Optimization Of Primary Annealing Temperature With Bigdye Reagent In Sequencing Reaction

Harumi Yuniarti, Astri Rinanti, Bambang Cholis S.

Abstract --- In this research, the annealing temperature was varied to determine the appropriate template sequence. The amplification process used the polymerase chain reaction (PCR) method in the Primary template, to separate the double DNA into a single chain. Furthermore, the cycling duration was compared with the pGEM_Standard. In this research, the process was heated for some time, and the temperature decreased to obtain an appropriate result. Bigdye-pGEM reagents were used to stick the separated molds to become single chains. Also, a primer pair with a large melting temperature difference tends to cause a reduction in the amplification process. The primary sequencing of M13 at 50°C produced a well-readable ampiclon on the observed electropherogram using ABI Prism 310 sequencer. The results shows that the sequencing test with the addition of bigdye reagent volume (without buffer) at 1x concentration and 25 times cycling duration formed high and clear peaks around 600bp. Shorter sequences occurred at lower concentrations, with the reagent used to determine the exact annealing temperature and how optimal the reagent brings up the sequence length that appears on the electropherogram.

Keywords --- Bigdye Reagent, Primary Sequencing, PCR, Sequencing, Annealing Temperature, Electropherogram,

1. INTRODUCTION

THE Sanger method was used to analyze the DNA sequencing with polymerase used as an enzyme subunit to synthesize the presence of dNTP. This method was also used to distinguish dNTP from ddNTP with ABI prism-310 sequencer optimized by setting and determining the composition of the reagent using several processes. The appropriate temperature for the process, and cycling duration to determine the number of amplification cycles related to the reagent injection process was sorted by the capillary electrophoresis (CE) pipe [1], [2], [3]. In previous studies, DNA sequencing was carried out using the BigDye Terminator v3.1 reagent volume dilution method. The cycling duration of 25 times and at 50°C using a phydit program, obtained clear sequencing results with 99.74% similarity [4]. The temperature is estimated for the primer to stick to the DNA mold in a stable state. High annealing temperature makes the primary bond difficult, resulting in less efficient PCR products. Conversely, low temperature causes primary annealing to the DNA in an unspecified place [5]. For this reason, the study continued with variations in the concentration of the reagent, which was expected to determine how optimal the reagent is able to reveal the sequence. Therefore, the selection of reagent concentrations and the correct temperature setting allowed the determination of the sequence length needed in observations, in accordance with efficiency and cost.

2. RESEARCH METHODOLOGYMATERIAL AND METHODS

DNA fragments in the ACGT base sequence (Adenine, Cytosine, Guanine, and Tymine) were used as templates (primers) and amplified using enzymes as well as materials complement the PCR reagent [6]. The amplification process using a PCR machine was carried out with a cycling duration of 25 times. The sequencing test was prepared for the composition of the reagent concentration using the pGEM-3Zf + reagent, by regulating the temperature at 96 °C for 1 minute for the initial denaturation to occur. Each cycle was tested using a temperature of 49°C, 50°C and 51°C, then compared with pGEM Standard. The next cycle sequencing was carried out in stages of successive heating, by denaturation at 96°C for 10 seconds, and 60°C extension for 4 minutes. Furthermore, it was stored at 4°C for purification, which was carried out using ethanol EDTA precipitation as a buffer running. In summary, the DNA sequencing process using the PCR (Polymerase Chain Reaction) was carried out in stages (Figure 1). The reagent concentration method was used to observe the sequences of the DNA process. The composition of the ingredients used are shown in Table 1,

Sequencing test preparation

In Table 1, the addition of 8 µL bigdye terminator v3.1 without buffer is considered the main reagent with concentration 1x, according to the initial procedure and a total volume of 20 µL in the capillary pipe (CE).

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![Figure 1. Stages of DNA sequencing process](image-url)
### TABLE 1

**VARIATION IN REAGENT CONCENTRATION**

<table>
<thead>
<tr>
<th>Reagent Concentration</th>
<th>Buffer</th>
<th>pGEM 3zf(+M13 Prime)</th>
<th>Water</th>
<th>ΔVolume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 x</td>
<td>-</td>
<td>1µL</td>
<td>4µL</td>
<td>7µL</td>
</tr>
<tr>
<td>0.5 x</td>
<td>2µL</td>
<td>1µL</td>
<td>4µL</td>
<td>9µL</td>
</tr>
<tr>
<td>0.25 x</td>
<td>3µL</td>
<td>1µL</td>
<td>4µL</td>
<td>10µL</td>
</tr>
</tbody>
</table>

However, the buffer was added to determine the optimal reagent used to produce a certain sequence length and its quality for concentrations of 0.5 times plus 4µL and 0.25 times plus 2µL. The preparations used to make concentrations of primary dilution with 70% ethanol, and 20 mL buffer TAPS were carried out as follows:

1. **Dilution of Primary M13** with a stock concentration of 59.4 nmol / mL to 3.2 pmol / µL (volume 20 mL) with the following calculation:
   
   \[ m_1 = 59.4 \text{ nmol/mL} \]
   
   \[ = 59.4 \times 10^{-3} \text{ pmol/10}^3\mu\text{L} \]
   
   \[ = 59.4 \text{ pmol/µL (stock concentration)} \]

   \[ V_2 = \text{Volume made is 20 mL} \]

   Used: \[ m_1 V_1 = m_2 V_2 \], then obtained:
   
   \[ V_1 = \{(3.2 \text{ pmol/µL}) (20µL): 59.4 \text{ pmol/µL}\} \]
   
   \[ = 1.08 \mu\text{L} \]

Thus, \[ V_{aqb} = (20 \mu\text{L} - 1.08 \mu\text{L}) = 18.92 \mu\text{L} \] (Volume of aquabides added)

2. **Preparing the buffer 5x** (the final concentration becomes 1x)
   
   a). **Buffer TAPS** (1x) of 20 mL with a formula for 100 mM TAPS. The pH is adjusted to 8 with 5N NaOH, as follows:
   
   \[ m_1 V_1 = m_2 V_2 \]
   
   then:
   
   \[ V_1 = \text{Volume added} \]
   
   \[ m_1 = \text{initial stock concentration} \]
   
   \[ = 1000\text{mM TAPS} \]
   
   \[ m_2 = \text{concentration to be made} \]
   
   \[ V_2 = \text{Prepared volume (20 mL)} \]

   This obtained:
   
   \[ V_1 = \{m_2 V_2 / m_1\} \]
   
   \[ = (100\text{mM}). (20 \mu\text{L}) / (1000 \text{mM}) \]
   
   \[ = 2 \mu\text{L} \]

   b). **1 mM EDTA**
   
   Obtained:
   
   \[ V_1 = \{m_2 V_2 / m_1\} \]
   
   \[ = (1 \text{mM EDTA}) (20\text{mL})/(1000\text{mM}) \]
   
   \[ = 0.02\mu\text{L} \]

   **Buffer 5x** = (2 + 0.02) mL = 2.02 mL

   In order for a total volume of 20 mL, it is necessary to add aquabides:
   
   \[ V_{aqb} = (20 - 2.02) \mu\text{L} = 17.98 \mu\text{L} \]

### 3. RESULT AND DISCUSSIONS

Annealing temperature control is performed to ensure proper primer to the template. The criteria at optimal temperatures are as follows:

- The temperature is characterized by the emergence of the amplicon result of good and clear sequencing with sufficient concentration.
- It is seen from the results of a clearer and less noisy electropherogram.
- The high peak in the electropherogram shows a sufficient yield concentration level.

The results of the electropherogram were not satisfactory with a sequencing test concentration of 1x on a 25x cycling duration with the addition of 8 µL reagent volume. Peak sequences between ± 150 to 200 bp for annealing temperatures of 49°C and 51°C, respectively, are shown in Figures 2 and 3. Based on the electropherogram results in Figure 2, it was shown that 49°C annealing temperature failed to form an amplicon. When the primer remained attached to the template at temperatures below Tm, mispriming tends to occur, ie the primer does not stick to a specific location or is unable to hybridize. For 1x concentration, at 50°C with 25 cycles, a clear amplicon, and relatively high peak was formed (Figure 5).

![Figure 2: 1x concentration (addition of 8 µL reagent volume) on a 25 times cycling for 49°C annealing temperature](image-url)

![Figure 3: 1x concentration (addition of 8 µL reagent volume) on a 25 times cycling for 51°C annealing temperature](image-url)
However, when the annealing temperature was increased to 51°C with 25 times cycling duration, an amplicon was also formed. This is indicated by the low average signal intensity and unreadable sequences (read N), because at 51°C the temperature was too high, therefore, the primer failed to stick to the template which contains G (guanine) and C (cytosin) greater or equal to 60% (Darmo Handoyo et al, 2001). Figure 4 shows the results of the electropherogram in terms of length of the sequence that appeared for 50°C annealing temperature, which completed with standards for variations in concentration of 1x, 0.5x, and 0.25 times. Based on the electropherogram in Figure 5, for a concentration of 1x, setting annealing temperature of 50°C for cycle of 25 times a clear amplicon, with relatively high peaks, was clearly observed ± 600 bp of ± 3000 bp. At a concentration of 0.5x the sequence was formed around ± 200 bp of ± 1000 bp (Figure 6), and at 0.25x the sequence formed ± 100 bp of ± 400 bp (Figure 7).

4. CONCLUSION

In conclusion, a setting of 50°C annealing temperature for 25 times cycling duration in the sequencing process using BigDye reagent (pGEM-BGT) produced electropherograms with clear amplicons. High peaks were relatively observed up to 600 bp while at lower concentrations, a relatively shorter number of sequences were formed. The selection of reagent concentrations and the right annealing temperature regulation greatly influenced the sequence formed.

REFERENCES.


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