Automatic Gram Staining for Sputum Slide

K.P. Ping[1], Rosyati Hamid[1], Faradila Naim[1], Nurul Wahidah Arshad[1], Zeehaida Mohamed[2]
[1]*Faculty of Electrical & Electronics Engineering, University Malaysia Pahang, Pekan, Pahang*
[2]*HUSM Kubang Kerian, Kota Bharu, Kelantan*
rosyati@ump.edu.my

***Abstract*—The Gram stain is the most important and universally used staining technique in the bacteriology laboratory. Gram staining method is used to do staining of the clinical material or the bacteria from colonies on laboratory media and provide a direct visualization of the morphology of the organisms based on their reactions to the chemical present in stains. A sputum sample slide need to be stained before the quality of the sputum sample is determined. However, due to human inconsistency, some of the slides are heavily stained with dark color whereas some of it is lightly stained. This inconsistency would create a difficulty for automated sputum quality system using image processing. Therefore, an automated gram staining for sputum slide is needed in order to standardize the slide staining. The automated Gram-staining will undergo staining, washing, and drying process. Each process periods are controlled by a timer built in the microcontroller. The analysis is done on the accuracy of the position of the slide stain, the consistency of the amounts of staining solution drop on the slide and the time efficiency of this automated system is compared to manual operation.**

***Index Terms*—Gram Staining; Sputum; Automated System.**

I. INTRODUCTION

Sputum is a material coughed up from the lungs and expectorated through the mouth. Sputum contains pus cells, squamous epithelial cells, gram-positive and gram-negative organisms. The sputum slide needs to undergo Gram staining before it is processed for quality testing. The Gram staining method is one of the most important staining techniques in microbiology. It was named after the Danish bacteriologist, who originally devised it in 1882 and then published in 1884, Hans Christian Gram. Gram staining is the most important and universally used differential staining technique in bacteriology laboratory. Based on their reaction to Gram stain, bacteria species can be divided into two large groups which are Gram-positive and Gram-negative. It differentiates bacteria based on the chemical and physical properties of their cell wall by peptidoglycan, which presents in a thick layer of gram-positive bacteria. Gram staining is always being used in a clinical microbiology laboratory for the first step in the identification of a bacterial organism. The bacteria present in an unstained smear are invisible when viewed using a light microscope. Once stained, the morphology and arrangement of the bacteria may be observed as well. Furthermore, it is also an important step in the screening of infectious agents in clinical specimens such as direct smears from a patient. Cerebrospinal fluid, sterile fluids, expectorated sputum or Bronchoalveolar lavages, and exudates are routinely stained directly [1]. Gram staining is currently manually performed by hand in microbiology laboratories. The time intervals between processes are adjusted by a clock, timer or a chronometer [2]. If the reagent timing is not set properly, staining quality may be affected. Therefore, due to human inconsistency, some of the slides are heavily stained with dark color whereas some of it is lightly stained. This inconsistency would create a difficulty for automated sputum quality system using image processing.

Therefore, automated staining machine is developed in order to standardize the sputum slide staining as well as to reduce the time consumption needed in the staining process in microbiology laboratories. In this study, an automated Gram-staining prototype is built to standardize the sputum slide staining. The standardization of the staining sputum slide will be performed with the automatic automated Gram-staining prototype and the results are presented.

Gram staining was developed empirically by Christian Gram in 1884 has been modified by some investigators. Gram staining is used routinely and by request in a clinical microbiology laboratory for the primary microscopic examination of specimens submitted for smear and culture [3]. Although the traditional manual gram-stain method is a simple technique, the quality of the staining result is highly dependent on a laboratory personnel's skill. The accuracy of the grain results depends on many factors such as human interpretive error, the thickness of smear inoculation, fixation of smear, the time duration of different staining components, and properties of certain microorganisms leading to the problems associated with under-decolorization or over-decolorization [4].

Automatic staining machines are developed for reducing the workload and increasing the staining quality in microbiology laboratories. A gram-staining machine controlled by micro-controller was developed where the staining, washing and drying periods are controlled by a timer built in micro-controller. In addition, non-microbiologist clinical laboratory scientists perform stat Gram stains using electro-optical technology to evaluate the material on the slide to optimize the staining process [4][5].

The advantage of the machine staining is the saving of working time. In manual staining for one slide, at least 4 minutes are required and the technician may not devote his time to another procedure. Besides, in machine staining, after pressing the start button, the technician is able to do other experiments and especially in a busy diagnostic laboratory [3].

In the study of Soo Chan Kim, Seung II Kang and Deok Won Kim (2003) from Korea Institute of Science and Technology, the result indicated by the automated stainer for acid fast bacilli (AFB) developed looks promising for use in the clinical mycobacteriology laboratory to minimize the personal variation during the staining process. From their research, they found that manual sputum staining is time-consuming and laboratory technicians with high workload do not have sufficient time to examine all AFB smear slide

properly. Hence, it may result in a lower detection rate for AFB in a sputum sample. Therefore, an automated AFB stainer can produce greater accuracy and reproducibility [6].

There are few steps involved in gram staining technique which are:

i. Staining process. Transfer a loopful of the liquid culture to the surface of a clean glass slide and spread over a small area.
ii. Washing process. To stain material from a culture growing on solid media, place a loopful of tap water on a slide; using a sterile cool loop transfer a small sample of the colony to the drop, and emulsify.
iii. Drying process. Allow the film to air dry. Fix the dried film by passing it briefly through the drying fan two or three times

Gram staining permits the separation of all bacteria into two large groups, those which retain the primary dye, crystal violet after treatment with iodine and alcohol acetone appear purple or bluish purple and are designated as Gram positive. Those bacteria which lose the crystal violet show the color of the counter stain employed. The commonly-used counter stain is safranin which gives a pink or red color to bacteria and these organisms are labelled as Gram negative [7].

In Microbiology & Parasitology Department of Hospital Universiti Sains Malaysia, Kubang Kerian, the gram staining process is done manually on each of a sputum slide. A sputum sample slide is prepared by placing a thin smear of a sputum on a clean glass slide, dry it in the air and fix it by passing through the flame of a burner. Then, the process of gram staining of a sputum slide begins. Firstly, the smear is covered with crystal violet and kept for one minute to dry. Then, it is washed with water. Next, some Lugol Iodine is dropped on the slide and left for one minute to dry and then washed with water. After that, the slide is decolorized with acetone and rocking gently for 10-15 seconds until the violet color comes off the slide. Then, it is washed with water immediately. Finally, the glass slide is counterstained with safranin and is stand for 30 seconds to dry and then wash with water.

This paper will discuss the development of the automatic gram staining. This project consists of two parts, the hardware development of the prototype and the software development using Arduino to control the process sequence of the gram staining. The software of the Arduino consists of a standard programming language compiler and a bootloader that executes on the microcontroller.

II. METHODOLOGY

Basically, the process sequence of the Gram staining is involving staining, drying and washing. The staining part is repeated with four different solutions which are crystal violet, Lugol iodine, acetone and safranin.

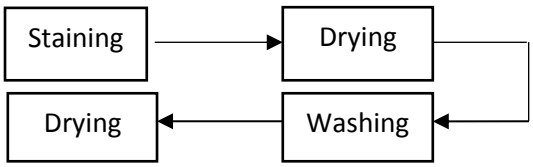

Figure 1: Block diagram of process sequence of gram staining

Figure 1 shows the block diagram of the process sequence of the Gram Staining. Firstly, there is staining process which transfers a loopful of liquid culture on the sputum slide and spread over the slide. Then, it will move to the second stage which is drying process. In this stage, drying fan will be used to dry the sputum slide. After that, the third process is washing process. The process rinses the tap water on the sputum slide and the sputum slide is then emulsifying. The last process will be the drying process again to dry the film then the slide is to be ready to examine under a microscope.

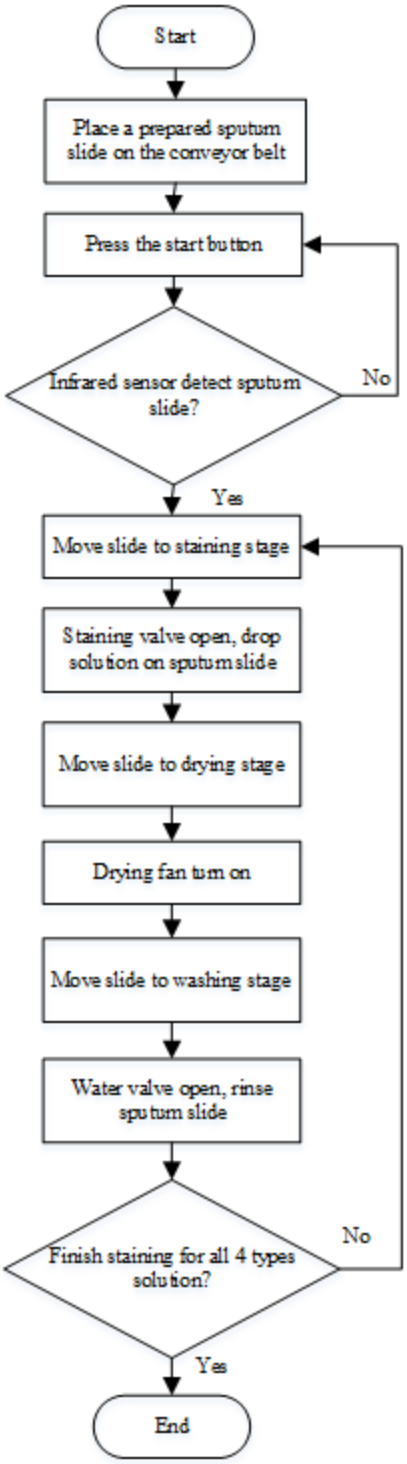

Figure 2: Automated gram staining for sputum slide sequence operation

Figure 2 shows the flowchart of the automated Gram Staining for sputum slide sequence operation in this project. First, the prepared sputum slide is placed on the conveyor

belt. Then, the start button is pressed to move the conveyor belt. When the infrared sensor is detecting the presence of the sputum slide, the conveyor will start moving to staining stage. After that, the staining solution valve will open to drop a loopful of liquid culture on the sputum slide and spread over the slide. The valve is then open at 90 degrees to drop the staining solution on the sputum slide. The dropping period is set in the program. The first solution is crystal violet and the slide is stained for 20 seconds.

After that, the valve of the staining solution is close and the conveyor belt transports the sputum slide to next stage which is drying process. The drying fan is turned on to allow the sputum slide to dry. Then, the conveyor is moving to the washing process. In this stage, the valve of the water tank is open to rinse the tap water on the sputum slide. The sputum slide is then emulsifying.

After 12 seconds, the water valve is closed and the system is then repeated 3 times for another 3 different solutions which are Lugol Iodine, acetone and safranin. Lugol iodine is stained for 20 seconds, acetone is stained for 5 seconds and safranin is stained for 10 seconds. After the washing stage of the last solution which is safranin, the sputum slide will be transported to the last process which is drying process. The drying fan is turned on to allow the sputum slide to dry. The staining, washing and drying process period are controlled by Arduino microcontroller. After the sputum slide is dried, the sputum slide is ready to do the primary microscopic examination.

A. Hardware Design

This automated Gram Staining consists of the mechanical part and electrical part as shown in Figure 3.

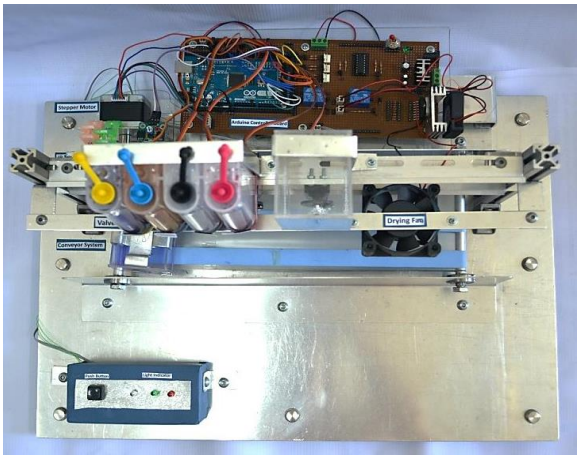

Figure 3: Hardware design of automated gram staining prototype for sputum slide

1) Mechanical Part

The mechanical part of the hardware design is shown in Figure 4. This mechanical design is consisting of three main parts, which are the conveyor system, the staining tank holder and the slide container. The conveyor system is the main part of the mechanical part. It is important to transport the slide from one stage to the next stage. The movement of the conveyor is driven by the stepper motor. The conveyor system is made up of one stepper motor, two roller rods, four bearings and one conveyor belt as shown in Figure 5. The length of the conveyor is 40cm, the width is 12.5cm and the height is 7cm.

The holder stand is made to hold the RC Servo motor, solution tanks and DC Fan as shown in Figure 6. The solution and water tank are screwed on the aluminium plate above RC servo motor so that a short pipe can connect between the base of the tank and the valve. The holes are drilled to fix the solutions valves with the RC Servo motors. The 12V DC brushless fan is screwed on the aluminium plate together with the valves. The height of the holder stand is 20cm, the width is 9cm and the length is 42cm.

The slide container as shown in Figure 7 is designed to hold sputum slide. So that, the sensor can detect the presence of the container as the presence of the slide. The slide container can hold and fix the position sputum slide to make sure the accuracy of the position on the conveyor. The excess staining solution and water will flow into the container when staining and washing process are done. When the container is full, the solution inside can be poured out. The dimension of the slide container is 5cm x 9cm x 4.5cm.

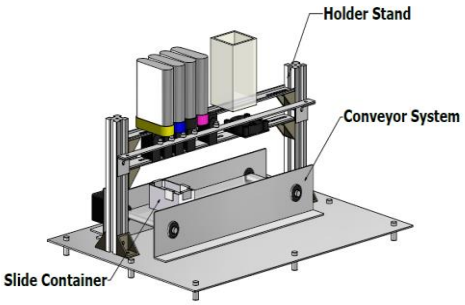

Figure 4: Mechanical part of the hardware design

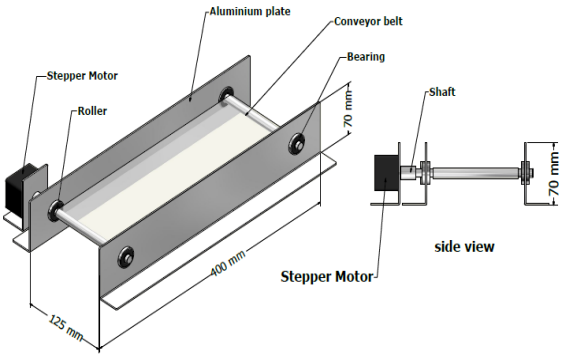

Figure 5: Conveyor system

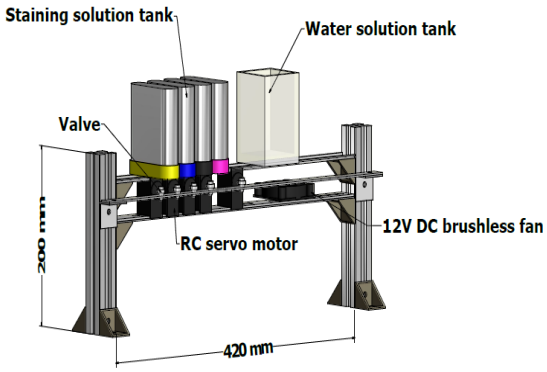

Figure 6: Staining tank holder

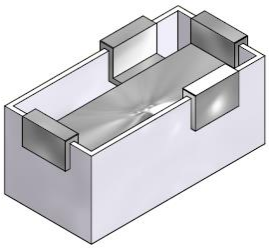

Figure 7: Slide container

2) Electrical Part

The electrical parts of this prototype are consist of an Arduino Mega 2560 as the main board to control the whole process, a IR01A Medium Range Infrared Sensor as the input to the program to detect the presence of the sputum slide, a Mineaba stepper motor to control the movement of the conveyor, five Servo Motors as actuators to open the valves of solutions and two DC fans used to dry the film and to cooling the motor driver IC.

The circuit for this prototype is designed and developed using software Proteus 7.7 sp2 schematic entry and PCB layout tools as shown in Figure 8. The circuit is connected to Arduino Mega 2560 with all the components used in this prototype.

B. Software Design

Arduino programming is the heart of this project. This is because all the components are controlled by Arduino. Arduino will give the command to output and starts the process after receiving the signal from the sensor when the presence of the sputum slide is detected. Arduino Mega 2560 is programmed by Arduino IDE 0014 and above version. It can be downloaded at Arduino official website.

Firstly, all the input and output pin in Arduino must be initialized. The sensor signal is attached and initialized at digital pin 22. Then, the output ports are initialized for five servo motors, stepper motor, DC fans and LEDs. RC servo motors and stepper motor are attached at PWM pin whereas 2 DC fans and 3 LEDs are attached to Digital pin of Arduino. The Arduino input port initialization programming is shown in Figure 9 and output port initialization programming are shown in Figure 10, Figure 11, and Figure 12.

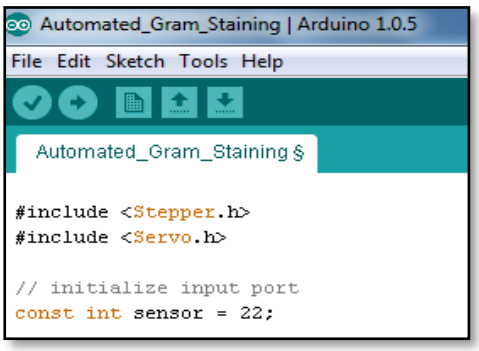

Figure 9: Input port initialization for IR sensor

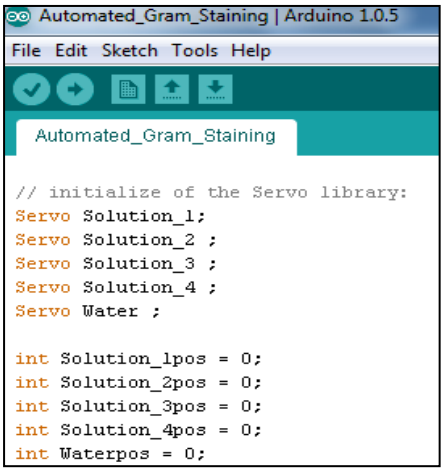

Figure 10: Output port and position initialization of RC servo motor

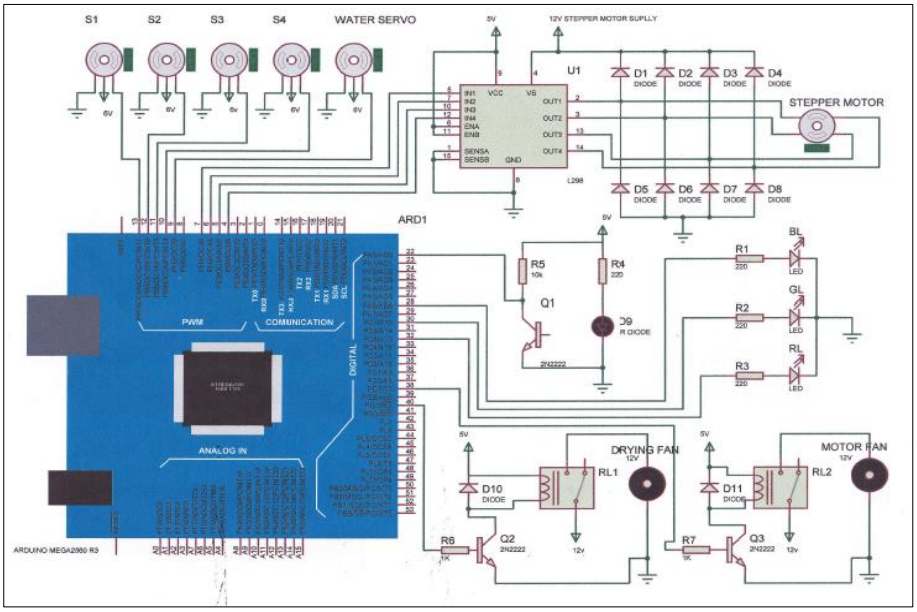

Figure 8: Schematic diagram of complete circuit

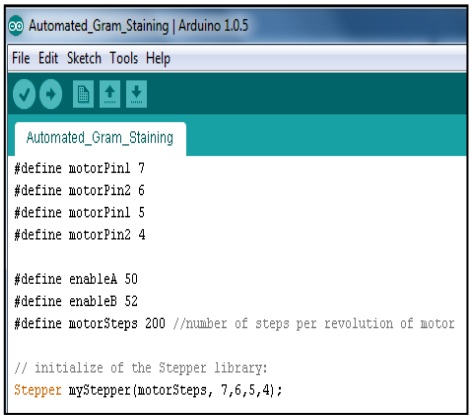

Figure 11: Output port initialization and steps per revolution set for stepper motor

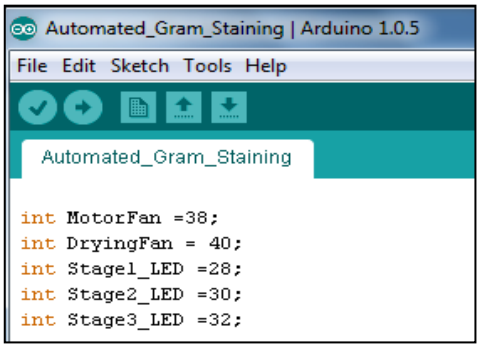

Figure 12: Output port initialization for 2 DC fans and 3 LEDs

Next, the declaration of the pin is important to receive the signal from the input and give the command to the output. The motor speed is set at 70 RPMS. The serial port is initialized at the bound rate 9600. The sensor is declared as input and RC servo motors, stepper motor, DC fans and LEDs are declared as output is shown in Figure 13.

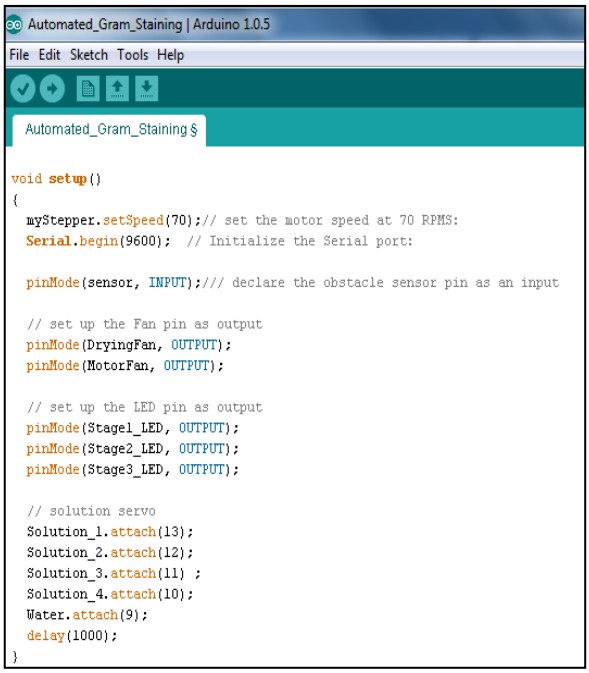

Figure 13: Declaration of the pin

After that, the program for output is developed. The number of steps is needed to count to run the stepper motor to the desired position. The example of the program to move stepper motor forward and backward for 1.5cm is shown in Figure 14.

Theoretical calculation

200 steps per revolution = 360°
1 steps = 360°/ 200 = 1.8°per step
200 steps per revolution = 2.2cm
1cm = 200/2.2 = 90.9 steps ≈ 91 steps

For this prototype, 1cm is approximate to 91 steps. Then 1.5cm is approximate to 140 steps.

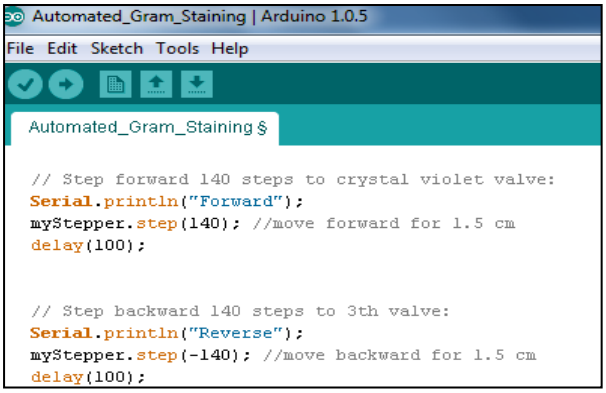

Figure 14: Program to move stepper motor forward and backward for 1.5cm

In this prototype, the solution valve is open at 90º. The period of the valve open can be set in the program as the delay is shown in Figure 15.

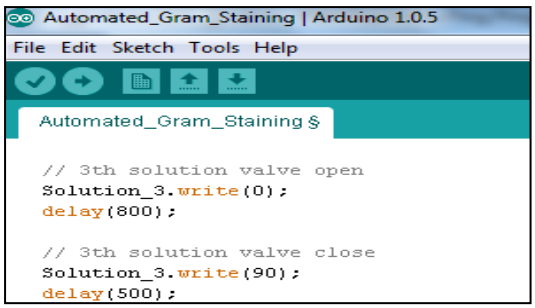

Figure 15: Program to open the solution valve

In order to turn on the DC fans or LEDs, signal HIGH (1) is set in the program. Meanwhile, signal LOW (0) is set to off the DC fans or LEDs. Both DC fans and LEDs are connected to digital pins of Arduino. The example of the program is shown in Figure 16.

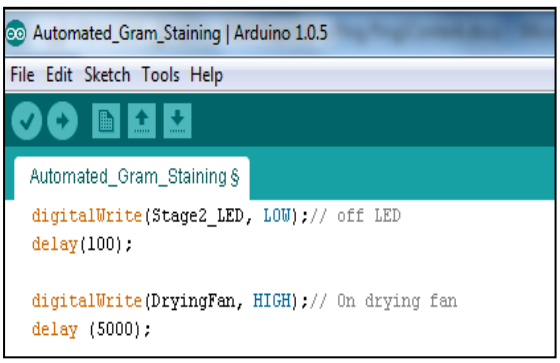

Figure 16: Program to turn on the LED and off drying fan

III. Result & Discussions

The outcome of this project result is analyzed in several aspects. The analysis is done on the accuracy of the position of the slide stain, the amounts of staining solution drop on the slide and the time efficiency of this automated system are compared to manual operation.

A. The Accuracy of the Position

The position is important to make sure the staining solution drop at the right position of the sputum slide. The length between each valve to DC Fan from start to the end is measured and shown in Figure 17. 10 slides were tested with this prototype with the full procedure to check on the position accuracy and it found that the position accuracy is 80%.

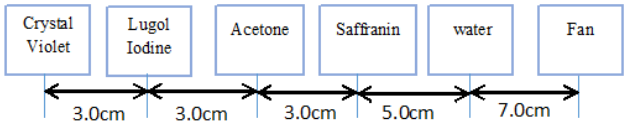

Figure 17: Length of each valve

B. The Amount of the Solution Drop

In this part, 10 slides are undergoing staining process to check the amount of each solution drop. The amount of every solution drop on the slide is measured using a syringe. The amount is measured and recorded in Table 2. From Table 2, it is found that the amounts of every staining solution drop on the slide are consistent. Therefore, the gram staining is uniform and standard.

Table 2
Results of Amount of Staining Solution Drop

Slide No.	Amount of Crystal Violet (*ml*)	Amount of Lugol Iodine (*ml*)	Amount of Acetone (*ml*)	Amount of Saffranin (*ml*)
1	0.20	0.20	0.20	0.22
2	0.24	0.20	0.20	0.20
3	0.20	0.20	0.20	0.20
4	0.20	0.20	0.20	0.22
5	0.22	0.20	0.20	0.20
6	0.20	0.20	0.20	0.20
7	0.20	0.20	0.20	0.23
8	0.20	0.20	0.20	0.20
9	0.20	0.20	0.22	0.20
10	0.20	0.2	0.20	0.20

C. Time Efficiency

Both manual and automated procedures on gram staining are done in 10 slides to compare in terms of time's efficiency. The results are tabulated in Table 3. It is found that the time needed for gram staining using automated gram staining is averagely about 1 min 41 seconds, whereas the manual procedure by one of the technologist needs averagely 4 min 4 seconds. Besides, the technologist time that using manually procedure is inconsistent. It is because the manual procedure is not only depending on the laboratory personnel skill, but also subject to distraction, fatigue, and forgetfulness. Therefore, it is good to have an automated system of gram staining which is consistent and time-saving.

Table 3
Results of Times Needed Using Automated Procedure vs. Manually Procedure

Slide No.	Timing using Automated System	Timing using Manually Procedure
1	1 min 40 seconds	3 min 40 seconds
2	1 min 41 seconds	5 min 20 seconds
3	1 min 43 seconds	3 min 30 seconds
4	1 min 40 seconds	3 min 48 seconds
5	1 min 42 seconds	3 min 10 seconds
6	1 min 40 seconds	4 min 05 seconds
7	1 min 44 seconds	4 min 33 seconds
8	1 min 40 seconds	5 min 34 seconds
9	1 min 40 seconds	3 min 23 seconds
10	1 min 40 seconds	3 min 33 seconds

IV. Conclusion

In this research, a new hardware prototype automated gram staining for sputum slide is developed. This prototype will automatically do gram staining on sputum slide by just pressing a start button. The automated gram stain prototype will move the sputum slide from one stage to one stage automatically. The Gram staining on sputum slide will be done standardizing in sequential manner operation. The prototype can solve the problem where some of the slides are heavily stained with dark color whereas some of it is lightly stained. It also can produce uniformly gram stain on sputum slide. Furthermore, it is time-saving and it can reduce busy workload in microbiology laboratories.

Acknowledgment

This research is supported by Universiti Malaysia Pahang Internal Grant of RDU1703142. The authors would also like to thank the Faculty of Electrical & Electronics Engineering Universiti Malaysia Pahang for providing the facilities to conduct this research and for financial support throughout the process.

References

[1] W. Lawrence Drew, Andres N. Pedersen And Jacques J. Roy. 1971. Automated Slide Staining Machine. *Applied Microbiology*. IEEE, 23(1): 17-20.

[2] O. A. N. Husain, J. M. Grainger And J. Sims. 1978. Cross contamination of cytological smears, with automated staining machines and bulk manual staining procedures. *Journal of Clinical Pathology*. 31: 63-68

[3] Felek, S. and Arslan, A. 1999. Gram Staining with an Automatic Machine. *Folia Microbiology*. 44(3): 333-337.

[4] River CW Wong, MPhil, Samuel SY Heung, MSc, YC Ho, MSc, KT Yip et al. 2010. Evaluation of PREVI Color Gram Automated Staining System on Positive Blood Culture Samples. *LABMEDICINE*. IEEE, 42(7).

[5] Ellen Jo Baron, Samantha Mix And Wais Moradi. 2009. Clinical Utility of an Automated Instrument for Gram Staining Single Slides. *Journal of Clinical Microbiology*. 48(6): 2014.

[6] Soo Chan Kim, Seung Il Kang, Deok Won Kim, Seung Cheol Kim, Sang-Nae Cho, Jung Ho Hwang, Young Kim, Sun-Dae Song, Young Ha Kim. 2003, Development and evaluation of an automated stainer for acid-fast bacilli. *Medical Engineering & Physics*. IEEE, 25: 341–347.

[7] Nur Shahida Nawi, Rosyati Hamid, Nurul Wahidah Arshad, Faradila Naim, Mohd Falfazli Jusof, Mohd Najib Razali. 2013. *The Detection and Summation of Squamous Epithelial Cells for Sputum Quality Testing*. Faculty of Electrical & Electronics Engineering, Universiti Malaysia Pahang, IEEE, 978-1-4673-6195-8/13,2013.