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“Achieving Resilient and Environmentally Sustainable Animal Industry in the post COVID-19 Pandemic Era”
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Case Study of Critical Point on RFLP (Restriction Fragment Length Polymorphism)

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Abstract
RFLP is an advanced method after PCR amplification that allows more specific results. The RFLP process starts from the DNA extraction process to the electrophoresis stage. The final RFLP product will show different DNA band profiles in the fragment lengths between individuals. Each of the stages has critical points that can lead to failure. Several obstacles occur during the RFLP process, such as primer design (Tm is greater than 58), PCR (DNA bands do not appear), RFLP (enzymes cannot cut perfectly) and electrophoresis (electric current voltage). This paper will discuss the critical points that influence the success of the RFLP and how to solve it.

Key words: RFLP; PCR; Critical Point

INTRODUCTION

RFLP (Restriction Fragment Length Polymorphism) is an advanced method after PCR amplification that allows more specific results. RFLP uses restriction enzymes to cut specific sites of the target DNA in each individual. The cutting results will show different DNA band profiles in the length of the fragments between individuals. RFLP is generally used in the analysis of genetic and phylogenetic relationships, the origin and history of the domestication of a species, selection, genetic drift and isolation of unique genes of wild species (Pagala and Nafiu, 2020).

PCR (Polymerase Chain Reaction) is a technique used in the laboratory to amplify nucleotides in vitro. PCR can produce millions of copies of a particular piece of DNA from a small amount of DNA. In PCR machine there are consist of a heating and cooling process called the thermal cycle. There are three stages of PCR, namely denaturation, annealing and extension. As molecular biology develops, the use of PCR is to genetic and disease identification, evolutionary biology, viral infections, bio-diversity applications, gene mutation and forensics (Fatchiyah et al., 2009).

The success of the RFLP process is influenced by several factors, one of which is the isolation of some DNA without degradation. RFLP requires a relatively large number of samples and purities of DNA. Besides that, several critical points can lead to failure. In this paper, it will be described what failures in some cases and the process of solving them.

MATERIALS AND METHODS

DNA Extraction
DNA extraction is the first stage of molecular research before moving on to the next step. DNA extraction is used to separate DNA from other materials such as cell membranes, carbohydrates, fats and other cellular components. The quantity and quality of DNA produced must be good, pure without contamination from RNA and protein. There are five stages in DNA extraction, namely 1) Isolation of cells; 2) Lysis of cell walls and membranes; 3) Extraction in solution; 4) Purification; 5) Precipitation. The principles of carrying out DNA extraction are centrifugation and precipitation (Faath, 2009). The success of the DNA extraction process often determines the results in the next process.
Primer

Primers are short nucleotides measuring 12-20 bases which are needed as the attachment point for the DNA polymerase enzyme in the process of DNA formation or elongation of a specific gene in vitro through PCR techniques. Primers function as precursors for DNA synthesis from DNA prints or as a barrier to target DNA fragments to be amplified (Riupassa, 2009; Maitriani et al., 2015).

Primer Design

Primer design includes a very important role in molecular biology activities. This activity will produce a specific primer that will be used in the DNA amplification process. Primer design can be done by using GeneBank data which can be accessed through a DNA database engine in the form of Ensembl or NCBI, the MEGA software application and the Primer Stats website which is useful for evaluating potential PCR primers. In the PCR process, a pair of primers is needed, each of which is located at both ends of the target DNA fragment, namely the forward primers and the reverse primers.

A good primer requirement that needed to obtain optimal result, are:

**Primer length:** a good primer has a length of 18 bp-26 bp, but 20 bp is optimal. The primer length used in different studies, but still minimizes the primer size. According to Kampke et al. (2001) primer length of 16 bp-28 bp intervals. In another theory, (Burpo, 2001; Borah 2011) use a primer limit of 18 bp-22 bp. The primer length is sufficient so that the primer can be bonded easily at the annealing temperature.

**GC content:** the amount of Guanine and Cytosine in the primer should be 40-60%, the optimal GC content is 50%. Calculate it with the formula:

\[
\text{GC content} = \frac{nG + nC}{|p|} \times 100
\]

**Explanation:**
- nG: The number of bases G in the primer
- nC: The number of bases C in the primer
- |p| : Primer length

Avoid the A/T bases on the 3 end of the forward and reverse primers. The primer pair has a G/C base at the end of the primer making the hybridization more stable and the G/C base bond stronger.

**Tm:** good melting temperature about 58-62°C. Primers with too low temperature, will stick elsewhere and produce an unspecified product. Whereas Tm above 65°C can reduce the annealing effectiveness so the DNA amplification process is not going well (Yustina et al., 2018). The distance between the forward and reverse Tm should not be more than 2°C.

**No hairpins and self annealing.** Hairpin is an intramolecular interaction in the primer because it can interfere with the PCR process. Self annealing is the presence of bonds with other similar primers.

Primer optimization

Primer optimization is carried out to obtain the appropriate PCR composition and conditions so that the PCR results are optimal. The efficient of time and materials that be used so that the detection process can be carried out quickly and precisely. The purpose of primer optimization is to increase the amplification product so a thick and clear band is obtained (Yuenleni, 2018; Joko et al., 2011). The optimization step can vary the PCR stages including time, temperature and PCR composition.

Temperature gradient is a step to find the right temperature during PCR optimization for optimal results. Temperature is one of the causes of successful implementation because the process of attaching the primer to the open DNA strand requires optimal temperature. The temperature gradient uses five temperature variations, there are 58°C, 59°C, 60°C, 61°C, 62°C. The results were analyzed by visually comparing the thickness of the bands.
The optimal ribbon is thick, single, clean and on target. If the best temperature is found, this will facilitate the annealing process.

**PCR (Polymerase Chain Reaction)**

The PCR process is an iterative cyclic process including denaturation, annealing and extension by the DNA polymerase enzyme and limited by a pair of primers. The DNA polymerase enzyme used was isolated from the bacterium Thermus aquaticus BM (Taq). This enzyme is able to survive up to a boiling temperature of 100°C (Feranisa, 2016). The basis of PCR cycles is 30-35 cycles, including (Nugroho and Rahayu, 2018; Fatchiyah et al., 2009):

- **Pre-denaturation**: steps carried out for 1-9 minutes at the beginning of the reaction to ensure complete denaturation and activate the DNA polymerase.
- **Denaturation**: occurs at high temperatures (94°C-96°C) causing double strand separation. The recommended temperature is 95°C.
- **Annealing**: the stage of primer attachment to the target DNA depends on the length of the strand, the amount of GC content and the primer concentration. In general, the annealing temperature used is between 50-65°C.
- **Extension**: the process of extending a new DNA strand, starting from the position of the primer attached to the target DNA will move from the 5’ end to the 3’ end of the single DNA strand. Extension temperature ranges from 70-72°C.
- **Post-extension**: ensuring that every single thread that remains is completely extended. The temperature used is around 70-72°C for 5-15 minutes.

**RFLP (Restriction Fragment Length Polymorphisme)**

This technique is used to compare the profiles of DNA bands from cutting restriction enzymes. Samples of several individuals who have genetic diversity will show a visible variation of the banding profile. Premixes that used for the RFLP process are:

1. PCR Product: 5 µL
2. Digest Buffer: 0.7 µL
3. DW: 0.9 µL
4. Restriction enzymes: 0.4 µL

Some enzymes cannot work if the buffer composition is not suitable. The use of different buffers caused the enzyme to work less optimally. Then, it was incubated in an incubator for 16 hours at 37°C during the cutting process. The separation of the cutting was done by agarose gel electrophoresis.

**Electrophoresis**

Electrophoresis is a separation method using the electrical voltage that generated by the electrodes to separate protein, DNA or RNA from molecules that are negatively charged towards to the positive pole. The determining factors in the electrophoresis process, namely (Harahap, 2018):

- **Sample**: The movement of molecules in an electric field is influenced by the shape, size, charge size and chemical properties of the molecule. Electrophoresis will show different DNA bands.
- **Buffer solution**: as a medium for providing electrolytes in the process of moving electricity.
- **Electrical voltage**: a stable source of electricity is needed to produce an electric current with a constant voltage.

The electrophoresis process begins with making agar from 2% agarose gel and put the printed agar into the electrophoresis machine until submerged by the buffer solution. Insert 5 µL of PCR product and insert 3 µL of marker into the well. A 100 V current source was used for 30 minutes.

The next step is staining, giving the sample color so easy to observe. The dye solvent comes from Diamond Nucleic Acid Dye. Staining assisted with a shaker for 30 minutes.
RESULT AND DISCUSSION

DNA Extraction

The success of the DNA extraction process can be measured through several processes, there are checking the presence of DNA bands using the electrophoresis method or measuring the concentration of dissolved DNA using the spectrophotometric method (Phillips et al., 2012). Figure 1 shows the DNA bands after checking by electrophoresis. The DNA concentration that can be used for PCR activities is 50 ng. According to Ningsih et al. (2017) that the minimum concentration of DNA samples is 25 ng/µL. DNA purity from the extraction process determines the thickness of the DNA band.

![Figure 1. DNA Band Checker](image)

Primer Design

Primer design is useful for producing specific primers that help in the process of DNA propagation (amplification). The success of DNA amplification depends on the accuracy of the primers, the primer design is assisted by a computer program (Handoyono and Rudiretna, 2000). Figure 2 shows that there is a warning on Tm of both forward and reverse when primers is greater than 58. However, it is safe on other terms. Therefore, primer optimization and temperature gradient were carried out.

![Figure 2. Checking the Primer Design Result](image)

PCR (Polymerase Chain Reaction)

The success of DNA amplification techniques using PCR can be determine by some factors, there are DNA samples, primer oligonucleotides, printed DNA, buffer solution composition, number of reaction cycles, enzymes used, technical and non-technical factors (Feranisa, 2016). In Figure 3, the DNA bands are not visible, because the lack of time spent on the primer attachment so that time needs to be added to the PCR process. The addition of annealing time was carried out for primer attachment to the length of the DNA fragment.
RFLP (Restriction Fragment Length Polymorphism)

RFLP occurs when fragment length varies between individuals was detected. Figure 4 shows the results of cutting with restriction enzymes. DNA bands appear to vary in each sample. However, in samples 10 and 12, the DNA bands in the second and third rows were brighter than the first DNA bands. This could be because the concentration of the PCR product was higher than the concentration of the enzyme. So when cutting, the restriction enzymes have not finished cutting the DNA band. To find out more clearly, it is necessary to do sequencing.

Electrophoresis

The result of DNA fragments are separated based on their length through an electrophoresis process with agarose gel. The visible result of electrophoresis is the formation of bands and shows pieces of the number of base pairs. The factors that influence electrophoresis are samples, buffer solutions and electric media. Figure 5 shows an illegible and unusable result, because the voltage in the electrophoresis process is unstable. So it is necessary to re-electrophoresis process.
CONCLUSION

The RFLP technique is a series of DNA extraction processes to visualization using electrophoresis. Several obstacles occur during the RFLP process, such as primer design (Tm is greater than 58), PCR (DNA bands do not appear), RFLP (enzymes cannot cut perfectly) and electrophoresis (electric current voltage). Therefore, it is necessary to pay attention to every critical point during the RFLP process, until the good cutting products are obtained.

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