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DESIGN OF ELECTROPHORESIS DEVICE FOR OPTIMATION OF DNA VISUALIZATION AND DNA CONCENTRATION USING SOFTWARE

DESAIN ALAT ELEKTROFORESIS UNTUK OPTIMASI VISUALISASI DAN KONSENTRASI DNA MENGGUNAKAN SOFTWARE

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ABSTRAK

Molekul DNA menunjukkan polarisasi yang kuat sehingga memungkinkan baik gerak elektroforesis berdasarkan muatan negatifnya maupun gerak dielektroforesis berdasarkan induksi polarisasi. Perancangan alat menggunakan kombinasi prinsip elektroforesis dan dielektroforesis dilengkapi perangkat lunak untuk mengukur konsentrasinya sangat diperlukan. Utamanya mengingat uji kualitatif DNA berbasis visualisasi pada gel elektroforesis bersifat sangat subyektif dan kurang terukur. Pengukuran konsentrasi DNA menggunakan spektrofotometer UV/VIS sangat tergantung oleh ketersediaannya di laboratorium. Penelitian bertujuan untuk mendesain piranti untuk mengukur konsentrasi DNA berdasarkan visualisasinya pada gel elektroforesis menggunakan perangkat lunak berbasis MatLab. Pengukuran konsentrasi DNA didasarkan visualisasinya pada gel elektroforesis lalu dibandingkan dengan hasil penghitungan spektrofotometer UV/VIS. Hasil penelitian menggunakan piranti tersebut memperlihatkan visualisasi DNA yang lebih optimal. Hasil pengukuran jumlah DNA menggunakan spektrofotometer memiliki kecenderungan yang sama dengan hasil pengukuran menggunakan perangkat lunak berbasis MatLab meskipun terdapat perbedaan nilai kuantitatif.

ABSTRACT

Molecules of deoxyribo nucleic acid (DNA) show a strong polarization allowing for both motions of the dielectrophoresis induced by polarization and electrophoresis based on its negative charge. Considering high subjective and less quantifiable result of the visualization based qualitative test of DNA on gel electrophoresis, designing the tool using a combination of the principles of electrophoresis and dielectrophoresis completed with a software for optimization of DNA visualization and to measure the concentration of small and large–sized DNA fragment is very needed. Accuracy of measurement of DNA concentration using a spectrophotometer UV /VIS is depend on its availability in the laboratory. The aim of this study was to design device for optimization of DNA visualization and measuring the concentration in the gel electrophoresis using MatLab- based software. Experiment using this software measured the concentration of DNA based on its visualization and compared it with calculation obtained from spectrophotometer UV/VIS. The research results showed that the amount of DNA analysed using a spectrophotometer tend to similar with the measurement results using the MatLab-based software although there was differences in quantitative values.

Keywords: DNA concentration; visualization; electrophoresis

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INTRODUCTION

Currently, the study of micrometer-scale biological particles from 10 nm to 100 m m as cells, proteins, viruses and DNA intensified considering its application in various fields. For the detailed understanding of the physical properties of DNA and for the realization of a variety of novel devices

such as an integrated lab-on-a-chip, the ability to stretch, orient or sort DNA molecules is a key prerequisite (Hoeb *et al*., 2007). The researchers do that by developing the potential electrostatic power using electrophoresis, dielectrophoresis, electroosmosis and electrofusion (Chou *et al*., 2002). Dielectrophoresis is a technique that utilizes non-uniform electric field to induce a dipole in the polarisation particle (Lapizco-Encinas *et al*., 2004; Castellarnau *et al*., 2006). Dielectrophoresis is usually used for large cells whereas DNA fragments of smaller size can be more easily concentrated using electrophoresis. This technique works in a pushing motion of neutral particles, but can be used on charged particles such as DNA that normally uses electrophoresis. Several studies have shown that DNA can be collected on the metal electrode on dielectrophoresis (Hoeb *et al*., 2007). Electrophoresis and dielectrophoresis potential growing demand for the use of DNA, proteins and viruses along with the discovery microelectroda (Kua *et al*., 2004; Bakewell and Morgan, 2006). Although this technique encourages neutral particle motion, but can be used on the charged particles. DNA molecules can accumulate on the electrode metal on dielectrophoresis. Hoeb *et al*.(2007) study the frequency dependence of the dielectrophoretic movement of DNA and compare it to the predictions of theory and the movement of polystyrene beads under identical conditions. Since dielectrophoresis works equally in field Alternating Current (AC), thereby reduce small ions trapping and electrochemical effects on the electrode, it tends to be used only for large DNA fragments in deionized water and Tris Ethylene diamine tetra acetic acid (EDTA) (Miles *et al*., 1989). The tool design uses a combination of dielectrophoresis phenomenas and electrophoresis to gaining better visualization of large and small DNA bands.

During this time, qualitative tests for DNA visualization commonly used agarosa and polyacrylamide gel. These assays determine the size of DNA band based on comparisons with a known concentration markers. Sometimes it is also used for measuring DNA concentration which is highly subjective and less scalable. Measurements of an accurate concentration of DNA is usually done using a spectrophotometer UV / VIS. A spectrophotometer is an instrument for measuring the transmittance (light that is passed) or absorbance (absorption to light) of an object as a function of wavelength. Measurement of a single wavelength on a series of samples can be based on a spectral region that is called infrared spectrophotometer, ultra violet, and so on. The UV spectrophotometer has a wavelength of 190-380 nm (Miles *et al.*, 1989; Day and Underwood, 2002). The absence of a laboratory spectrophotometer often causes measurements of DNA concentration not easy to do. The research we've done, provide an alternative way of measuring DNA-based visualization on agarose gel after the run on devices combined electrophoresis and dielectrophoresis applications without using a spectrophotometer. The measurement results will compared further with measurements using a spectrophotometer UV / VIS.

METHODS

This research will be conducted at the Genetics Laboratory Biology Department, Laboratory of Instrumentation and Electronics Department of Physics, State University of Diponegoro.

The materials used in this study were Janjan fish (*Oxyurichthys microlepis*), Bloso fish (*Glossogobius circumpectus*) and green crab (*Scylla serrata*). Buffers used in this research were digestion buffer (100 mM Sodium Chloride, 10 mM Tris Cl pH 8, 25 mM Ethylene Diamine Tetraacetic Acid (EDTA) pH 8), 10% Sodium Dodesyl Sulphate (SDS), phenol, chloroform, sodium acetate 3M pH 5.2, Tris EDTA buffer (TE) pH 8, absolute ethanol, ethanol 70%, gel electrophoresis, Tris acetate EDTA buffer (TAE) 0.5 x , the lysozyme enzyme, agarose gel, loading buffer, ethidium bromide, ice cubes, toilet paper, and distilled water.

Steps of DNA isolation and electrophoresed was modificated from (Sambrook *et al*., 1898; Ausubel *et al*., 1995). The DNA material taken from the fresh or frozen fish meat. A sample of 1 g of cell walls are digested using 6 ml of digestion buffer, 1 mL lysozyme enzyme 10 mg / ml and 1 ml 10% SDS. The suspension was incubated at 50 ° C for 12 hours and homogenized every 30 minutes. The supernatant as a results of incubation in the lysis stage then

added with phenol: chloroform with a ratio of 1:1. Suspension was homogenized for 10 seconds and centrifuged at 6,000 rpm for 5 minutes. Supernatant was added with 3M sodium acetate pH 5.2 as much as 1 / 10 volume of the supernatant obtained then homogenized. Supernatant was added with absolute ethanol as much as 2 times the volume of the supernatant and sodium acetate and homogenized thoroughly and incubated at - 20 ° C for two days. All the suspension was centrifuged again at 10,000 rpm for 5 minutes. Supernatant was added with 70% ethanol at room temperature (30^{o}C) and centrifuged again. DNA pellets were air dried and added with TE buffer pH 8. DNA solution was stored at a temperature of -20^{o}C.

Visualization of DNA performed using electrophoresis in a current of 110 V for 10-15 minutes based on Sambrook *et al*., 1989 and Ausubel *et al*., 1995. The DNA samples viewed using agarose gel with a concentration of 1%. Agarose dissolved in 0.5 X TAE buffer. The results of gel electrophoresis observed by UV transilluminator. Visualization of DNA bands is documented with a digital camera.

Measurement of DNA concentration using Spectrophotometer

Spectrophotometric done by diluting the DNA pellet with 1:1000 dilution to volume of 3 ml and put into cuvet. Shells are made using the same diluent. Cuvet placed into the spectrophotometer and tested at a wavelength of 260 nm to measure the concentration of DNA (Sambrook *et al*., 1989; Ausubel *et al*., 1995).

RESULTS AND DISCUSSION

The idea to create our own instrument for molecular electrophoresis separation of DNA fragments of cells that had been imported, have inspired the design and construction of electrophoresis device. Employment potential of these instruments need to be tested and optimized through the isolation and visualization applications on the bacterial DNA in order to obtain the best results as expected and refine existing ones. Optimization of DNA visualization conducted with dielectrophoresis phenomenon was in accordance with the initial study in 1950 by Pohl. Dielectrophoresis (DEP) was used to move neutral polarizable matter in a nonuniform electric field. Starting with Washizu *et al.* in 1990 in Hoeb *et al*., (2007) numerous groups have demonstrated that DNA can aggregate on planar metallic electrodes or insulating constrictions via dielectrophoresis

Software Design

Development of new methods can be done based on literature studies include a theoretical and mathematical study accomplished with adaptation, innovation and synthesis appropriate to the problems emerge. In this research, the development of image restoration methods using statistical mechanism approach could be applied for complex images to improve the performance of image restoration. The main reason of using statistical methods due to difficulties of restored the random noise by ordinary filters. It is necessary to develop stochastic and statistical methods to restore complex image, which has a magnitude and phase components (Goldfarb and Yin, 2005). Statistical methods models according to model the energy function in a complex pixel image will be updated with the energy value of its neighbors. While in this study, statistical methods model is assumed as Markov random field (MRF), because the MRF can be used as an estimate of the probability distribution of the object by comparing the pixels that will be updated with 8 pixel neighbors. Selection of Metropolis Hastings algorithm Markov Chain Monte Carlo (MH-MCMC) is done because these algorithms can be implemented on a probabilistic sample and to estimate parameters (Gemen and Gemen, 1984; Tanaka and Yoshiike, 2003). In addition to the Metropolis Hastings will serve as a decision-making process (accept and decline) of pixels that will be updated by performing calculations on the energy difference between neighboring pixels are pixels that will be updated. As an optimization of this algorithm by using simulated annealling able to estimate the free energy and optimization of Markov Chain Monte Carlo (MCMC) to achieve convergence (Haario *et al*., 2004; Murthy, 2005). Image restoration in this study were done separately between magnitude and phase components.

The testing method made by the implementation of Metropolis-Hastings algorithm, Markov Chain Monte Carlo (MH-MCMC) into the model of statistical methods and then analyzed the results (Haario *et al*., 2004; Murthy *et al*., 2005). After the implementation of the algorithm was examined by using a binary image, gray scale images, and complex imagery (InSAR or MRI). The test here is done subjectively and objectively, subjectively testing done by asking the opinions of the user (expert) by showing the image before restoration and after

restoration. While objectively by looking at parameters that have been generated at the image restoration process.

Design of Electrophoresis Instrument

Electrophoresis is a separation technique based on the size of DNA molecules, using an electric field. The electric field will cause the negatively charged DNA moves toward the positive pole through a medium such as agarose gel. Electrophoresis instrument as illustrated in Fig 1. designed to be easily used in laboratories in accordance with student needs. Dielectrophoresis device is constructed using local components which are available in Indonesia market.

The main part of this instrument as illustrated on Fig. 2 consists of system power supply 5v, 12v and 100V dc; system programmable counter timer. The 2-digit system of the viewer as well as systems with adjustable pulse amplitude of 100V.

The electrophoresis bath as shown in Fig. 3 was made of transparent acrilic. This material will facilitating visualization of DNA fragments.

Design of electrophoresis instrument was viewed on Fig. 4. The instrument scheme consists of the black and white color scheme. Both schemes use the existing components in the diagram block of electrophoresis. In the cholor scheme, design of 4 x 16 LCD module is done as a timer or time display, menu instrument and other information. Modules that have been created are then connected with the AVR 8535 minimum system module kit. Further tests carried out with AVR 8535 minimum system and 4 x 16 LCD display by making the program listing. This is done to display text and characters on the 4 x 16 LCD using a AVR 8535 microcontroller. Tests have been able to display text on the LCD screen and the word sharpness can be adjusted by turning its VR 10 Kπ.

Design and simulation of the oscillator

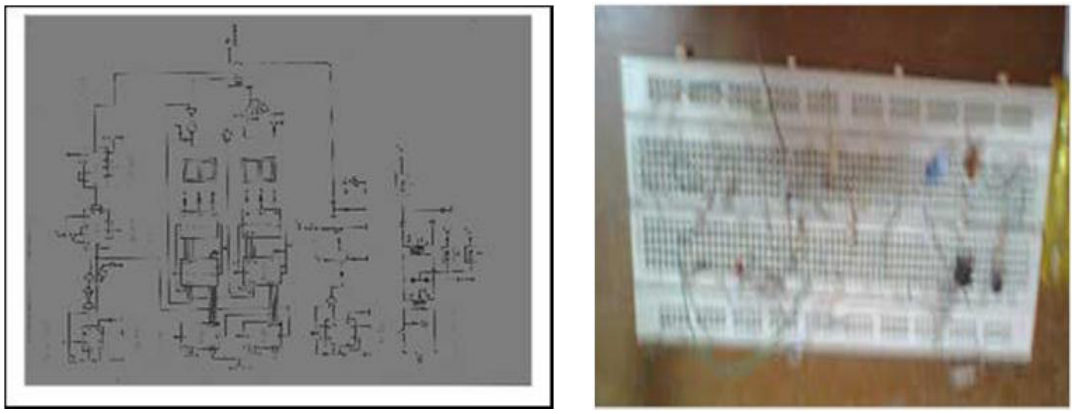

Figure 1. The design of the dielectrophoresis instrument

Figure 2. The main part of the instrument

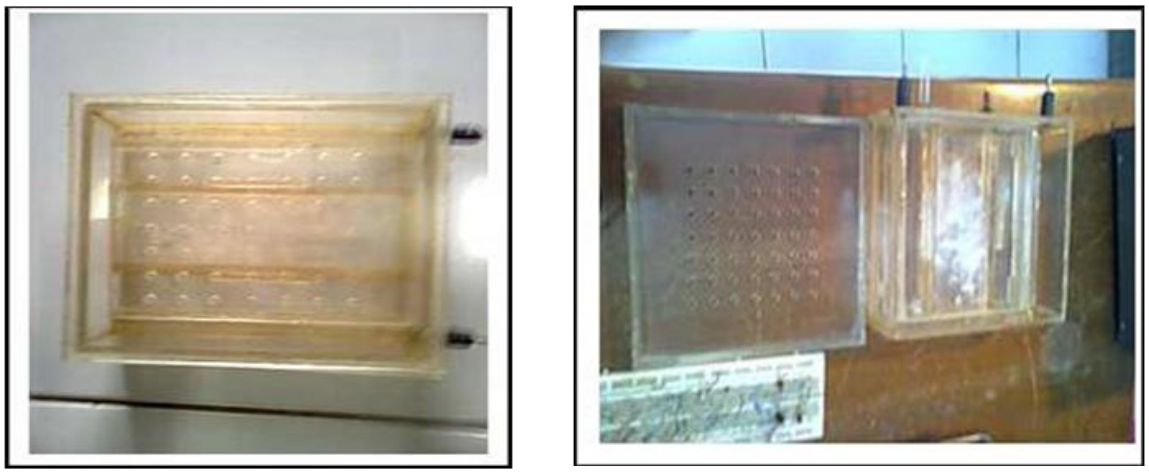

Figure 3. The electrophoresis bath

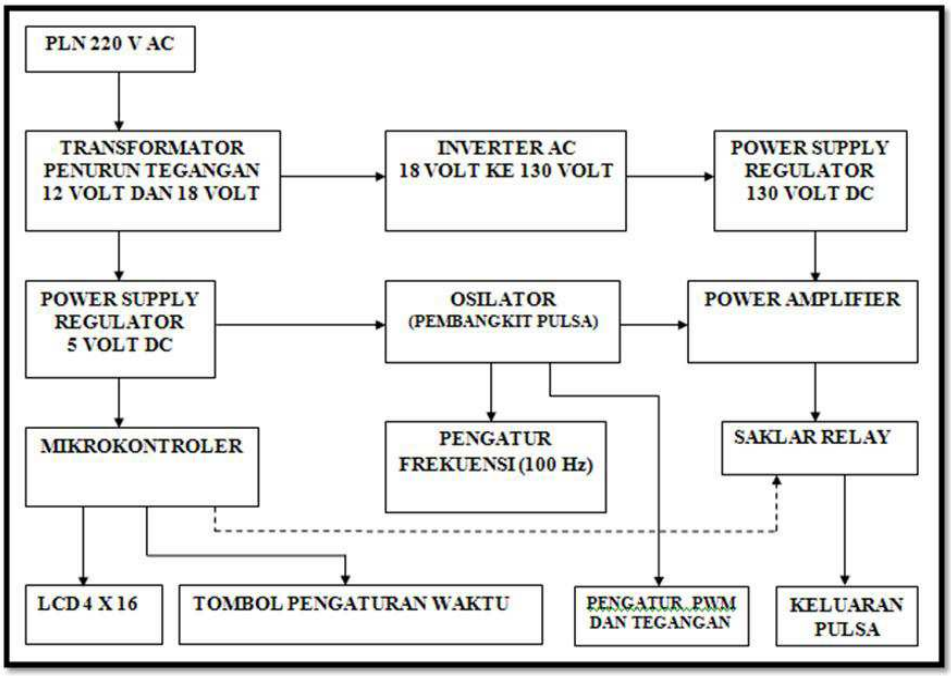

Figure 4. Design of electrophoresis instrument

pulse basic circuit was the next stage performed on instrument design. This is done by adjusting the frequency and PWM pulse width using a 1 C 555. The following step is to manufacture a box pulse oscillator module for electrophoresis with the 1C circuit 555 in accordance with the specified circuit. The research results obtained the box of oscillator module that can be set their width pulse. The next steps is manufacturing the inverter circuit or voltage towing and 130 volt voltage regulator circuit equipped with the lowering voltage. The result is the completion of the power supply regulator and voltage towing of 130 volts DC.

The next stage of instrument design is the creation of a power amplifier circuit to strengthen the pulse of the oscillator pulse 1 C L555. Design of a series of relay switches and button settings do afterwards followed by creating a pointer scale voltmeter circuit voltage.

Stage of instrument completion and optimization is done by making electrophoresis program listings comprehensively that include the timer, discs and minutes of the instrument. Once this phase is completed and then will followed by the performance test for programs and instruments, primarily using multitester and oscilloscope.The characterization of istrument design was illustrated on Table 1.

Instrument characterization results have

Table 1. Characterization of instrument design

Input Block 1 (Step down)		
Input (220 volt)	Output AC (12 volt)	Output AC (18 volt)
228	12,68	18,46
Block 2 (Regulator)		
Input AC (12 volt)	Output penyearah	Output (regulator 5 volt)
12,68	14,88	4,98
Block 3 (Inverter)		
Input AC (18 volt)	Output AC (130 volt)	
18,46	136,7	
Block 4 (Regulator 130 volt DC)		
Input AC (130 volt)	Output DC (volt)	Output Regulator DC (volt)
136,7	177,8	147,5
Block 5 (Ocillator)		
Input regulator dc (5 volt)	Output silator (Hz)	
4,98	100,97	
Block 6 (Microcontroller)		
Timer stopwatch (minutes)	Timer microcontroller (minutes)	
1	1,0056	
Block 7 (Pulse output)		
Output frequency (Hz)	Output tegangan AC square wave (volt)	
101,62	0-140	

Figure 5. The performance of electrophoresis instrument design (The air bubbles was marked by white arrow).

produced the expected electrophoretic devices. Test electrophoresis using timing devices for 10 minutes and a current of one hundred volts on electrophoresis tank filled with buffer Tris acetic acid EDTA (TAE) has shown that the electrophoresis instrument capable of running properly marked by air bubbles on the edge of the tank that the instrument performance can be seen on Fig. 5. The TAE buffer is a solution that is able to mediate the migration of DNA so that the negatively charged DNA will move toward the positive pole.

Optimation of DNA Visualization

Electrophoresis process will separate the DNA from other components based on differences in size. The DNA molecules colored transparent and makes it difficult to be seen visually. Optimation of DNA visualizayion was done using electrophoresis device. Ethidium bromide (EtBr) was a commonly chemical substrances coloring the DNA. Ethidium bromide will intercalated (insert into the DNA) so that the DNA will be casting a transparent colored fluorescent orange (fluorescent) in the ultraviolet rays that can be visualized using a UV

transilluminator. The addition of loading buffer with dye ethidium bromide in electrophoresis process serves to increase the density, so that the DNA will be at the bottom of agarose gel wells. Bands of the DNA molecules will move along the gel after electrophoresis. The DNA measurements performed qualitatively by visualization on electrophoresis. This device will running DNA as negatively charged molecules migrate toward the positive pole in an electric field through a gel medium. Smaller size of the DNA molecule move faster. The DNA conformation not only affect the speed of electrophoretic migration of DNA but also affected by dielectrophoresis dipole (Albert *et al*., 1991, Regtmeier *et al*., 2007; Regtmeier *et al*., 2010). An orange color that looks at the visualization of DNA on gel electrophoresis as shown in the visualization of DNA bands in the UV transilluminator electrophoresis (Fig. 6) is the result of the fluorescence mechanism for DNA irradiated with ultraviolet light. The brighter of the color indicates the concentration of DNA (mg / ul) were higher. The brightness of the color indicates the concentration of DNA in a qualitative way.

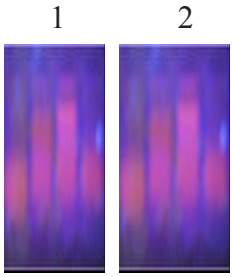

Figure 6. The DNA visualization using ethidium bromide dye (1 : DNA Janjan fish (*Oxyurichthys microlepis*), 2 : DNA green crabs (*Scylla serrata*))

The DNA concentration based on spectrophotometer UV/VIS

The concentration of deoxyribonucleic acid (DNA) were measured using a spectrophotometer UV / VIS at a wavelength of 260 nm would indicate the maximum absorbance of UV light by the DNA molecule. Table 2. and Table 3. shows the DNA measurement results obtained using MatLab software compared with the measurements using a spectrophotometer UV / VIS.

Table 2. The quantity of DNA using MatLab-software application

Color intensity		MatLab Program	
		a value	b value
		band-1	band-2
Color	R	188,039	175,453
	G	96,514	76,275
	B	152,337	166,646

Table 3. The quantity of DNA using Spectrophotometer

DNA Band	Spectrophotometer measurement (μg/ml)
Band-1(a)	2550
Band-2 (b)	2250

Quantitative application using a MatLab program was following qualitative methods of measuring DNA-based visualization on agarose gel (Tables 1). This is specifically to overcome the limited availability of spectrophotometers in the laboratory. MatLab is a language for technical computing with high ability. They combines computing, visualization, and programming in a single unit that is easy to use where the problem and the solution is expressed in mathematical notation known. The research result shows that the interface concentration with R, G, B turns red indicates an increase and a decrease in the value of the corresponding measurement results due to DNA concentration using a spectrophotometer light beam results in the area of the fluorescence was red (Tables 2). Calculation of the DNA concentration is visually based on the intensity of the red color. The greater DNA concentrations marked by the red orange glow that indicates the number of ethidium bromide intercalated into the DNA chain. Compare to the results of measurements using MatLab-based software program, it is consistent with the fact that ultraviolet radiation at a wavelength of 254 nm is absorbed by the DNA will be transmitted to the orange-red dye ethidium bromide. Thus, energy is re-emitted by the radiation spectrum in the orange-red region at a wavelength of 590 nm. All molecules can absorb radiation in the UV-visible because they contain electrons, either single or in groups, which can excitated a higher energy level. Wavelength at which absorption took place, depending on how strong the electrons bound in a molecule (Sambrook *et al*., 1989; Riyadi, 2009). The concentration of deoxyribonucleic acid (DNA) measured by spectrophotometer UV / VIS at a wavelength of 260 nm indicated the maximum absorbance

of UV light by the DNA molecule. The DNA molecules can only absorbs wavelengths of 260-280 as a wave UV excitation.

Appearance concentration of the R, G, B turned out to be complex though is quantitative. Rated R will show an increase when the concentration of DNA are also getting bigger, while the value of B on the downside when the DNA concentration increases. This further reinforces the above results that the greater concentration of ethidium bromide in the DNA will cause the blue and ultraviolet increasingly absorbed thereby increasing the intensity of the fluorescent emission in the red-orange region. The results obtained show that the measurements of DNA amount using a spectrophotometer have the same trend with the DNA concentration determine by the program based on MatLab.

CONCLUSION

The results of the DNA concentration measurements based on visualization was in accordance with the calculation of the spectrophotometer. The concentration of the DNA indicated by the red orange luminescence is absorp more UV. The measurement of DNA concentration based on their visualization using the software showing coherent result with spectrophotometer calculations.

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REFFERENCES

Alberts B, D.Bray, J Lewis , M. Ralf, K. Roberts, and JD Watson. (1991). *Molecular Biology of The Cell.* Garland Publ. London.

Ausubel, F., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl. (1995). Short Protocols in Molecular Biology. A Compedium of Methods from Current Protocols in Molecular Biology. 3nd Ed . Wiley & Sons. Inc. USA. 2-10.

Bakewell D.J. and H. Morgan. (2006). Dielectrophoresis of DNA: time- and frequency-dependent collections on microelectrodes. IEEE *Trans Nanobioscience* 5(2) : 139 – 146

Castellarnau M., Errachid A., Madrid C., Juárez A.and J. Samitier. (2006). Dielectrophoresis as a Tool to Characterize and Differentiate Isogenic Mutants of *Escherichia coli*. *Biophysical Jour.,* 91(10) : 3937-3945

Chou, C, Tegenfeldt J.O., Bakajin O., Chan S.S.,. Cox E.C, Darnton N., Duke T. and R.H. Austin. (2002). Electrodeless Dielectrophoresis of Single- and Double-Stranded DNA. Abstract. *Biophysical Jour.* 83 (4) : 2170-2179

Day, R. A. dan A. L. Underwood. (2002). Analisa Kimia Kuantitatif. Erlangga, Jakarta.

Gemen S. and D. Gemen. (1984). Stochastic Relaxation, Gibbs distributions and Bayesian Restoration. *IEEE Transactions of Pattern Analysis and Machine Intelligence, 6 721*

Goldfarb D and Yin W (2005). Second-Order Cone Programing Methods For Total Variation Based Image Restoration. *AMS*.

Haario, H., Laine, M., Lehtinen, M., Saksman, E. and Tamminen, J. (2004). Markov chain Monte Carlo methods for high dimensional inversion in remote sensing. *J. R. Statist. Soc. B 66(3), 591-608*

Hoeb M., Rädler J.O., Klein S., Stutzmann M. and M.S. Brandt. (2007). Light-Induced Dielectrophoretic Manipulation of DNA. *Biophysical Jour.,* 93(3): 1032-1038

Kua, C.H, Lam Y.C., Yang , C. and K. Youcef-Toumi. (2004). Review of bio-particle manipulation using dielectrophoresis. Nanyang Tech Univ, Singapore.; Massachusetts Inst. of Tech., Cambridge, Massachusetts.p. 1-7

Lapizco-Encinas, B.H., Simmons, B.A, Cummings, E.B. and Y. Fintschenko. (2004). Insulator-based dielectrophoresis for the selective concentration and separation of live bacteria in water. *Electrophoresis* 25 : 1695–1704

Miles R., Wang A., Bettencourt K., Nasarabadi S., Belgrader P., Davidson J.C. and R.P. Mariella Jr. (1989). Manipulation of DNA for Use in Microfluidic Devices. UCRL-JC-132459 Preprint. Lawrence Livermore. Nat. Lab.

Murthy K. P. N. (2005). Bayesian Restoration of Digital Images Employing Markov Chain Monte Carlo", *eprint arXiv:cs/0504037*

Regtmeier J., Duong T.T., Eichhorn R., Anselmetti R, and A. Ros. (2007). Dielectrophoretic Manipulation of DNA: Separation and Polarizability. *Anal. Chem*., 79(10) : 3925 -3932

Regtmeier J., Eichhorn R., Bogunovic L., A. Ros and Anselmetti D. (2010). Dielectrophoretic Trapping and Polarizability of DNA : The Roll of Spatial Conformation. *Anal. Chem*., 82:7141-7149

Sambrook, J., Fritsch, E. R. & T. Maniatis.(1989). Molecular Cloning A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory Press, USA.

Tanaka K and N Yoshiike. (2003). Statistical-Me-

chanical Approach to Probabilistic Image Processing. *Workshop on Statistical Mechanical Approach to Probabilistic Information Processing*.