



OPTIMIZATION OF CONCENTRATION AND SOAKING TIME OF HARRIS HEMATOXYLIN IN DNA BAND EXAMINATION USING ELECTROPHORESIS

OPTIMASI KONSENTRASI DAN LAMA WAKTU PERENDAMAN HEMATOKSILIN HARIS PADA PEMERIKSAAN PITA DNA MENGGUNAKAN ELEKTROFORESIS

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ABSTRACT

Background: Deoxyribonucleic acid (DNA) molecules can be effectively visualized when stained and observed under ultraviolet light during the electrophoresis process. The commonly used dye Ethidium bromide (EtBr) is considered hazardous due to its potential to cause mutations, cancer, and congenital disabilities. Various alternative dyes have been reported, one of which is hematoxylin. Hematoxylin compounds do not have mutagenic potential and are easier to apply than EtBr. However, there is no optimal variation in concentration and duration of staining for clear and effective visualization of DNA bands. **Purpose:** To find the concentration and staining time of harris hematoxylin staining for DNA bands from agarose gel electrophoresis. **Method:** An experimental method with a group comparison statistical design. The amplified DNA I6S rRNA gene from *Escherichia coli* (584 bp), which had undergone electrophoresis, was stained using harris hematoxylin dye at 0.01%; 0.02%; 0.03%; 0.04%; and 0.05% concentrations and immersion times soaking times of 10, 15, 20, and 30 minutes variations. The intensity of the DNA bands was analyzed using ImageJ. The staining power of the experimental groups was compared to the intensity of control dye and given a grading score of 1 - 4. The experiment was repeated twice, and the mean grading score was calculated. The highest mean value was considered the most optimal value. **Result:** A concentration of 0.02% showed relatively constant staining intensity for each soaking time. A mean value of 3.5 was obtained for a 0.01% concentration for 15 minutes. A 0.03% and 0.04% concentrations for 20 minutes. **Conclusion:** The highest mean value of 4 was obtained for Harris hematoxylin at 0.05% for 15 minutes.

ABSTRAK

Latar belakang: Pada proses elektroforesis, molekul DNA dapat divisualisasikan dengan baik saat diwarnai dan dilihat dengan media sinar ultraviolet. Pewarna *Ethidium bromida* (EtBr) merupakan pewarna yang umum digunakan, namun dinilai berbahaya karena berpotensi menyebabkan mutasi, kanker, dan cacat bawaan. Berbagai perwarna alternatif telah dilaporkan, salah satunya hematoksilin. Senyawa hematoksilin tidak memiliki potensi mutagen dan lebih mudah diaplikasikan dibandingkan EtBr. Namun, dalam penggunaannya belum terdapat variasi konsentrasi dan lama waktu perendaman yang optimal untuk mewarnai pita DNA hasil elektroforesis dengan baik dan jelas. **Tujuan:** Mengetahui konsentrasi dan lama waktu perendaman yang optimum pada hematoksilin haris untuk mewarnai pita DNA hasil elektroforesis gel agarose. **Metode:** Eksperimen dengan desain *static group comparison*. DNA hasil amplifikasi gen I6S rRNA dari *Escherichia coli* berukuran 584 bp yang telah melewati proses elektroforesis diwarnai menggunakan pewarna hematoksilin haris pada konsentrasi 0,01%; 0,02%; 0,03%; 0,04%; dan 0,05% dengan variasi lama waktu perendaman 10, 15, 20, dan 30 menit. Hasil pita DNA yang terbentuk dianalisis intensitasnya menggunakan aplikasi *imageJ*. Intensitas pewarnaan kelompok percobaan dibandingkan dengan intensitas yang terbentuk pada pewarna EtBr, kemudian diberi skor penilaian pada rentang 1-4. Percobaan dilakukan 2 kali pengulangan, kemudian dihitung *mean grading* dari setiap kelompok percobaan. Nilai *mean* terbesar dianggap sebagai nilai yang paling optimum. **Hasil:** Konsentrasi 0,02% menunjukkan intensitas pewarnaan yang cenderung konstan di setiap lama waktu perendaman. Kemudian, ditemukan *mean* sebesar 3,5 untuk konsentrasi 0,01% selama 15 menit. Konsentrasi 0,03% dan 0,04% selama 20 menit. **Kesimpulan:** Nilai *mean* tertinggi sebesar 4 diperoleh pada hematoksilin haris 0,05% selama 15 menit.

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INTRODUCTION

Polymerase Chain Reaction (PCR) is a method for enzymatic replication/amplification of *Deoxyribonucleic acid* (DNA) fragments without needing an in-vitro setting. The PCR process involves several cycles: denaturation, annealing, and extension/elongation. Each cycle duplicates the DNA target into millions of copies (Nurhayati and Darmawati, 2017). After the amplification stage, DNA fragments can be detected through various methods, including gel electrophoresis (Fatchiyah *et al.*, 2018).

Electrophoresis separates molecules using an electric field (electro) as the driving force for the molecules and a porous support matrix (phoresis). The electrophoresis principle within an electric field, positively charged biological molecules will migrate toward the negative electrode (Fatchiyah *et al.*, 2018). Several factors can influence electrophoresis, including (1) The sample, (2) The concentration of agarose gel, (3) The buffer solution, and (4) The electric voltage used during the electrophoresis process (Harahap, 2018). The migration rate of samples in electrophoresis is influenced by their charge, size, and molecular shape. The magnitude of a DNA molecule's charge is directly proportional to its migration rate, which is also referred to as electrophoretic mobility. The larger the charge of the molecule, the greater its electrophoretic mobility (migration speed) (Artati and Lubis, 2017). The effective pore diameter within the gel is determined by the concentration of agarose used. Higher agarose concentrations form smaller pore sizes (Lee *et al.*, 2012).

The type of buffer solution used also plays a significant role. Buffer solutions are essential when charged molecules undergo electrophoresis through the separation medium. Various types of buffers can be utilized in DNA electrophoresis, including *Tris-Acetate EDTA* (TAE) buffer with a pH of 8.0, *Tris-Borate* (TBE) buffer, and *Tris-Phosphate* (TPE) buffer with a pH around 7.7 - 7.8 (Green and Sambrook, 2020). TBE buffer is recommended when high resolution is needed for DNA fragments smaller than 2 kb, while TAE buffer is suggested for DNA fragments larger than 3 kb or in cloning processes. Voltage also significantly impacts the outcomes of electrophoresis. Running electrophoresis at higher voltages can accelerate the separation of molecules (Harahap, 2018). However, excessively raising the voltage can generate excess heat, causing the gel to melt (Sinaga *et al.*, 2017).

DNA molecules can be effectively visualized, when stained and observed under ultraviolet light (Sismindari *et al.*, 2019). One commonly used dye for this purpose is *Ethidium bromide* (EtBr) (Artati, 2016). The effectiveness of this dye relies on the type and strength of molecular binding sites to DNA and the thermodynamic parameters involved in the binding or intercalation process. Complex dye molecules can non-covalently bind to DNA through intercalation, groove

binding, and electrostatic interactions (Yuwono and Safitri, 2017). EtBr can intercalate between base pairs, allowing DNA molecules to be visible under ultraviolet illumination. It remains the most favored dye among researchers due to its affordability, effectiveness, and sensitivity. However, previous studies have reported that EtBr is considered a serious biohazard due to its high potential for mutagenicity, carcinogenicity, and teratogenicity, requiring careful waste handling (Saeidnia and Abdollahi, 2013). Several alternative dyes have been recommended, including SYBR Gold, SYBR Green, crystal violet, and methylene blue (Green and Sambrook, 2019a). Other options include GelRed at a concentration of 100x (Huang *et al.*, 2010), PicoGreen (Japaridze *et al.*, 2015), methyl green (Prieto *et al.*, 2015), and hematoxylin (Abdullah, 2019). These alternatives provide safer options for DNA staining, reducing the potential risks associated with using EtBr.

Hematoxylin ($C_{16}H_{14}O_6$) is one type of histological stain used for tissues. This dye is extracted from the wood of the *Haematoxylum campechianum* tree. In principle, hematoxylin can serve as an alternative DNA stain because it can bind to the phosphate side of DNA and works through electrostatic attraction. The cationic charge in hematoxylin alum will attach to the negative phosphate groups on the DNA ribose backbone, resulting in the staining of cell nuclei rich in chromatin with colors ranging from blue to purple (Ortiz-Hidalgo and Pina-Oviedo, 2019). Based on the research conducted by Abdullah (2019), it was found that hematoxylin at a concentration of 0.01% and a staining duration of 40 minutes did not yield optimal color results. Therefore, further investigation is required to study the effect of different concentrations and soaking times of hematoxylin on DNA band staining in agarose gel electrophoresis. The research was carried out by staining DNA with hematoxylin to achieve optimal results at concentrations of 0.01%; 0.02%; 0.03%; 0.04%; and 0.05%, with varying soaking times in agarose gel for 10, 15, 20, and 30 minutes.

MATERIAL AND METHOD

This research was conducted in May 2023 at the Molecular Biology Laboratory in the Department of Medical Laboratory Technology, Bandung Ministry of Health Polytechnic. DNA isolation was performed, followed by amplification using a PCR thermal cycler, electrophoresis, and gel staining using harris hematoxylin dye.

DNA isolation

The DNA isolation was carried out by harvesting up to 200 μ L of *Escherichia coli* culture grown in nutrient broth media, utilizing the spin column method with the *Geneaid Kit* (Geneaid, 2020). In each reaction, a total of 50 μ L of DNA template was generated using this kit.

The isolation procedure was repeated three times to guarantee a sufficient quantity of DNA template for subsequent amplification, ultimately resulting in a total yield of 150 μ L of DNA template. The purity of DNA template was not recalculated because a reagent kit from the manufacturer was used. Its purity had been tested and confirmed for routine testing.

DNA amplification

The DNA amplification process was carried out using the *Promega Kit* for 24 reactions. In each reaction, 40 μ L of the master mix reagent and 5 μ L of DNA template were pipetted into a PCR microtube (Promega, 2021). The amplification process was performed using a PCR thermal cycler under the following conditions: initial denaturation at 94°C for 3 minutes (1 cycle), denaturation at 94°C for 20 seconds (35 cycles), annealing at 56°C for 20 seconds (35 cycles), extension at 72°C for 30 seconds (35 cycles), and final extension at 72°C for 10 minutes. Subsequently, the amplification products (amplicons) obtained from the PCR microtubes were combined into a single microtube.

The electrophoresis process

First, a 1.5% agarose gel was prepared. 0.75 grams of agarose gel were weighed and dissolved in 50 mL of 1x TAE buffer in a 250 mL erlenmeyer flask. The mixture of agarose gel was then heated until it completely dissolved (indicated by it becoming clear). An electrophoresis chamber was prepared, and tape was attached to the ends of the chamber. A comb was placed at one end of the chamber, positioned almost touching the base of the chamber.

The temperature of the agarose solution was checked by touching the erlenmeyer flask until it cooled to around 60°C. The agarose solution was then poured into the electrophoresis chamber and solidified for approximately 15 minutes. The comb was carefully removed, and the tape at the end of the chamber was peeled off. Next, the electrophoresis apparatus was prepared, and the gel was placed inside the electrophoresis chamber. The chamber was filled with 1x TAE buffer solution to cover the agarose gel. 6 μ L of each DNA sample and DNA marker were pipetted onto the gel. Cables were connected from the power source to the electrophoresis tank. The power was turned on, and the voltage and running time were set to 150 volts for 30 minutes. The 'run' button was pressed to start the electrophoresis (Ismaun *et al.*, 2021).

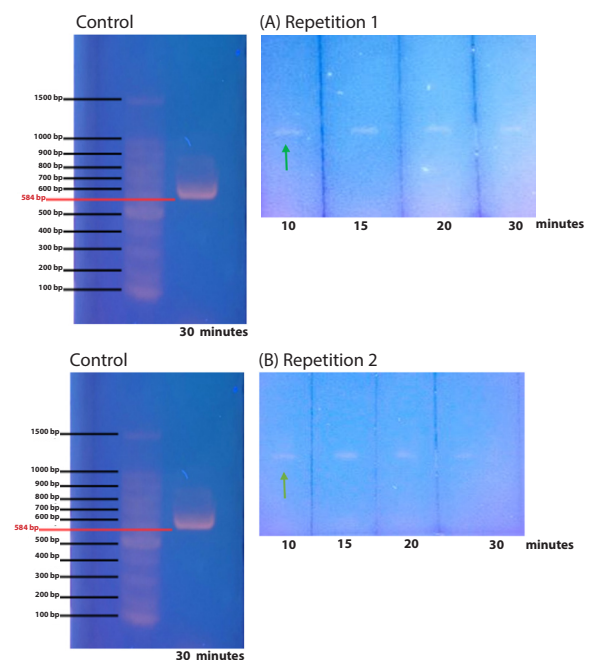
The DNA staining

The amplified DNA of the 16S rRNA gene from *E. coli*, sized 584 bp, underwent electrophoresis, and was subsequently stained with harris hematoxylin dye using various concentrations (0.01%; 0.02%; 0.03%; 0.04%; and 0.05%) and different durations of gel immersion (10, 15, 20, and 30 minutes). The resulting DNA bands were then analyzed for their intensities using *ImageJ*

software and compared to a control. DNA from agarose gel stained with 0.01% EtBr for 30 minutes. The observed intensities were assigned grading values from 1 to 4. The grading was based on the intensity data obtained from the control, repeated 11 times. To determine the grading categories, the data from the control intensities were used to create four categories using the 1.5 *Standard Deviations* (SD) formula (Azwar, 2016). The experiment was repeated twice for each experimental group. The grading results from each repetition were then calculated to obtain the mean value. The highest mean value was used to determine the most optimal concentration and duration of gel immersion. This research received ethical approval with reference number 04/KEPK/EC/VI/2023 from the ethical committee at Bandung Ministry of Health Polytechnic.

RESULT

In Figure 1 shows the DNA staining results using 0.01% EtBr for 30 minutes and 0.01% harris hematoxylin, at various immersion times, namely 10, 15, 20, and 30 minutes. In this study, a 100 bp marker was used, and the DNA band of the 16S rRNA gene from *E. coli*, sized 584 bp, was observed to be stained in both the control EtBr staining and the harris hematoxylin staining. In Figure 1, the size of the DNA marker is indicated by a black arrow, while the red arrow shows the size of the DNA sample. The DNA band stained with EtBr appeared as an orange line, while the band stained with harris hematoxylin appeared as a pale yellow color. Staining with harris hematoxylin was repeated two times.



Concentrations: (a) Samples stained in the first repetition; (b) Samples stained in the second repetition

Figure 1. The comparison of DNA staining results using EtBr and harris hematoxylin at a 0.01%

Table 1. The results of the DNA band intensity assessment

Concentration (%)	Immersion time (minutes)	Repetition				Mean grading
		I		II		
		Intensity	Grading	Intensity	Grading	
0.05	10	2.27	4	1.00	2	3
0.04		0.60	1	0.65	2	1.5
0.03		1.09	3	0.64	1	2
0.02		0.11	1	0.23	1	1
0.01		2.76	4	0.56	1	2.5
0.05	15	3.39	4	1.51	4	4
0.04		1.01	2	0.60	1	1.5
0.03		1.28	3	0.52	1	2
0.02		0.21	1	0.15	1	1
0.01		2.53	4	1.04	3	3.5
0.05	20	0.00	1	0.89	2	1.5
0.04		2.31	4	1.34	3	3.5
0.03		1.58	4	1.11	3	3.5
0.02		0.15	1	0.15	1	1
0.01		3.69	4	0.42	1	2.5
0.05	30	1.32	3	0.82	2	2.5
0.04		1.06	3	1.36	3	3
0.03		1.15	3	2.07	4	3.5
0.02		0.14	1	0.36	1	1
0.01		4.63	4	0.57	1	2.5

Note: The intensity values represent the quantification of DNA band intensities obtained using the *ImageJ* application

Throughout all concentrations, clear differences in staining results between the two repetitions were not observed, thus necessitating the quantification of band intensities using the *ImageJ* application. The quantification results using the *ImageJ* application are presented in Table 1.

Table 1 presents the intensity values and grading scores for each treatment. The obtained grading scores were then calculated to determine the mean values. As shown in Table 1, the highest mean values for each concentration variation are highlighted in green, while the highest values in this experiment are highlighted in red. The highest mean value was obtained for the agarose gel immersion for 15 minute using 0.05% harris hematoxylin concentration.

At 0.01% concentration, the highest mean value was obtained for 15 minute gel immersion time. At 0.02% concentration, the intensity of staining formed tended to be constant at each gel immersion time. Meanwhile, for 0.03% and 0.04% concentrations, the highest mean intensity of staining was found 20 minute gel immersion time. Based on these results, it could be

concluded that 0.05% harris hematoxylin concentration with 15 minute gel immersion time provided the best condition for producing highly distinct DNA band color intensities (resulting in a mean grade of 4).

DISCUSSION

DNA band intensities were quantified using the *ImageJ* application to determine the influence of variations in harris hematoxylin concentration and immersion time during the electrophoresis process. The *ImageJ* application is an image processing and analysis program widely used for various purposes. Its primary advantage lies in transforming qualitative data into quantitative data and this data can be presented in the form of graphs. The *ImageJ* application can also be employed to measure the results of electrophoresis gels quantitatively (Ferreira and Rasband, 2012.). In this study, the intensities used were adjusted relative intensities, adapted to account for various factors affecting the staining (Lukemiller, 2010).

Based on the research findings above, the *ImageJ* values from each experimental group exhibited significant variation. This variation can be attributed to two main factors. First, differences in pipetting conditions or errors during the first and second repetitions may have occurred. Variations in pipetting can result in differences in DNA sample volume, leading to variations in the thickness of the formed bands. Second, there may be no significant differences in the staining reactions with the variations in harris hematoxylin concentrations used. In this study, immersion concentrations ranged from 0.01% to 0.05%. The concentration range is very close, making it difficult to distinguish the formed colors significantly. These factors may contribute to the observed variations in *ImageJ* values among the experimental groups, affecting the interpretation and analysis of the research results. It is crucial to be aware of these potential sources of variation to ensure the reliability and validity of the study's conclusions. Further investigations or adjustments in the experimental design may be necessary to mitigate these issues and achieve more precise and consistent results.

Upon analyzing the research results, certain limitations were identified in this study, specifically the need for more stability of harris hematoxylin staining when used repeatedly in the DNA staining process. Throughout the experimental series, variations in color intensity were observed between the first and second immersions. Even when attempting a third experiment, the color completely disappeared. This condition is related to using the same harris hematoxylin for three immersions with different durations, causing the hematoxylin to be exposed for an extended period to the agarose gel. The mechanism involves the diffusion of hematoxylin molecules with conjugated double bonds into the agarose pores. Additionally, dipole reactions between agarose and hematoxylin contribute to reducing dye concentration with increasing immersion time, especially during the 30 minutes immersion with three repetitions. Therefore, in future research, it is recommended to use fresh hematoxylin staining for each application to minimize changes in dye concentration that could impact consistent and accurate staining results (Green and Sambrook, 2019b).

In principle, hematoxylin has the potential as an alternative stain for DNA due to its ability to bind to the phosphate side of DNA molecules and interact through electrostatic attraction. In this case, the cationic charge present in hematoxylin molecules will interact with the negative charges on the phosphate groups of the DNA chain, enabling hematoxylin to label cell nuclei containing chromatin with colors ranging from blue to purple (Llewellyn, 2009). Hematoxylin was diluted with distilled water in various concentrations to optimize its staining effect in this research. It is essential to note that hemalum can undergo the hydration of its molecules. This phenomenon is related to solid ionic substances that dissociate into individual cations and anions when

dissolved in water. These ions will be hydrated by several water molecules (Mustika *et al.*, 2020).

Similarly, when hemalum binds with water, the molecules of the hemalum solution undergo hydration. The hydration of hemalum solution molecules can affect the solution's stability, the solubility of its components, and the strength of the bond between hematein and aluminum salts. Hydration can also influence the interaction of hemalum with its target staining, such as interactions with the phosphate groups in cell DNA when used as a nuclear stain. After several uses, hematoxylin may lose its affinity for the target components. This loss of dye affinity can reduce hematoxylin's ability to bind with DNA and produce consistent color intensity. Research conducted by Kiernan in 2018 supported this observation, where an increase in pH led to a rise in nuclear staining intensity by hemalum. Still, the negative charge on the dye weakened due to the increased protonation of DNA phosphate groups (Kiernan, 2018). Consequently, during repeated staining, a weakening of staining intensity was observed.

Harris hematoxylin can be an alternative to substitute EtBr for several reasons. Firstly, hematoxylin can specifically bind to DNA, enabling the detection of DNA fragments similar to what EtBr does. Harris hematoxylin is considered safer compared to EtBr. EtBr is known to be carcinogenic and potentially hazardous to both humans and the environment. On the other hand, Harris hematoxylin is relatively safer and is not classified as dangerous to human health. This characteristic makes Harris hematoxylin a viable stain for DNA electrophoresis analysis.

Other researchers can use Harris hematoxylin to ensure safer laboratory practices and minimize potential health and environmental risks associated with EtBr. Furthermore, the specific binding properties of Harris hematoxylin make it a reliable choice for DNA staining, offering comparable results to EtBr while maintaining a safer working environment. Indeed, it is essential to consider that using Harris hematoxylin as a substitute for EtBr also has some limitations. One of the drawbacks is that hematoxylin may be less sensitive in detecting low levels of DNA compared to EtBr, which is known for its high sensitivity. Additionally, Harris hematoxylin tends to produce weaker colors that are not easily visible on agarose gels after electrophoresis. Furthermore, Harris hematoxylin is less stable when used for repeated staining. Therefore, it is necessary to use fresh hematoxylin to achieve satisfactory staining results.

Harris hematoxylin can be a viable alternative to substitute EtBr in DNA electrophoresis staining, primarily due to its safer nature. However, it is crucial to consider the strengths and limitations of each dye, as well as specific experimental conditions and objectives, before choosing to use harris hematoxylin as a substitute for EtBr. Researchers should carefully evaluate the specific requirements of their experiments and choose the most suitable dye accordingly. While

Harris hematoxylin provides safer staining practices, researchers may need to weigh its lower sensitivity and weaker staining against the benefits of its safer properties. Overall, selecting the appropriate dye for DNA electrophoresis is a critical decision that can have an impact on the accuracy and reliability of research findings.

CONCLUSION

Based on the observations and discussions of the experiments, it can be concluded that the optimum condition for various concentrations and immersion times for harris hematoxylin in staining DNA bands after electrophoresis is 0.05% for 15 minutes. This combination yielded the most satisfactory and consistent results, providing a clear and distinct visualization of DNA bands. Other researchers can consider this optimized condition for future DNA electrophoresis experiments using Harris hematoxylin as the staining agent.

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