METHYLENE BLUE AS ALTERNATIVE DNA STAINING IN ELECTROPHORESIS

Fusvita Merdekawati[1], Ira Gustira Rahayu[2] Yogi Khoirul Abror[2]*

[1*)] Departement of Medical Laboratory Technologist Poltekkes Ministry of Health Bandung, Cimahi City, West Java 40514, Indonesia, Email : fusvitamerdekawati@gmail.com;

[2] Departement of Medical Laboratory Technologist Health Poltekkes Ministry of Health Bandung, Cimahi City, West Java 40514, Indonesia

Abstract, Background: Electrophoresis is a technique for separating DNA molecules based on their size by using an electric charge. To make electrophoresis fast, precise, effective. Ethidium bromide (EtBr) is used as an intercalator dye. However, EtBr is mutagenic and carcinogenic therefore has significant disadvantages both from an environmental and human health perspective. To avoid the disadvantages of EtBr, scientists have started looking for alternative nucleic acid dyes. One of them is the use of fluorescent dyes which have high speed and sensitivity for DNA visualization.

Method: The type of research conducted was Experimental research. The design of this study was made with variations in agarose concentration (1%, 1.5% and 2%), methylene blue concentration (0.00625%, 0.0125% and 0.025%), agarose gel contact time from electrophoresis with Methylene Blue and long destaining time (10, 20 and 30 minutes) ectrophoresis conditions in the form of length of time (1 hour, 1.5 hours and 2 hours) and voltage variations (100 volts and 150 volts) of running electrophoresis.

Result: The best appearance of the Sars CoV 2 DNA band at 100 bp and 250 bp using a concentration of 0.025% methylene blue, 1.5 % agarose concentration, 20 minutes of staining and 30 minutes of destaining. The electrophoresis process takes place with a voltage of 100 volts for 60 minutes.

Conclusion: The best appearance of the DNA band using a concentration of 0.025% methylene blue, Therefore, it is necessary to conduct further research regarding the sensitivity of Methylene Blue

Keywords: Methylene Blue, Alternative Staining, Electrophoresis

Background

Clinical Laboratory is a health laboratory that carries out clinical specimen examination services to obtain information about individual health, especially to support efforts to diagnose disease, cure disease, and restore health (Ministry of Health of the Republic of Indonesia, 2013). Various methods have been developed in the field of laboratory examination to produce fast and accurate diagnostic methods with a high level of specificity and sensitivity, one of which is by using a molecular-based approach, namely Polymerase Chain Reaction (PCR) (Rosenblum et al., 2015).

PCR has high sensitivity and specificity, wide detection capability, simplicity, and reproducibility. This method is also very useful for analysis of large numbers of specimens and for measuring viral load. By using PCR can detect even a single copy of the gene (Abbas et al., 2005; Alves et al., 2016). Detection using PCR consists of nucleic acid isolation, DNA amplification and visualization of PCR products using agarose gel (Gerrit J. Viljoen, Louis H. Nel, 2005).

Nucleic acid isolation aims to separate DNA or RNA from other materials such as proteins, fats, and carbohydrates (Nurhayati, Betty., 2017). The isolated DNA was then amplified using PCR to

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produce PCR products. The resulting PCR product was then analyzed using agarose gel electrophoresis (Reaction & Handoyo, 2001).

Electrophoresis is a technique for separating DNA molecules based on their size by using an electric charge (Nurhayati, Betty., 2017). To make electrophoresis fast, precise, effective, ethidium bromide (EtBr) is used as an intercalator dye. As a positively charged compound, EtBr precipitates into DNA base pairs, binds to DNA and facilitatesmonitoring and identification of nucleic acid bands under ultraviolet (UV) light (Phil Turner, Alexander Mclennan, 2005).

However, EtBr is mutagenic and carcinogenic and therefore has significant disadvantages both from an environmental and human health perspective. To see the DNA that has been stained with EtBr also requires UV light, where UV light with short wavelengths is dangerous, in addition to handling waste from the gel that has been stained with EtBr must also be disposed of as a hazardous material and using special equipment, which is an expensive effort (Sigmon & Larcom, 1996)

To avoid the disadvantages of EtBr, scientists have started looking for alternative nucleic acid dyes. One of them is the use of fluorescent dyes which have high speed and sensitivity for DNA visualization. However, these fluorescent dyes are often less stable than EtBr and more expensive than EtB (Hilal & Taylor, 2008). Other alternative DNA dyes include ethyl violet, acridine orange, Crystal violet, SYBR Safe, SYBR Green I, GelRed, BlueView, DAPI and methylene blue. GelRed, DAPI, SYBR Green I have almost the same sensitivity as EtBr, but require high costs because the price is more expensive than EtBr (Bio-Rad, 2018; Cong et al., 2010; Nafisi, 2006; Noor Azuwa Hamdan, Rahizan Issa, 2012; Silvestrini et al., 2015)

An alternative dye for DNA staining that is safe, inexpensive and easy to find is Methylene Blue. Methylene Blue is a heteroaromatic tricyclic compound that can bind strongly to DNA. Methylene Blue is usually used for treatment and dye in laboratory tests (Silvestrini et al., 2015; ThermoFisher, 2018). This compound stains DNA through an intercalation process, similar to the binding of ethidium bromide to DNA. The advantages of using Methylene Blue are its low toxicity, not carcinogenic, cheaper than ethidium bromide, can be reused for 3-6 months, and can reduce the risk of exposure to UV radiation because the staining results can be observed using visible light. (visible light) (Nafisi, 2006).

Research on the use of methylene blue as a DNA dye has been carried out previously with the title of optimizing the use of methylene blue as a substitute for ethidium bromide in DNA from agarose gel electrophoresis. From the results of his research, the optimal concentration of methylene blue was 0.0125% with a contact time of 25 minutes, which was carried out at a concentration of 2% agarose gel and electrophoresis conditions were carried out at 150 volts for 30 minutes (Yayah Winarti, 2017). In this study, Measles cDNA was able to color the size of 650 base pairs. However, if viewed from the results of the research, it has not optimized all the factors that can affect the results of DNA staining using agarose gel electrophoresis such as running electrophoresis conditions, gel concentration, staining contact time, destaining contact time and reading of electrophoresis results (EDVOTEK, 2003).

Electrophoresis running conditions such as time and voltage used during the electrophoresis process affect DNA migration. The longer and larger time and voltage, the DNA migration will be faster than the shorter and smaller time and voltage. The longer electrophoresis time will increase the separation between DNA fragments. It is very important to analyze the position of DNA fragments, especially if the position of the observed DNA bands is close together. However, the electrophoresis process must be stopped when the loading dye migration rate reaches 3.5-4 cm from the agar tip.

The concentration of agarose gel will affect the resistance of the gel produced. Agarose gel in high concentration will produce a gel that has smaller pores and is stronger than the low concentration of agarose gel. A high concentration of agarose gel can also slow the rate of DNA migration, while a low concentration of agarose gel will produce a gel that is easy for DNA migration to pass. The choice of agarose concentration used must also be adjusted to the length of the PCR product or DNA produced from the PCR process.

The staining contact time is the time required for DNA and dye to bind so that the DNA can finally be visualized. The dye will insert between adjacent DNA base pairs without interfering

the arrangement as a whole. These dyes can interact with DNA through several different mechanisms depending on the nucleotide sequence, the strength of the ionic solution, and the MB/DNA concentration ratio (Noor Azuwa Hamdan, Rahizan Issa, 2012; Silvestrini et al., 2015; Synthesis & Blue, n.d.) . On this basis, the researchers wanted to conduct further research on the optimum conditions of methylene blue as an alternative dye for DNA in agarose gels

Methods

Experiment Design

The type of research conducted is experimental research. The design of this study was made with variations in agarose concentration (1%, 1.5% and

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2%), methylene blue concentration (0.00625%, 0.0125% and 0.025%), agarose gel contact time from electrophoresis with Methylene Blue and long destaining time (10, 20 and 30 minutes) and electrophoresis conditions in the form of length of time (1 hour, 1.5 hours and 2 hours) and voltage variations (100 volts and 150 volts) of running electrophoresis.

The time of the research was carried out in June 2020 - December 2020 which was carried out at the Molecular Biology Laboratory, Health Analyst Department, Health Polytechnic Bandung

Tools and Material

A set of agarose gel electrophoresis equipment, micropipette, white tip, uv transluminator, plastic container, stopwatch, beaker, Erlemeyer flask, analytical balance, hotplate, ball pipette, gloves, measuring flask, measuring cup and tissue

Methylene blue, Syber safe, DNA PCR products, agarose gel, TAE, DNA markers, loading dye and aquadest

Data Collections

The data used in this study are primary data obtained from the visualization of DNA from Methylene Blue staining in the form of measurement of the surface area of the band formed

Agarose Gel Making (1, 1.5 and 2 %) Concentrations

1. Make 250 ml of 1x TAE buffer solution by mixing 5 ml of 50x TAE into 245 ml of Aquadest.
2. Agarose gel 1, 1.5 and 2% agarose gel were made then dissolved in 1x TAE buffer and boiled until completely dissolved.
3. Prepare an electrophoresis tray, attach tape on each end of the electrophoresis tray. (Make sure that the tape is firmly attached and that there are no holes at either end of the tray).
4. Install the electrophoresis comb at one end of the tray with the position almost touching the bottom of the tray.
5. Check the temperature of the agarose solution by sticking an erlenmeyer to your hand, if the temperature has dropped to about 60°C, pour the agarose solution into the electrophoresis tray, allow the solution to turn into a solid gel.
6. Taking the comb carefully, remove the tape from the ends of the tray.

Electrophoresis Running

1. Insert the tray containing the agarose gel into the electrophoresis tank which has been filled with the remaining 1x TAE buffer solution. (Make sure that the agarose gel is completely submerged in TAE).
2. Prepare about 5 cm of parafilm paper near the electrophoresis tank.
3. Pipette 6 l of DNA sample, 6 l of DNA marker, and 6 l of negative control. The DNA used in this study was a PCR product from SARCOV2
4. It is homogenized on parafilm paper, then inserted into the agarose gel well.
5. Connect the wire from the current source to the electrophoresis tank (make sure that the wire connected to the negative pole is near the well, while the wire connected to the positive pole is far from the well; if not, change the position of the tray/gel in the opposite direction).
6. Turn on the current source, set the voltage (100 volts and 150 volts) and running time (1 hour, 1.5 hours and 2 hours)
7. Run electrophoresis (run) by pressing the run button
8. Electrophoresis will stop when the set time is up, which is indicated by an alarm sound. The current source is turned off and the tub is removed from the electrophoresis tank.

Staining Solution Preparation

1. Made as much as 100 ml of Methylene Blue stock solution with a concentration of 1%, by weighing 1 g of ethylene Blue powder then dissolved in 100 ml of distilled water, then filtered. After that, 100 ml of Methylene Blue solution was made with various concentrations of 0.00625%, 0.0125%, and 0.025%.
2. Preparation of 100 ml of 0.00625% Methylene Blue solution was carried out by pipetting 625 l of 1% Methylene Blue stock solution into the flask Measure 100 ml, then add with aquadest to the mark, then homogenize.
3. Making 100 ml of 0.0125% Methylene Blue solution is done by pipetting 1.25 ml of 1% Methylene Blue stock solution into a 100 ml volumetric flask, then adding aquadest to the mark, then homogenized.
4. Preparation of 100 ml of 0.025% Methylene Blue solution was carried out by pipetting 2.5 ml of 1% Methylene Blue stock solution into a 100 ml volumetric flask, then adding aquadest to the mark, then homogenized.

DNA Staining

1. Agarose gel which has been added with 5 L of Syber safe, after the electrophoresis process is complete, it is visualized under UV light. This gel was used as a comparison for agarose gel which was stained with methylene blue
2. Soaking agarose gel in each solution of Methylene Blue with concentrations of 0.00625%, 0.0125%, and 0.025%. The immersion time at each concentration was varied (10, 20 and 30 minutes). Then carried out deep destaining with time variations using aquadest (10, 20 and 30 minutes)

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DNA Band Visualization

1. Place the agarose gel that has been colored by cyber safe on top of the UV transilluminator.
2. Turn on the UV transilluminator, observe the visualized DNA bands.
3. The results of staining using Methylene Blue were observed using visible light

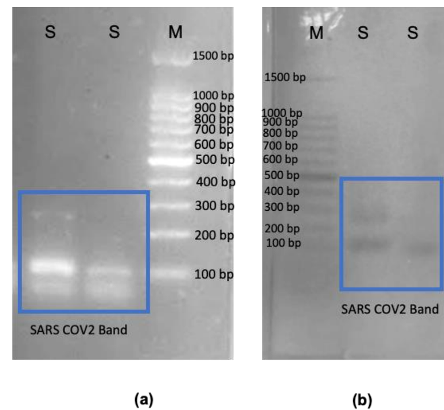
Result and Discussion

This research, with the title Methylene Blue as an alternative DNA staining in gel electrophoresis, has received a Certificate of Eligibility for Research Ethics from the Research Ethics Commission of the Health Polytechnic of the Ministry of Health Bandung with No. 16/KEPK/EC/X/2020 dated 15 October 2020.

This research was conducted to determine the optimum conditions for Methylene Blue in staining DNA from electrophoresis. In this study the DNA used was PCR products from SARCOV2 compared with cyber safe used.

This PCR product from SARCOV2 will produce four DNA bands, each of which is less than 100 bp, 100 bp, 250 bp and 350 bp in size. The DNA marker used in this study is 100 base pairs, which will produce 11 DNA bands with their respective sizes: 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 and 1500 base pairs. Based on the insert kit, it was stated that the optimal agarose gel for 100 bp DNA marker was 2% under the condition of running electrophoresis for 3 hours with 80 voltage.

The first stage of the research is to carry out an electrophoresis process where the agarose gel is colored with cyber safe as a comparison against Methylene Blue. At this stage the agarose gel used is the same, namely 2% while the running time of the electrophoresis is varied but with the same voltage, which is 100 volts.



Picture 1. (a) Visualization of Sars CoV2 DNA bands at 100 and 250 bp stained with cyber safe, agarose concentration of 2%, running electrophoresis for 1.5 hours and 100 vott. (b) Visualization of Sars CoV 2 DNA bands at 100 and 250 bp stained with 0.025% Methylene Blue, agarose concentration of 1,5 %, running electrophoresis for 1 hours and 100 volt. M for marker and S For Sample

Based on the results in Figure 1a, it can be seen that the DNA markers are clearly separated, producing 11 DNA bands, the smallest PCR product can also be visualized and the DNA band of Sars CoV2 detrmined in 100 and 250 bp.

Compared with Figure 1b is the best appearance of the Sars CoV 2 DNA band at 100 bp and 250 bp using a concentration of 0.025% methylene blue, 1.5 % agarose concentration, 20 minutes of staining and 30 minutes of destaining. The electrophoresis process takes place with a voltage of 100 volts for 60 minutes.

The volume of the sample added is 10 microliters. Because at a volume of 5 microliters there is still no visible DNA band. Therefore, it is necessary to conduct further research regarding the sensitivity of Methylene Blue dye

Based on literature studies and previous research, it is stated that Methylene Blue can stain DNA although its sensitivity is smaller than the comparison dye. Methylene Blue binding mechanism to DNA occurs through semi-intercalation and electrostatic bonds. Intercalation bonds occur when a planar or heteroaromatic Methylene Blue aromatic ring structure is inserted between adjacent DNA base pairs without disturbing their overall arrangement. When intercalation occurs, the vertical distance between DNA base pairs is further apart, resulting in a change in the degree of rotation (twist angle) of the DNA structure. The electrostatic bond occurs between the cationic ligand in Methylene Blue with a negatively charged phosphate group in DNA. This interaction is non-specific and results in external binding along the

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DNA strand

Conclusion

The best appearance of the DNA band at 100 bp and 250 bp using a concentration of 0.025% methylene blue, 1.5 % agarose concentration, 20 minutes of staining and 30 minutes of destaining. The electrophoresis process takes place with a voltage of 100 volts for 60 minutes. The volume of the sample added is 10 microliters. Therefore, it is necessary to conduct further research regarding the sensitivity of Methylene Blue dye

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