elongation of 72℃ for 15 seconds. The repetition cycle is 30 times.[7]

2.7 Analysis of PCR Results

The results of DNA amplification were then analyzed using agarose electrophoresis technique (Advance Mupid Ex-U, Japan), with a concentration of 2%. Running electrophoresis was done with a voltage of 100 V for 25 minutes. Visualization of the results of the electrophoresis was carried out with UV Transilluminator Gel Doc (UVITEC Fire Reader V10, UK) to see the DNA band.

2. Results

2.1 Primer Design

The results of the primer design with in silico analysis using the "BLAST primer" from the NCBI website produced four primer candidates. Subsequent analysis with DINAmelt and Mfold (Figures 1 and 2) yielded three primary candidates, called primers A, B, and C (Table 1). These three primers are used in the PCR reaction optimization process.

Table 1. Primer candidate for BCL-2

	Sequence (5'-3')	Length	Tm	GC%
	Primer A			
Forward	GTGGATGACTGAGTACCTGAAC	21	61.9	55
Reverse	GAGACAGCCAGGAGAAATCAA	22	619	55
	Primer B			
Forward	GGAGGATTGTGGCCTTCTTT	20	59.90	55
Reverse	GTTCAGGTACTCAGTCATCCAC	20	59.97	60
	Primer C			
Forward	CGGGAAGTACGGGAATCGAG	20	59.97	60
Reverse	TACAAGGTTTTGGGCTGCCA	20	60.11	50

2.2 Annealing Temperature Optimization

The first PCR optimization is done by annealing temperature optimization with a temperature gradient at 55-65℃ to get the optimal temperature for the primer.

Optimal annealing temperature results are at 56.9℃ (figures 3 and 4, well C). Primer A does not produce DNA bands in the optimization, so this primer is not used for further optimization.

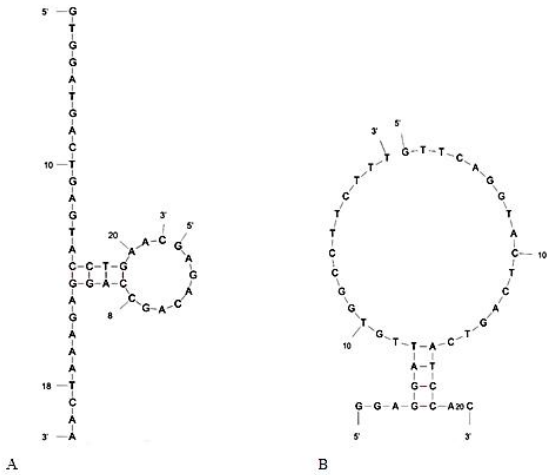

Figure 1. Results of DINAmelt analysis for primer A (left) and Primer B (right).

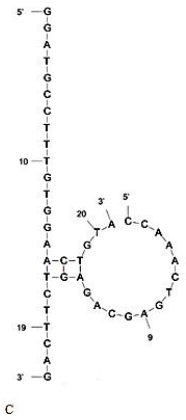

Figure 2 Results of DINAmelt analysis for primer C.

2.3 Primer Concentration Optimization

The primer concentrations used were 400-800 nM for B and C primers. The result showed 800 nM is the optimal concentration, based on DNA band visualization using the UV Transilluminator Gel Documents. The DNA band for 800 nM looked thicker and clearer than the 400 nm concentration. The higher the concentration the PCR reaction can run more optimally because of the high primary quantity[7]. Target DNA bands range from 19-22 bp.

2.4 Validation of PCR Reaction Optimization Results

The results of the optimization are then validated using biopsy samples from patients with cervical tissue abnormalities. Sample validation using primers B and C with concentrations of 400 and 800 nm. There was no DNA band from this validation.

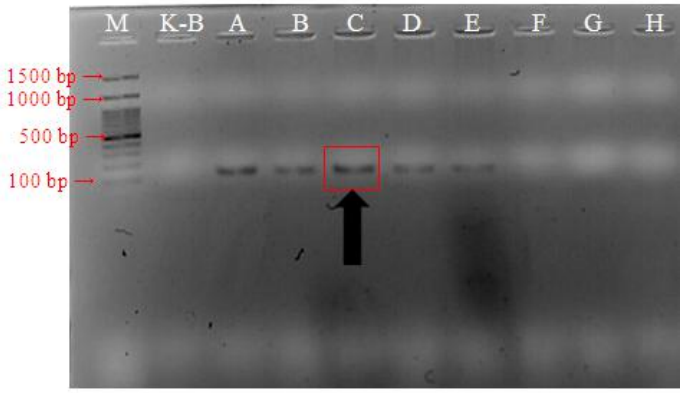

Figure 3. Optimization Results for primer B with 55-65℃ annealing temperature. M: DNA Marker, A: annealing temperature 55℃, B: annealing temperature 55.5℃, C: annealing temperature 56.9℃, D: annealing temperature 58.9℃, E: annealing temperature 61.1℃, F: annealing temperature 63.1℃, G: annealing temperature 64.5℃, H: annealing temperature 64.5℃ and K-B: Negative Control. DNA bands obtained at a size of about 72 bp.

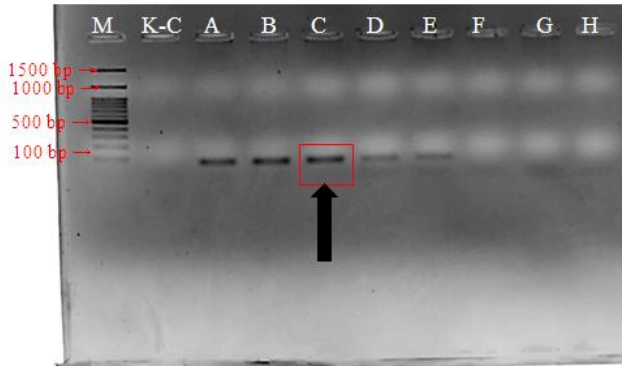

Figure 4 Optimization Results for primer C with annealing temperature 55-650C. M: DNA Marker, A: annealing temperature 55, B: annealing temperature 55.5, C; annealing temperature 56.9, D: annealing temperature 58.9, E: annealing temperature 61.1, F: annealing temperature 63.1, G: annealing temperature 64.5, H: annealing temperature 64.5. K-C: Negative Control. The arrows show the thickest and brightest visualization of the DNA band in column C.

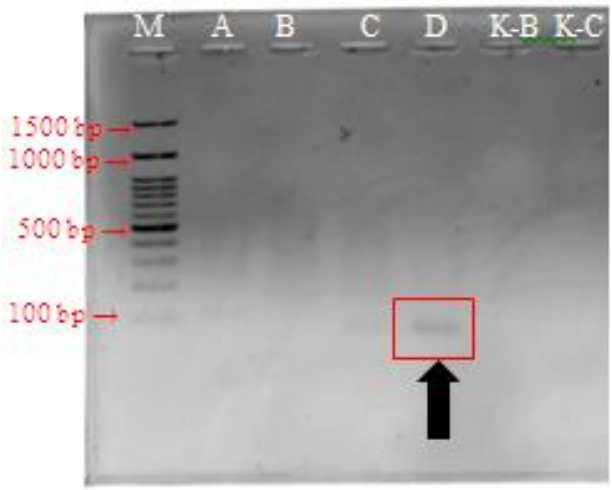

Figure 5. The results of primer concentrations optimization for primer B and C. M: DNA Marker, A: primer B with 400 nm concentration, B: primer B with 800 nm concentration, C: primer C with 400 nm concentration and D: primer C with 800 nm concentration. K-B: negative control for primer B and K-C: negative control for primer C. The arrow shows the visualization of DNA bands for primer C with a concentration of 800 nm. The length of the DNA band 108 bp seems to match the marker.

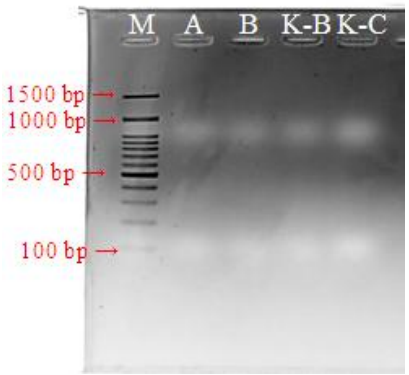

Figure 6. PCR reaction validation with patient samples. M: DNA Marker 100 kb, A: primer B with concentration 800 nm, B: primer C with concentration 800 nm sample DNA of patients, KB: negative control for primer B and KC: negative control for primer C. There were no DNA bands are visible.

3. Discussion

The results of the primer design for the BCL-2 gene showed similarity with 100% identity value in humans based on an analysis with BLAST. Many studies[8–12] using the BCL-2 gene for disease identification and therapy studies have been carried out that use the BCL-2 gene as a target. The use of PCR was chosen because PCR has a high level of specificity and does not require a long time to multiply DNA.

Appropriate criteria for good primers by looking at several parameters such as the large number of G-C bonds formed and the absence of complementary 3 'which can increase bond formation between primers (primary dimers). Analyzing the secondary structure of the amplicon by using Mfold as a prediction to find out the secondary structure in the primary binding. The results of the UNAfold analysis showed that amplicons A, B, C, and D did not have a secondary structure.

The three primer candidates have a length of about 19-22 bp. If the length of the primer is more than 30 bp makes the primer unspecified. These primers have GC values between 55-65%, which is good for the primers. This in-silico analysis needs to be done to get a specific primer so that template replication can occur.

Primer candidates A, B, and C showed good criteria with GC values between 55-57% and lengths around 19-22 bp. In silico analysis was done for four primer candidates (table 1). This analysis conducted using DINAmelt and Mfold yielded three candidate primers that were matched with good primer criteria (primer A, B, and C). The primer D has three G-C bonds that can interfere with the process of attaching primers to the DNA template.[13]

Primer A optimization with the gradation of annealing temperature from 55 to 65°C resulted no DNA bands from the PCR reaction. The use of agarose with 2% concentrations improves the quality of DNA separation during electrophoresis to obtain clear visualization results from the DNA band to facilitate data analysis and a clear band showing the absence of a dimer primer. No DNA bands can be seen from the results of visualization for primer A optimization. This can be caused by several factors such as the results of in silico analysis obtained. The in silico analysis for primer A showed that this primer has three hairpin structures and the PCR mismatch reaction.

Optimization of PCR reaction with primers B and C using salivary DNA samples obtained from healthy subjects reacted optimally at 57 by producing an amplicon size for B primers around 108 bp and C primers around 72 bp. Temperature optimization is done to determine the best PCR reaction to produce DNA bands with high concentrations and specificity. The optimal primer concentration is 800 nM for all pairs of primers used (primers B and C). This can be seen from the visualization of the gel documentation system, which shows DNA bands that are thick and clear at this concentration. The higher the concentration the PCR reaction can run more optimally because of the primary quantity[14]

The results of PCR reaction validation with patient samples did not show the presence of DNA bands during visualization. This is presumably because the concentration of DNA produced is low, so the PCR reaction does not work optimally. The recommended PCR device used states that the minimum concentration of DNA template used is around 10 ng[7]. Differences in the concentration of DNA isolated from saliva and patient samples cause differences in the results obtained. Optimization of the PCR reaction for the doubling of the BCL-2 gene can then be carried out for research on the role of the BCL-2 gene in the event of a disease.

4. Conclusion

Based on this optimization result of the PCR reaction using a gradation of annealing temperature obtained 57 as optimal annealing temperature and 800 nM as optimal primer concentration. The optimal primers for in-house PCR reaction for BCL-2 are primer C (5'-GGATGCCTTTGTGGAACTGTA-3 ') and (5'-CCAAACTGAGCAGAGTCTTCAG-3 ') with ~ 72 bp amplifiers. The absence of DNA bands from the visualization of PCR results validation of patient samples is thought to be due to low DNA concentration so that no PCR reaction occurs for the doubling of the BCL-2 gene. The important thing to consider in designing a primer is in silico analysis with good primary criteria such as the absence of a secondary structure in the primer attachment area, the specificity of the primer to recognize and stick to the target gene as desired.

References

1. Sengupta, P., Chattopadhyay, S. & Chatterjee, S. G-Quadruplex surveillance in BCL-2 gene: a promising therapeutic intervention in cancer treatment. *Drug Discov. Today* **22**, 1165–1186 (2017).

2. Czabotar, P. E., Lessene, G., Strasser, A. & Adams, J. M. Control of apoptosis by the BCL-2 protein family: Implications for physiology and therapy. *Nat. Rev. Mol. Cell Biol.* **15**, 49–63 (2014).

3. Bruni, L. *et al.* Human Papillomavirus and Related Diseases Report. *ICO/IARC Inf. Cent. HPV Cancer (HPV Inf. Centre)* 307 (2019).

4. Markham, N. R. & Zuker, M. DINAMelt web server for nucleic acid melting prediction. *Nucleic Acids Res.* **33**, 577–581 (2005).

5. Zuker, M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res.* **31**, 3406–3415 (2003).

6. Research, Z. *Quick -DNA Miniprep Plus Kit*. (Zymo Research Corp., 2019).

7. Bioline. MyTaq ™ HS DNA Polymerase. www.bioline.com (2018).

8. Perini, G. F., Ribeiro, G. N., Pinto Neto, J. V., Campos, L. T. & Hamerschlak, N. BCL-2 as therapeutic target for hematological malignancies. *J. Hematol. Oncol.* **11**, 1–15 (2018).

9. Amir, A. Ekspresi Gen Family Bcl-2 dan Ekspresi Gen Protein Kanal Ion Vdac1 pada Oligozoospermia. *J. Kesehat. Andalas* **3**, 123–127 (2014).

10. Correia, C. *et al.* Emerging understanding of Bcl-2 biology: Implications for neoplastic progression and treatment. *Biochim. Biophys. Acta - Mol. Cell Res.* **1853**, 1658–1671 (2015).

11. Yu, Y. *et al.* Significance of c-Myc and Bcl-2 Protein Expression in Nasopharyngeal Carcinoma. *Arch. Otolaryngol. - Head Neck Surg.* **129**, 1322–1326 (2003).

12. Loreto, C. *et al.* The role of intrinsic pathway in apoptosis activation and progression in Peyronie's disease. *Biomed Res. Int.* **2014**, (2014).

13. Judelson, H. Guidelines for Designing Primers. 1–5 (2012).

14. Thermofisher. PCR Cycling Parameters — Six Key Considerations for Success. 8 (2018).