A paper stick test kit for rapid detection of total microbial contaminants in fresh milk

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Abstract. The way to determine the total microbial contaminants in fresh milk is by the Total Plate Count (TPC) method. The TPC method requires 48 hours, high analytical skills, and high analytical costs. The aim of this research was to design a rapid detection tool for microbial contaminants in fresh milk called Paper Stick Test Kit (PSTK), which is fast, cheap, and easy. The stages of research designing PSTK are: (a) making PSTK, (b) testing the optimal time for using PSTK, and (c) making standard color maps. The results of this research are: (a) the best PSTK is made from cardboard with color indicators made from a mixture of bromothymol blue 0.3 g/250 ml solvent and NaOH 0.01 N; drying PSTK is best done in an oven with a temperature of 100°C, for 20 minutes; (b) the optimal time of application of PSTK in fresh milk is 20 seconds; and c) standard color maps are based on the correlation between microbial contaminant levels in fresh milk and color levels in PSTK dipped in microbial contaminated fresh milk.

1 Introduction

Biosensor research aims to produce technology to detect the level of microbial contaminants in food, including milk, quickly, cheaply, and easily. [1] The development of microbial detection tools has challenges in terms of speed and accuracy.[2] Development of biosensors to detect microbes in food based on the presence of fluorescent antibodies, enzymes (ELISA method), and polymerase chain reaction (PCR). [3, 4] Another technique developed is a biosensor based on the presence of organic acids produced by microbes. The presence of chemical compounds produced by microbes can be used as a marker for the presence of certain microbes so that they can be counted. [5, 6, 7, 8]

The performance of this research is based on the fact that milk is a nutrient-rich substrate for microbes. Microbes in milk will utilize the nutrients present in milk for growth and development. One of the nutrients utilized by microbes is sugar lactose. The sugar will be broken down through metabolism in microbial cells to obtain energy. This metabolism produces metabolites including organic acids which are excreted outside the cell.[9, 10,] Metabolite compounds produced by microbes excreted outside the cell can be used as biological markers (biosensors) for the presence of microbes and the possibility of their quantification.

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The existence of organic compounds as biosensors can be applied to food technology.[11] Organic acids will affect the level of acidity (pH) of milk. One of the biosensor principles uses the principle of binding organic acids produced by microbes through physical adsorption by utilizing Van der Wals forces. Organic acids will react with indicators that produce certain signals including color changes.[12] The longer the microbial population in milk will increase and the accumulation of organic acids will increase. Increasing the number of organic acids will increase the acidity of the milk or lower the pH of the milk. [9] This research forms the basis for research on the manufacture of paper stick test kits. The level of acidity of fresh milk will be related to the level of total microbial contaminants.

Research in 2008 showed the same trend data between parameters of total microbial contaminant level, organic acid concentration as total acid, and pH. It can be said that these parameters have a positive correlation. The data is used as a basis for research on the manufacture of paper stick test kits. Organic acid level as total acid level. The total acid level has an effect on the pH level. The pH level is related to the level of total microbial contaminants in fresh milk. Organic acids are produced by microbes as an indicator of the level of microbial contaminants. Organic acid compounds change the color of the indicator compound. The level of color change indicates the concentration of organic acids produced by contaminating microbes. The indicator compound attached to the paper media (paper stick) is used to measure the level of organic acids as total acid in fresh milk which produces a color change.

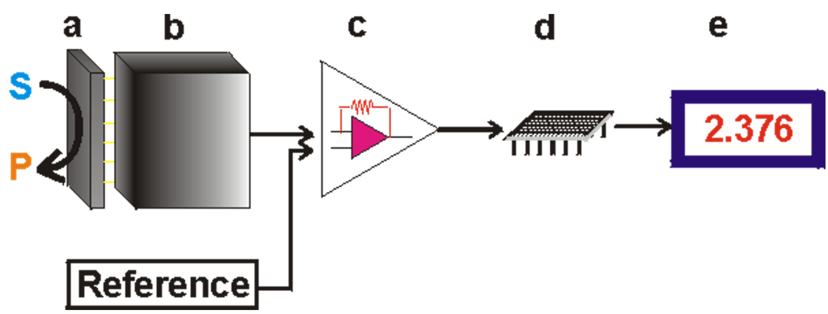

Fig. 1. How the paper stick test kit works (a) the substrate has been converted into a product (metabolite) recognized by the indicator, (b) the strengthening of the color change in the indicator (c) the color level is translated based on the standard color map reference (d) the conversion of the color level to the level contaminant microbial population, (e) number of contaminant microbes.[13]

The total microbial contaminant level of fresh milk has a positive correlation with the concentration of organic acids. Total organic acids affect the pH level. The color level on the paper stick test kit is proportional to the level of microbial contaminants. The relationship between these two parameters is mapped as a standard map of microbial contaminants in fresh milk. A standard map is used to show the level of total microbial contaminants in fresh milk based on the color change that occurs on the paper stick test kit. Total microbial analysis of fresh milk in general by indirect microbial counting using contaminant microbial culture on agar media. The medium used is Nutrient Agar (NA). On NA media, microbes grow to form microbial colonies. Each microbial colony originates from a single cell that divides to form a collection of similar microbes (pure culture). One microbial colony is counted as one cell. To avoid the cell density being too high, dilution was carried out. The dilution level will be taken into account as the dilution factor in the calculation of total microbes in fresh milk. The microbial count in this way is known as the total microbial count (Total Plate Count/TPC). The total microbial count in fresh milk in the above way requires an incubation period of 48 hours. After 48 hours of incubation, the total microbial count can only be counted. Calculation of total microbes by Total Plate Count requires a long time, media costs, equipment, and high analytical skills.

This study aims to produce a tool to measure total microbial contaminants in fresh milk including a paper stick test kit to measure the total microbial population, the optimal time for the paper stick test kit to measure total microbes, making standard color maps.

2 Materials and Methods

2.1 Research Materials

The research materials were cow's milk taken from various breeders, 80 g HVS paper, cardboard or manila paper, photo paper, and cotton buds. This research required chemicals such as alcohol, NaOH, phenolphthalein indicator, bromothymol blue indicator, Nutrient Agar, Plate Count Agar, distilled water, and printing paper. In addition, filter paper, tissue rolls, plastic bags, plastic container boxes, and stirrers are also needed.

2.2 Research methods

Paper stick test kit (PSKT) is made with three stages of the process:
First: The process of determining the temperature and drying time of PSTK material paper.

At this stage, the aim is to determine the proper temperature and drying time using an oven after the PSKT raw material paper is dyed with PSTK formula solution for 10 seconds. The parameters observed at this stage were dry and undamaged paper. The treatment used are:

a. Oven temperature used (50 °C, 75 °C, and 100 °C).
b. Drying time (0, 5, 10, 15, 20 minutes).

Second: The process of PSKT making.

At this stage, the aim is to make the right PSTK from the type of paper as a raw material for PSTK, concentration of NaOH, and concentration of bromothymol blue indicator.

a. NaOH concentration (0.01 N, 0.1 N, 1N).
b. Bromothymol blue indicator concentration (0.1g 0.2g, 0.3g for every 250 ml of water).
c. The type of paper used to make PSTK (HVS 80 g, cardboard, photo paper, and cotton buds).

Parameters observed were: the sensitivity of the PSTK to changes in the pH level during application which included parameters: speed of color formation, color sharpness, color interval, and color fastness in PSTK.

Third: Creation of a standard color map.

At this stage a total microbial measurement [14], pH[15] was carried out, observing the level of color formed when immersing PSTK in milk 0, 30, 60, 90, 120, 150, 180, 210, 240 minutes

3 Results and Discussion

3.1 Making Paper Stick Test Kit (PSTK)

PSTK is made of 80 g HVS paper, cardboard, photo paper, and cotton buds. The indicator formula is made of NaOH and bromothymol blue (BTB) indicator. The concentration of NaOH used varied from 0.01 N, 0.1 N, and 1N. While Bromothymol blue varied 0.1 g/250 ml, 0.2 g/250 ml, 0.3 g/250 ml. NaOH functions as a regulator increasing the pH of the milk substrate. Increased acidity to a more basic condition before microbial contaminants that produce organic acids occur. Increasing the pH value with NaOH needs to be done so that the color interval produced by the BTB indicator is wider so that the PSKT sensitivity will be higher. Bromothymol blue is an indicator that functions to produce a color change when it reacts with acids produced by contaminant microbes in fresh milk. The color spectrum produced by BTB has an interval from yellow at pH 7 to green at pH 6.6. Fresh milk from milking at the beginning of time has a pH below 7.0 so that in this condition when given the BTB indicator it will turn blue. Therefore, it is necessary to design a new formula between BTB and NaOH on 80 g HVS paper, cardboard, photo paper, and cotton buds, so that PSKT can work optimally to produce a wide spectrum of color changes when dipped in milk. These are strategies to make biosensor more effective and effisien.[17] This color spectrum change correlates with the level of concentration of organic acids[16] produced by contaminant microbes in fresh milk. That's reveals the level of microbial contaminant populations in fresh milk correlates with the concentration of organic acids produced, and the level of concentration of organic acids correlates with the pH level of fresh milk. PSTK was made by dipping 80 g HVS paper, cardboard, photo paper, and cotton buds into NaOH solution for 5 seconds until saturated, draining, and drying in an oven at 100 ^{o} C, for 20 minutes. Then 80 g of HVS paper, cardboard, photo paper, and cotton buds were dipped in bromothymol blue solution for 10 seconds and drained. The paper is dried in an oven at 100 ^{o} C for 20 minutes. PSTK is ready to be tested

Testing the sensitivity of PSTK was carried out on microbial-contaminated solutions or milk that had varying pH levels. The variations in pH prepared were pH 6.0; 6,2; 6,4; 6,6; 6.8 and 7.0. The color change trial was carried out by immersing the PSTK in a pH 6.4 solution. The acidity level of the milk used in the PSTK test is pH 6.6 because this pH is the level of acidity most often found in milk produced by breeders.

The color formation speed test was carried out by observing the colors formed on PSTK paper: 80 g HVS paper, cardboard, photo paper, and cotton buds after 10 seconds of dipping in the PSTK formula solution. The observed color is the color that lasts for a while which is considered a stable color. The time for color formation to occur varied from one type of paper to another as presented in Table 1. The stable color formation on 80 g HVS paper occurred after 3 seconds. On cardboard, photo paper, and cotton buds it takes 5, 10, and 4 seconds respectively. Formation of stable colors on 80 g HVS paper requires a short time (3 seconds) because the paper is thin, and is not coated with other materials. While the formation of stable colors on photo paper takes the longest time (10 seconds) because this paper is coated with a protective material. Color formation on cardboard takes 5 seconds, meaning 2 seconds longer than the time required on 80 g HVS paper. This is because the cardboard is thicker so the water absorbed in the material takes longer, and it takes longer time to complete the color formation reaction. Color formation on cotton buds is faster (4 seconds). Even though the cotton buds are thicker, they have a higher porosity which allows for a faster color formation reaction.

Paper as a PSTK material varies in type, thickness, and porosity whether or not there is a layer on the surface of the paper. After 10 seconds of immersion in a solution with a standard pH, a color change reaction occurs. PSTK was dipped in a standard pH solution of 6.6 resulting in a green color change in PSTK.

The level of blue sharpness on each paper varies depending on the type of paper. On HVS 80 g paper it produces a less clear/less sharp blue color. The color change in PSTK from cardboard material produces a color change with moderate clarity or sharpness (3) in the formula with 0.01 N NaOH 0.3 g BTB indicator. The level of clarity or color sharpness produced on cardboard paper is almost equivalent to the color sharpness of cotton buds (Table 2). The colors produced in PSTK in formulas with 0.1 N NaOH and 1 N at various indicator concentrations and various types of paper produce clear color sharpness (4) to very clear (5).

Table 1. Speed of Color Formation of Paper Stick Test Kit/PSTK (seconds)

Indicator		PSTK Materials	Speed of Color Formation in PSTK
NaOH 0.01 N	BTB 0.1 g	HVS Paper	10 ± 0,00
		Cardboard	30 ± 0,00
		Photo Paper	45 ± 0,00
		Cotton Buds	5 ± 0,00
	BTB 0.2 g	HVS Paper	10 ± 0,00
		Cardboard	30 ± 0,00
		Photo Paper	45 ± 0,00
		Cotton Buds	5 ± 0,00
	BTB 0.3 g	HVS Paper	10± 0,00
		Cardboard	30 ± 0,00
		Photo Paper	45 ± 0,00
		Cotton Buds	5 ± 0,00
NaOH 0.1 N	BTB 0.1 g	HVS Paper	6 ± 0,00
		Cardboard	7 ± 0,00
		Photo Paper	15 ± 0,00
		Cotton Buds	4 ± 0,00
	BTB 0.2 g	HVS Paper	6 ± 0,00
		Cardboard	7 ± 0,00
		Photo Paper	15 ± 0,00
		Cotton Buds	4 ± 0,00
	BTB 0.3 g	HVS Paper	6 ± 0,00
		Cardboard	7 ± 0,00
		Photo Paper	15 ± 0,00
		Cotton Buds	4 ± 0,00
NaOH 1 N	BTB 0.1 g	HVS Paper	4 ± 0,00
		Cardboard	5 ± 0,00
		Photo Paper	10 ± 0,00
		Cotton Buds	4 ± 0,00
	BTB 0.2 g	HVS Paper	4 ± 0,00
		Cardboard	5 ± 0,00
		Photo Paper	10 ± 0,00
		Cotton Buds	4 ± 0,00
	BTB 0.3 g	HVS Paper	4 ± 0,00
		Cardboard	5 ± 0,00
		Photo Paper	10 ± 0,00
		Cotton Buds	4 ± 0,00

Table 2. Sharpness of Color Paper Stick Test Kit (PSTK)

Indicators		PSTK Materials	Sharpness of Color Paper Stick Test Kit
NaOH 0.01 N	BTB 0.1 g	HVS Paper	1 ± 0,00
		Cardboard	1,33 ± 0,58
		Photo Paper	1 ± 0,00
		Cotton Buds	2 ± 0,00
	BTB 0.2 g	HVS Paper	1,33 ± 0,58
		Cardboard	2,67 ± 0,58
		Photo Paper	1 ± 0,00
		Cotton Buds	2,67 ± 0,58
	BTB 0.3 g	HVS Paper	1,33 ± 0,58
		Cardboard	3 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	3 ± 0,00
NaOH 0.1 N	BTB 0.1 g	HVS Paper	4 ± 0,00
		Cardboard	4 ± 0,00
		Photo Paper	4 ± 0,00
		Cotton Buds	4 ± 0,00
	BTB 0.2 g	HVS Paper	4 ± 0,00
		Cardboard	4 ± 0,00
		Photo Paper	4 ± 0 ,00
		Cotton Buds	4 ± 0,00
	BTB 0.3 g	HVS Paper	4 ± 0,00
		Cardboard	4 ± 0,00
		Photo Paper	4 ± 0,00
		Cotton Buds	4 ± 0,00
NaOH 1 N	BTB 0.1 g	HVS Paper	5 ± 0,00
		Cardboard	5 ± 0,00
		Photo Paper	5 ± 0,00
		Cotton Buds	5 ± 0,00
	BTB 0.2 g	HVS Paper	5 ± 0,00
		Cardboard	5 ± 0,00
		Photo Paper	5 ± 0,00
		Cotton Buds	5 ± 0,00
	BTB 0.3 g	HVS Paper	5 ± 0,00
		Cardboard	5 ± 0,00
		Photo Paper	5 ± 0,00
		Cotton Buds	5 ± 0,00

Description: 1. Not very clear, 2. Not clear; 3. Medium. 4. Clear. 5. Very Clear.

Table 3. Observation of the Paper Stick Test Kit (PSTK) Color Interval

Indicators		**PSTK Materials**	PSTK Color Range
NaOH 0.01 N	BTB 0.1 g	HVS Paper	1 ± 0,00
		Cardboard	1 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	1 ± 0,00
	BTB 0.2 g	HVS Paper	1 ± 0,00
		Cardboard	1,67 ± 0,58
		Photo Paper	1 ± 0,00
		Cotton Buds	2,33 ± 0,00
	BTB 0.3 g	HVS Paper	1 ± 0,00
		Cardboard	2,67 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	3 ± 0,00
NaOH 0.1 N	BTB 0.1 g	HVS Paper	1 ± 0,00
		Cardboard	1 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	1 ± 0,00
	BTB 0.2 g	HVS Paper	1 ± 0,00
		Cardboard	1 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	1 ± 0,00
	BTB 0.3 g	HVS Paper	1 ± 0,00
		Cardboard	1 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	1 ± 0,00
NaOH 1 N	BTB 0.1 g	HVS Paper	1 ± 0,00
		Cardboard	1 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	1 ± 0,00
	BTB 0.2 g	HVS Paper	1 ± 0,00
		Cardboard	1 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	1 ± 0,00
	BTB 0.3 g	HVS Paper	1 ± 0,00
		Cardboard	1 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	1 ± 0,00

Description: 1. Very narrow, 2. Narrow. 3. Medium. 4. Wide., 5. Very wide.

Another parameter tested was the color interval formed on PSTK when immersed in a standard solution of pH 6.3 and pH 6.8. The pH level is used as a color interval test that is formed because at this pH the acidity level of milk produced by breeders occurs at both the lowest pH level and the highest pH level. Color interval testing was carried out by comparing the sharpness of the color formed after PSTK was dipped in a standard solution of pH 6.3 and pH 6.8 for 10 seconds.

Another important parameter tested was the color interval formed on PSTK when immersed in a standard solution of pH 6.3 and pH 6.8. The pH level is used as a color interval test that is formed because at this pH the acidity level of milk produced by breeders occurs at both the lowest pH level and the highest pH level. Color interval testing was carried out by comparing the sharpness of the colors formed after PSTK was dipped in a standard solution of pH 6.3 and pH 6.8 for 10 seconds.

Table 4. Observation of the Color Resistance of the Paper Stick Test Kit (PSTK)

Indicators		PSTK Materials	PSTK Color Resistance
NaOH 0.01 N	BTB 0.1 g	HVS Paper	1 ± 0,00
		Cardboard	3 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	5,33 ± 0,58
	BTB 0.2 g	HVS Paper	1 ± 0,00
		Cardboard	3 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	5,33 ± 0,58
	BTB 0.3 g	HVS Paper	1 ± 0,00
		Cardboard	3 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	5,33 ± 0,58
NaOH 0.1 N	BTB 0.1 g	HVS Paper	1 ± 0,00
		Cardboard	3 ± 0,00
		Photo Paper	1 ± /0,00
		Cotton Buds	5,33 ± 0,58
	BTB 0.2 g	HVS Paper	1 ± 0,00
		Cardboard	3 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	5,33 ± 0,58
	BTB 0.3 g	HVS Paper	1 ± 0,00
		Cardboard	3 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	5,33 ± 0,58
NaOH 1 N	BTB 0.1 g	HVS Paper	1 ± 0,00
		Cardboard	3 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	5,33 ± 0,58
	BTB 0.2 g	HVS Paper	1 ± 0,00
		Cardboard	3 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	5,33 ± 0,58
	BTB 0.3 g	HVS Paper	1 ± 0,00
		Cardboard	3 ± 0,00
		Photo Paper	1 ± 0,00
		Cotton Buds	5,33 ± 0,58

Description: 1 = 0-10 minutes; 2 = 11-20 minutes; 3 = 21-30 minutes; 4 = 31- 40 minutes; 5 = 41- 50 minutes; 6 = 51 – 60 minutes

Data from observations of the color intervals formed are presented in Table 3. From the test results, it is known that the color intervals produced are influenced by the type of PSTK paper material, the concentration of NaOH, and the concentration of the indicator used. Types of paper as PSTK materials that are good to use are cardboard and cotton buds because they have good absorption of indicators and standard solutions so that they produce sharp color changes and have intervals to moderate. The color change interval is very important as a requirement for PSTK so that at different acidity levels you can see the level of color that occurs so that it can be distinguished.

The color interval is also affected by the concentration of the indicator. The higher the concentration of bromothymol blue used, the wider the color interval will be. Thus the use of a larger bromothymol blue indicator would be more recommended for the formula to produce PSTK. However, the use of NaOH at concentrations of 0.1 N and 1N resulted in very narrow color intervals. Therefore the recommended concentration of NaOH is 0.01 N.

Analysis was also carried out on the parameters of stable peak color resistance. The longer the time the peak color is formed or the more stable the color that is formed will be recommended as the right time for an accurate PSTK reading. This parameter is very important in the application of color change readings in PSTK besides the length of time the peak color forms after immersion in a standard solution which is presented in Table 4. The length of the peak color needs to be considered so that in PSTK readings it does not exceed the maximum time the peak color is formed. Peak color will fade when the recommended time is exceeded. The color that is formed will fade after holding out for a certain time.

The results of the color fastness analysis are presented in Table 4. The color fastness is largely determined by the PSTK material paper. The thickness and porosity of the paper in holding and accommodating the indicators as well as the standard liquid or fresh milk tested will determine the stable peak color resistance. Cardboard and cotton buds are PSTK materials capable of producing longer-lasting colors than 80g HVS paper and photo paper.

The stability of the stable peak color formed on PSTK made of cardboard was 21-30 minutes. The color reading on PSTK should be carried out no more than 30 minutes after the time of formation of a stable peak color. PSTK made of cardboard or manila paper has a peak color formation time of 3 minutes, therefore the color level reading is carried out 3 minutes after the PSTK is removed from the standard solution or milk being tested. PSTK made from cotton buds has a stable peak color time of around 50 minutes. The PSTK material from cotton buds has a high mass and porosity so that it is able to hold more indicators and solutions tested so that the stable peak color persistence will be longer compared to 80 g HVS paper, photo paper, and cardboard/manila paper.

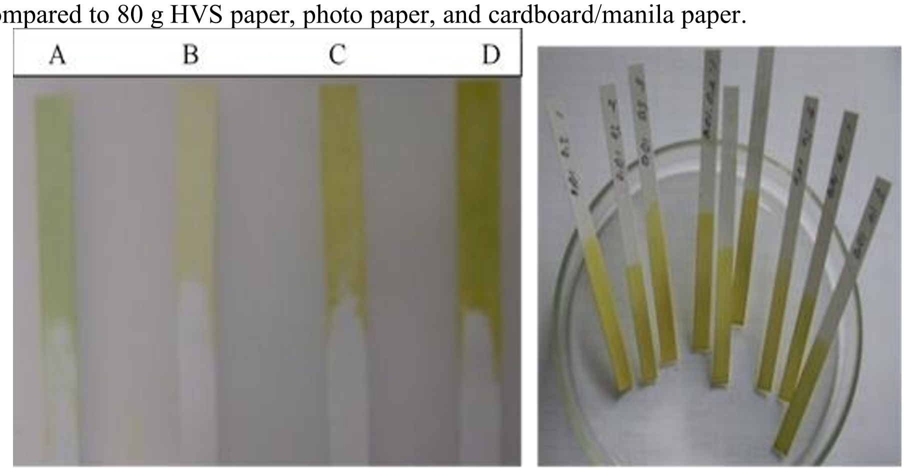

Fig. 2. The prototype of the Paper Stick Test Kit/PSTK after being dipped in fresh milk had different levels of total microbial contaminants and different pH levels. A, is PSTK dipped in fresh milk with the highest total microbial contaminants and the lowest milk pH, in contrast to PSTK D.

From the results of the research at this stage, it can be presented the initial PSTK research model Figure 2. This model will be further improved in terms of appearance and size aspects so as to obtain a good appearance that supports the optimal performance of PSTK.

3.2. Optimal Time for Application of Paper Stick Test Kit in Fresh Milk

Trials using the paper stick test kit on fresh milk showed the performance of the instrument. The paper test kit has the ability to absorb the liquid phase of the milk being tested. The ability to absorb milk is slower than the absorption of standard pH test solutions. Based on the paper test kit application test data on fresh milk, the optimal time needed for absorption to produce a stable color to read is 20 seconds. The optimal time test data for the paper test kit application is presented in Table 5.

Table 5. Optimal length of time for test kit application in fresh milk

NO.	Length of Application Time (seconds)
1	18
2	17
3	18
4	17
5	19
6	19
7	21
8	22
9	22
10	21
11	20
12	22
13	21
14	22
15	19
Mean	19.87 ± 1.85

3.3. Standard Color Map creation

A standard color map is made based on the relationship between the colors formed on the Paper Stick Test Kit when it is dipped in fresh milk containing a certain amount of total microbes. The color change in fresh milk that has been tested using a paper stick test kit shows a color difference in the paper stick test kit dipped in fresh milk with low, medium, and high levels of contamination. Changes in the color of the paper stick test kit when tested on fresh milk generally show four color changes that can be distinguished from the paper stick test kit. The three color changes on the paper stick test kit consistently correlate with the level of total microbial contaminants in fresh milk tested with the paper stick test kit (Figure 3).

A standard color map made based on a paper stick test kit on fresh milk and standardized as a standard color map can be shown in the figure below.

<1x10^6 cels/ml	**1x10^6 cels/ml**	**4x10^6 cels/ml**	**8x10^6 cels/sml**	**>8x10^6 cels/ml**

Fig. 3. Color map of the standard paper stick test kit

4 Conclusions

The most suitable material for making a paper stick test kit is cardboard/Manila paper. This paper has more optimal absorption of the indicator formula so as to produce sharper colors. The optimal indicator formula used is NaOH 0.01 N, bromothymol blue 0.3 g/250 ml of solvent (aquadest). The optimal time for applying the paper test kit to fresh milk is 20 seconds. A standard color map is made based on the relationship between the level of contaminants in fresh milk and the color change of PSTK dipped in milk with a certain level of contaminants.

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