# Development of Dairy Cow Mastitis Test Kit Based on Immunochromatographic Strip Using Monoclonal Antibody Against *Staphylococcus aureus*

# การพัฒนาชุดตรวจโรคเต้านมอักเสบในโคนมด้วยชุดตรวจแถบสีอิมมูโนโครมาโตกราฟิกโดยใช้โมโนโคลนอลแอนติบอดีต่อเชื้อ *Staphylococcus aureus*

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**บทคัดย่อ**: งานวิจัยนี้มีวัตถุประสงค์เพื่อพัฒนาชุดตรวจแถบสีสำหรับระบุเชื้อก่อโรคเต้านมอักเสบในน้ำนมดิบของแม่โค อาศัยเทคนิคภูมิคุ้มกันวิทยาโดยใช้โมโนโคลนอลแอนติบอดีต่อเชื้อ *Staphylococcus aureus* ทำการทดสอบความจำเพาะเจาะจงของโมโนโคลนอลแอนติบอดีต่อเชื้อ *S. aureus* และทดสอบการเกิดปฏิกิริยาข้าม ในเชื้อก่อโรคเต้านมอักเสบกลุ่มอื่น ๆ ได้แก่เชื้อ *Enterococcus faecalis, Streptococcus agalactiae* และ *Escherichia coli* ด้วยเทคนิคอินไดเรคเอนไซม์ลิงค์อิมมูโนซอร์เบนท์เอสเซ (indirect enzyme-linked immunosorbent assay: indirect-ELISA) ที่จำนวนเชื้อก่อโรค $10^6$, $10^7$, $10^8$ และ $10^9$ CFU/mL พบว่า ระดับความเข้มข้นของโมโนโคลนอลแอนติบอดีที่เหมาะสมในการทำปฏิกิริยาคือ 1: 2,500 สามารถจับกับเชื้อ *S. aureus*, *E. faecalis, S. agalactiae* และ *E. coli* ที่จำนวนเชื้อ $10^9$ CFU/mL ได้เท่ากับ 100, 30, 13 และ 10% ตามลำดับ เมื่อนำโมโนโคลนอลแอนติบอดีพัฒนาเป็นชุดตรวจแถบสี โดยนำมาติดฉลากกับ colloidal gold ขนาด 40 nm (pH 9.0) สามารถเชื่อมติดกันได้สูงสุดที่ค่าการวัดดูดกลืนแสง 532 nm จากนั้นนำโมโนโคลนอลแอนติบอดี ความเข้มข้น 1 mg/mL ปริมาตร 0.5 µL หยดบนแผ่นกระดาษไนโตรเซลลูโลสเมมเบรน ที่ตำแหน่งทดสอบ และหยด goat anti-mouse IgG (H+L) ความเข้มข้น 0.8 mg/mL ปริมาตร 0.5 µL ที่ตำแหน่งควบคุมปฏิกิริยา จากนั้นนำชุดตรวจมาอบแห้งที่อุณหภูมิ 37 °C นาน 14-16 ชั่วโมง หลังจากประกอบชุดตรวจทดสอบสำเร็จ จึงนำชุดตรวจไปทดสอบด้วยการหยดตัวอย่างเชื้อก่อโรคชนิดต่าง ๆ ที่จำนวน $10^9$ CFU/mL ในสารละลายบัฟเฟอร์ พบว่า เฉพาะตัวอย่างที่มีเชื้อก่อโรค *S. aureus* แสดงแถบสีชมพูม่วง ในตำแหน่งควบคุมและตำแหน่งทดสอบ ขณะที่เชื้อก่อโรคชนิดอื่นแสดงแถบสีชมพูม่วงในตำแหน่งควบคุมเท่านั้น ดังนั้นชุดตรวจระบุเชื้อก่อโรคเต้านมอักเสบที่พัฒนาขึ้นนี้เป็นชุดทดสอบที่มีความจำเพาะต่อ *S. aureus* และใช้เป็นชุดตรวจต้นแบบในระดับห้องปฏิบัติการเพื่อระบุการติดเชื้อ *S. aureus* ที่รวดเร็วสำหรับวินิจฉัยเชื้อก่อโรคเต้านมอักเสบได้

**คำสำคัญ**: โคนม โรคเต้านมอักเสบ สแตฟิโลค็อกคัส ออเรียส โมโนโคลนอลแอนติบอดี ชุดตรวจแถบสี

**Abstract**: This study aimed to develop an immunochromatographic strip test based on immunological techniques using monoclonal antibodies against *Staphylococcus aureus*, an infection mastitis pathogen in raw milk. Specificity of monoclonal antibodies to *S. aureus* and cross-reactivity to other potential mastitis pathogens, such as *Enterococcus faecalis*, *Streptococcus agalactiae*, and *Escherichia coli* were evaluated at the bacterial levels of $10^6$ - $10^9$ CFU/mL, using indirect enzyme-linked immunosorbent assay (indirect-ELISA). The results have shown that the optimal concentration of monoclonal antibodies in the indirect-ELISA test was 1:2,500, which could bind *S. aureus*, *E. faecalis*, *S. agalactiae* and *E. coli* ($10^9$ CFU/mL) with binding 100, 30, 13 and 10 %, respectively. To develop the strip, monoclonal antibodies were linked with colloidal gold (size 40 nm, pH 9.0), at the wavelength of 532 nm. After that, 1 mg/mL of monoclonal antibodies (0.5 µL), and 1.0 mg/mL of goat anti-mouse IgG (H+L) (0.5 µL), were coated on nitrocellulose membrane at the control line and test line, respectively. The strip was then incubated at 37 °C for 14-16 hours and assembled with the other components to create a prototype of the immunochromatographic strip test. To test the specificity of the strip test device, suspension of bacterial cells prepared at $10^9$ CFU/mL in running buffer was dropped onto the sample pad. The samples containing *S. aureus* formed purple-pink bands at the control line and the test line, while the samples containing other bacteria created a purple-pink band only at the control line. In conclusion, the developed mastitis detection kit was highly specific to *S. aureus* and could be used as a laboratory-level of the prototype for rapid determination of *S. aureus* infection in investigation causing mastitis.

**Keywords**: Dairy cattle, mastitis, Staphylococcus aureus, monoclonal antibody, immunochromatographic strip

## Introduction

According to the Cooperative Promotion Department of Thailand data, raw milk production during 2015 and 2020 was up to 1,371,133 tons, which was valued at approximately 25 billion Thai Baht. This suggests that raw milk production generate a significant economic value for the nation's agriculture and dairy farmers. Nevertheless, the farmers have still been facing many problems, one of which is mastitis, which has been causing damage to the dairy farming industry. Mastitis is caused by infection and inflammation in the cow's udder, leading to decrease in yield and milk quality. This results in a loss of income and increased expenses for the treatment of the disease and care of the infected animals, which were 40.45 Baht per milking cow per month (Sirinupongsanun and Simasatitkul, 1997). Inflammation in the udder tissue can be either symptomatic or asymptomatic. For the latter, milk production decreases while the symptoms of the disease are difficult to observe. The common tool for preliminary mastitis testing is the California mastitis tests (CMT), which is based on observing the changes in color and viscosity of milk. The chemical used in CMT was an anionic detergent, which would expose the DNA of white blood cells, causing the samples to appear more viscous. In other words, if the viscosity of blood in CMT increases, there is an asymptomatic infection in the cow (Barkema *et al*., 1998). Mastitis detection using CMT can determine infection and approximate

degrees of infection, but it does not identify specific pathogens. The bacterial pathogens causing mastitis can be divided into two types: contagious and environmental pathogen. Pathogens that are contagious can spread from one animal to another, whereas pathogens that are environmental can spread to the farm environment and infect cows through direct contact. A recent report shows that mastitis in Northern Thailand is mainly caused by bacteria *Streptococcus agalactiae*, *Streptococcus uberis* and *Streptococcus dysagalactiae* (Leelahapongsathon *et al*., 2014). Furthermore, other species such as *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis* also cause mastitis in the cow (Hamann, 2010). In particular, the prevalence of *S. aureus* infection in dairy cattle subclinical mastitis in Chiang Mai province is 8 % (Pumipuntu *et al*., 2017). Identification of specific pathogens can be performed in laboratory using polymerase chain reaction (PCR) (Boerlin *et al*., 2003), detecting nucleotides in natural samples by metagenomics DNA sequencing (Tringe and Rubin, 2005), or loop-mediated isothermal amplification (LAMP) (Sowmya *et al*., 2012). These methods require a complicated process, an expensive budget, and a long time to obtain the results. Therefore, detection of specific pathogens using immunological techniques with the cohesion of antigen and specific monoclonal antibody, which are simple, inexpensive, and rapid, is a promising approach. Thus, this study aimed to develop an immunochromatographic strip using monoclonal antibody against *S. aureus* to identify the cause of mastitis.

## Materials and Methods

### Species confirmation of bacterial strain

Four species of microorganisms were used, including *Staphylococcus aureus* AU17, which is the target microorganism, *Streptococcus agalactiae* SA35, which is a representative of contagious pathogenic streptococci, *Enterococcus faecalis* and *Escherichia coli* TISTR117, which are the representatives of environmental pathogens.

Firstly, the microorganisms were examined for their morphology under a light microscope. The 4 species were examined for their biochemical properties, including growth on Baird-Parker egg - yolk agar, coagulase production and hemolytic activity on Columbia ANC agar + 5 % sheep blood. Then, the species of each bacterial strain was confirmed using 16S rRNA gene sequencing (performed by Macrogen (Korea) through U2Bio Sequencing Service). Finally, the growth rates of the 4 species were measured to determine the numbers of the pathogens to be used in the immunological techniques.

### The specificity test of monoclonal antibody

#### 1. Optimization of monoclonal antibody concentration-based indirect-ELISA technique

Mouse anti-*S. aureus* monoclonal antibody concentration was optimized using the indirect - ELISA technique. Firstly, a 100 µL portion of $10^6$ - $10^9$ CFU/mL *S. aureus* AU17 was coated on a microtiter plate and incubated at 4 °C for 14 - 16 hours. Secondly, the excess cells were washed three times with 250 µL of 1XPBS + 0.05% Tween - 20 (PBST; Millipore, USA). Thirdly, the plate was blocked using 200 µL of 5 % BSA (Millipore, USA) and incubated at 37 °C for 2 hours, and then washed with 250 µL of PBST for three times. Then, the 1.5 mg/mL of the primary antibody, which is mouse anti-*S. aureus* monoclonal antibody (mouse anti-*S. aureus* mAb) (Fitzgerald, USA), was added

at the ratios of 1: 1,000, 1: 2,500 and 1: 5,000 and the microliter plate was incubated at 37 °C for 1 hour before washing with 250 µL of PBST for three times. Next, 0.8 mg/mL of the secondary antibody, which is peroxidase-conjugated affinipure goat anti-mouse IgG (H + L) (Jackson ImmunoResearch, Suffolk, UK), was added at the ratio of 1: 5,000 at 37 °C for 1 hour before washing with 250 µL of PBST for three times. After that, 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Surmodics, USA) was added, and the color change was observed at 10 minutes after substrate addition. Finally, the reaction was stopped by adding 50 µL of 2 M $H_2SO_4$. The result was read at 450 nm using a microplate reader (AccuReader, Metertech, Taipei, Taiwan, ROC).

**2. Specificity and sensitivity test using indirect-ELISA technique**

Specificity of mouse anti-*S. aureus* mAb (Fitzgerald, USA) was tested using *S. aureus* AU17 and other bacteria such as *E. faecalis*, *S. agalactiae* SA35 and *E. coli* TISTR117. The sensitivity of the test was investigated using the reactions between mouse anti-*S. aureus* mAb and each bacterial strain ($10^6$-$10^9$ CFU/mL) using of the indirect-ELISA technique, following the experimental steps.

**Immunochromatographic strip design**

**1. Preparation of the colloidal gold-linked mouse anti-*S. aureus* mAb**

The pH of 40 nm colloidal gold (Kestrel Bio Sciences, Thailand) was adjusted to 4-10 using 0.2 M $K_2CO_3$ (LOBA Chemie, India) and added onto the microliter plate at 100 µL each. After that, 10 µL of 0.1 mg/mL antibody (Fitzgerald, USA) was added before incubating at room temperature for 30 minutes under an agitating condition (30 rpm). Then, 0.5 % (w/v) BSA was added to block non-specific binding of the antibody and incubated for another 1 hour. Next, 10 µL of 3 M NaCl was added and observed for color changes. The colloidal gold (100 µL) prepared at the optimum pH and 0.1-0.5 µg of mouse anti-*S. aureus* mAb were added onto the microtiter plate respectively, before the incubation at room temperature for 30 minutes under an agitating condition (30 rpm). After that, it was blocked with 0.5 % (w/v) BSA before another 1-hour incubation. Next, 3 M NaCl (RCI Labscan Limited, Thailand) was added and formation of flocculation was observed.

Mouse anti-*S. aureus* mAb (200 µL of 0.1 mg/mL) was added into a conical tube containing 20 mL colloidal gold (pH 9) before incubating it at room temperature for 30 minutes under an agitating condition (30 rpm). After that, 0.5 % (w/v) BSA was added and further incubated for 1 hour. Finally, the conical tube was centrifuged. The pellet was kept in 1,000 µL of gold storage buffer and stored at 4 °C. The result of the conjugation between the antibody and the colloidal gold was observed at the absorbance range of 400 - 600 nm using a spectrophotometer. The conjugate was electrophoresis viewed through run on 1% of agarose gel run at 100 V for 35 minutes.

**2. Coating preparation of immunochromatographic test strip**

The detection kit composes of 4 parts: nitrocellulose membrane, conjugated pad, wicking pad and sample pad. The first step of assembling was to attach the nitrocellulose membrane (CN95; GE healthcare, USA) on the backing card (6 cm). After that, 0.5 µL of affinipure goat anti-mouse IgG (H-L) and 0.5 µL of mouse anti-*S. aureus* mAb were dropped on the control line and the test line,

respectively, before incubation at 37 °C for 14 - 16 hours. The conjugate pad, which is a glass fiber (GF33; GE healthcare, USA), was sprayed with mouse anti-*S. aureus* mAb-colloidal gold conjugated, before it was incubated at 37 °C for 14-16 hours. In the last step of assembling, the wicking pad (Whatman #470; Whatman®, UK) was placed next to the control line. The conjugated pad was then attached over the test line and covered by the sample pad (Millipore Surewick™ C048; Millipore, USA). The detection kit was cut into the size of 60 x 3.8 mm (Figure 1)

**3. Efficiency test of immunochromatographic strip**

The immunochromatographic strip was tested with *S. aureus* AU17, *E. faecalis*, *S. agalactiae* SA35 and *E. coli* TISTR117 and running buffer was used as a negative control. Sensitivity was examined using cells prepared at $10^6$-$10^9$ CFU/mL in a PBS solution.

## Results and Discussions

**Species confirmation of bacterial strain**

The result of culturing of *S. aureus* AU17 on Baird-Parker egg-yolk agar showed that the colonies of *S. aureus* AU17 were black, surrounded by opaque zone. The cells, examined under the microscope, were Gram-positive cocci, arranged in grape-like clusters. The biochemical test results gave positive reaction in coagulase test and showed β-hemolysis on Columbia ANC agar 5 % Sheep blood. The culture was confirmed as *S. aureus*, having the characteristic consistent to those of *S. aureus* listed by Boerlin *et al*. (2003). Furthermore, the DNA sequencing result showed 99.92 % similarity, which was close to *S. aureus* nucleotide sequence (Accession No. NR115606.1). For other species, morphological characteristics were examined in the same way. The nucleotide sequencing results showed that

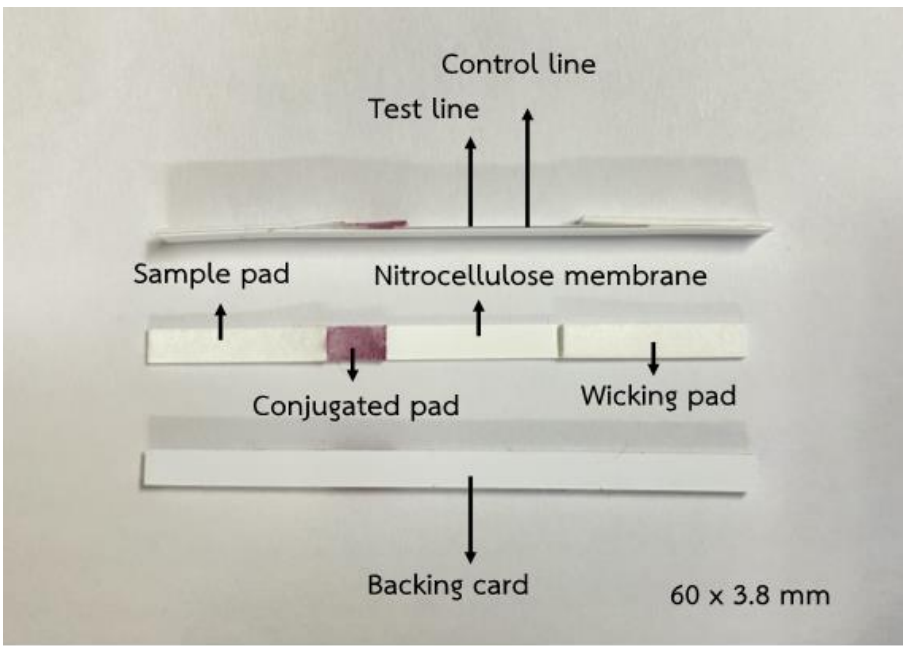


Figure 1. Components of the immunochromatographic test strip

they were *E. faecalis*, *S. agalactiae* and *E. coli*, with the similarity of 100, 99.77 and 99.25 %, respectively.

**Specificity test of monoclonal antibody by using immunological techniques**

**1. Optimization of antibody concentration**

The 1.5 mg/mL of mouse anti-*S. aureus* mAb (clone; M310128) produced by Fitzgerald, USA, was diluted into 0.5 % BSA (in 1XPBS + 0.05 % Tween-20) in the ratio of 1 : 1,000, 1 : 2,500 and 1 : 5,000. Peroxidase-conjugated affinipure goat anti-mouse IgG (H + L) (0.8 mg/mL), which was the secondary antibody, was used at 1 : 5,000. The absorbance results at 450 were 1.031, 0.942 and 0.215, respectively. The concentration of 1 : 2,500 was the optimal concentration because the antibody used was less than 1 : 1,000 and the attachment was better than 1 : 5,000 (Belkessam *et al*., 2010).

**2. Specificity and sensitivity test using indirect-ELISA technique**

The 1.5 mg/mL of mouse anti-*S. aureus* mAb prepared at the ratio of 1 : 2,500 and 0.8 mg/mL of peroxidase-conjugated affinipure goat anti-mouse IgG (H + L) prepared at the ratio of 1 : 5,000 were used in the sensitivity test with *S. aureus*, *E. faecalis*, *S. agalactiae* and *E. coli*. ($10^6$-$10^9$ CFU/mL). The highest absorbance at 450 nm was observed at a cell concentration of $10^9$ CFU/mL for the strain.

Specificity testing of mouse anti-*S. aureus* mAb subclass IgG3 against *S. aureus* AU17 showed that the binding ability was 100 % because mouse anti-*S. aureus* mAb subclass IgG3 was specific to protein A on the region of peptidoglycan on the cell surface of *S. aureus*. Furthermore, the mouse anti-*S. aureus* mAb subclass IgG3 was able to bind with *E. faecalis*, *S. agalactiae* SA35 and *E. coli* TISTR117. The non-specific binding abilities were 30, 13 and 10 %, respectively (Figure 2). Such binding occurred because they were able to loosely bind with other epitopes which were similar to protein A on cell surface (Guidry *et al*., 1991; Guo *et al*., 2018).

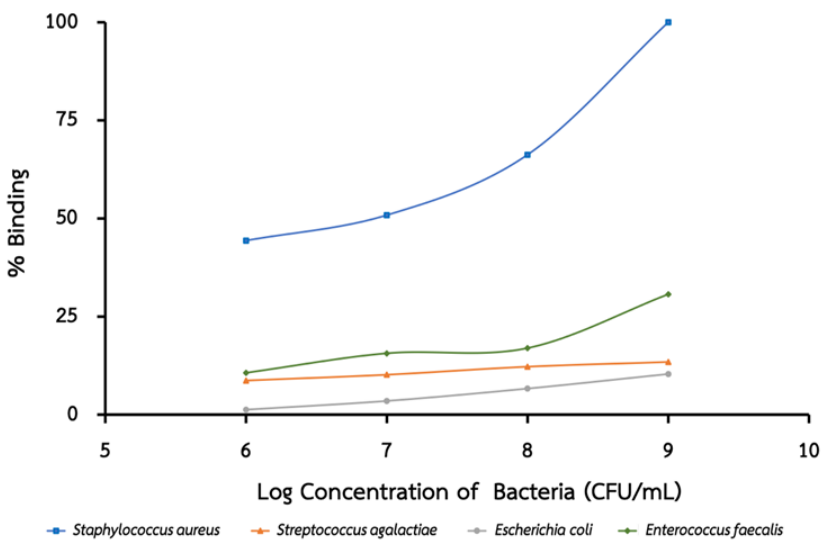


Figure 2. Percentage of binding ability between mouse anti-S. *aureus* mAb and various bacterial species

**Immunochromatographic strip design**

**1. Optimization of pH and antibody concentration**

The pH scales ranging from 4 to 9 were evaluated for conjugation of antibody and colloidal gold. The pH 4 and 5, resulted in color changing of colloidal gold from red to gray. After centrifuging at 13,000 rpm for 15 minutes, it was found that it created hard pellet, which made it not applicable. The pH 6 - 8 was close to the optimum point, resulting in faded color on the strip. The optimum pH of colloidal gold was found to be pH 9, which resulted in soft pellet after centrifugation, and showed purple-red color on the strip.

The colloidal gold prepared at pH 9 was examined with 1-5 µg/mL of mouse anti-*S. aureus* mAb. The results showed that 1 µg / mL was the optimum concentration for colloidal gold conjugation because flocculation was not found. Colloidal gold linked to mouse anti-*S. aureus* mAb was measured using a spectrophotometer in the range of 400 - 600 nm. The strongest absorbance was 532 nm, which gave the highest OD. While the wavelengths lower and higher than 532 nm yielded low OD (Figure 3). The result of this study was revealed in accordance with the previous study of Niu (2014), which showed 20 nm of colloidal gold conjugated with 1.0 mg/mL of mouse anti-*S. aureus* mAb gave the highest OD at 520 nm. Moreover, Upadhyay and Nasa (2018) found that 16 nm of colloidal gold conjugated with at least 0.015 mg/mL concentration of anti-staphylococcal enterotoxin A (SEA) mAb, gave the highest OD at 520 nm. The study of Srisrattakarn *et al.* (2020) showed that 13 nm of gold nanoparticles conjugated with 0.18 mg/mL of anti-protein A polyclonal antibody (anti-protein A pAb) yielded the highest OD at 521 nm. The result of electrophoresis showed the bands of conjugation between the mouse anti-*S. aureus* mAb and the colloidal gold (Figure 4). The formed bands were due to anionic monoclonal antibodies conjugated with gold nanoparticles moved towards the anode, whereas without monoclonal antibody conjugated with gold nanoparticles no band was found (Tomás *et al*., 2019).

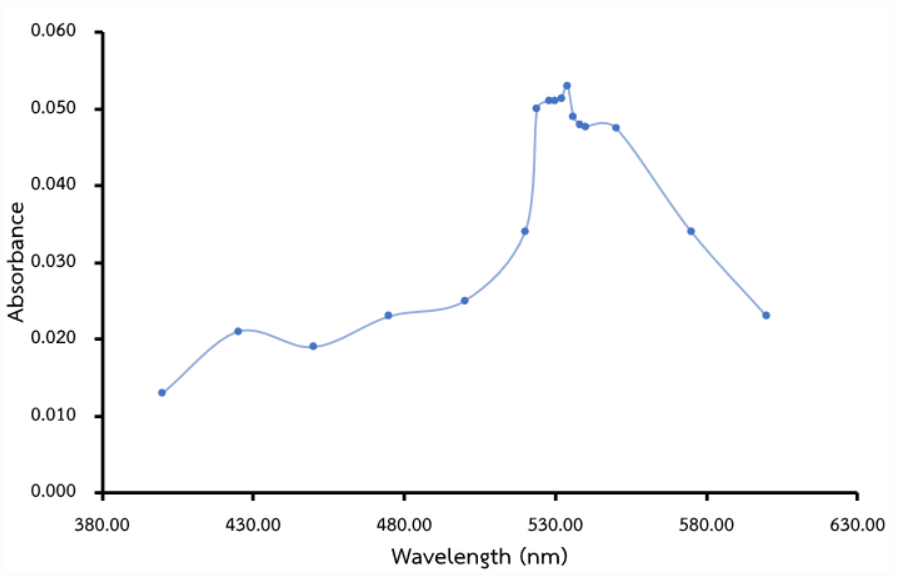


Figure 3. UV-visible spectra of colloidal gold-antibody conjugates

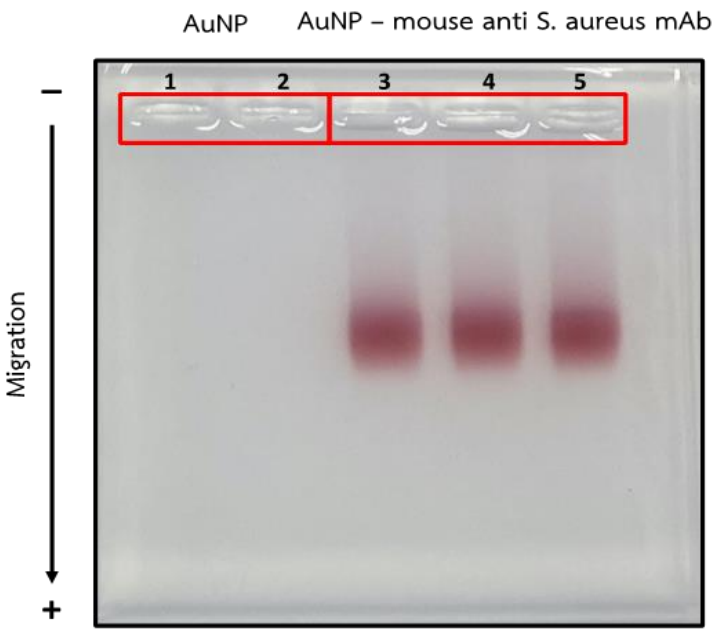


Figure 4. Detection of conjugated colloidal gold by electrophoresis (Lanes 1 and 2 were unconjugated with colloidal gold. Lanes 3 to 5 were mouse anti-S. *aureus* mAb conjugated with colloidal gold)

2. Efficiency test of immunochromatographic strip

*S. aureus* AU17 (prepared at $10^9$ CFU/mL) was suspended in running buffer and examined with the immunochromatographic strip. The positive result showed a purple-pink signal on the test line when the colloidal gold linked with mouse anti-*S. aureus* mAb was attached to *S. aureus* AU17 (colloidal gold-mAb-antigen complex). The complexes were then moved towards the test line and bound to mouse anti-*S. aureus* mAb, which was coated on the test line.

Subsequently, the signal on the control line was formed, due to the colloidal gold linked with mouse anti-*S. aureus* mAb were bound to AffiniPure Goat Anti-Mouse IgG (H + L), which was coated on the control line. In contrast, the pure running buffer without specific pathogen showed a purple-pink signal only on the control line (Figure 5).

The immunochromatographic test strip with *S. aureus* AU17 showed a positive result as the antibody specifically reacted to the target. The test with the other species such as *E. faecalis*, *S. agalactiae* SA35 and *E. coli* TISTR117 showed negative results as they did not have reaction with the *S. aureus* antibody, resulting in no signal formed on the test line, suggesting that the antibody was not specific to them. For the concentration of sensitivity of this developed immunochromatographic strip, it was found that $10^9$ CFU/mL was the lowest concentration that could be detected. The concentration lower than $10^9$ CFU/mL did not yield positive result because the antibody did not have as high binding capacity as a similar antibody in the study of Wiriyachaiporn *et al.* (2013), in which at least $10^6$ CFU/mL was detected. Another study by Srisrattakarn *et al.* (2020), in which pAb was used against *S. aureus* AU17, showed that the lowest detectable concentration was $10^7$ CFU/mL.

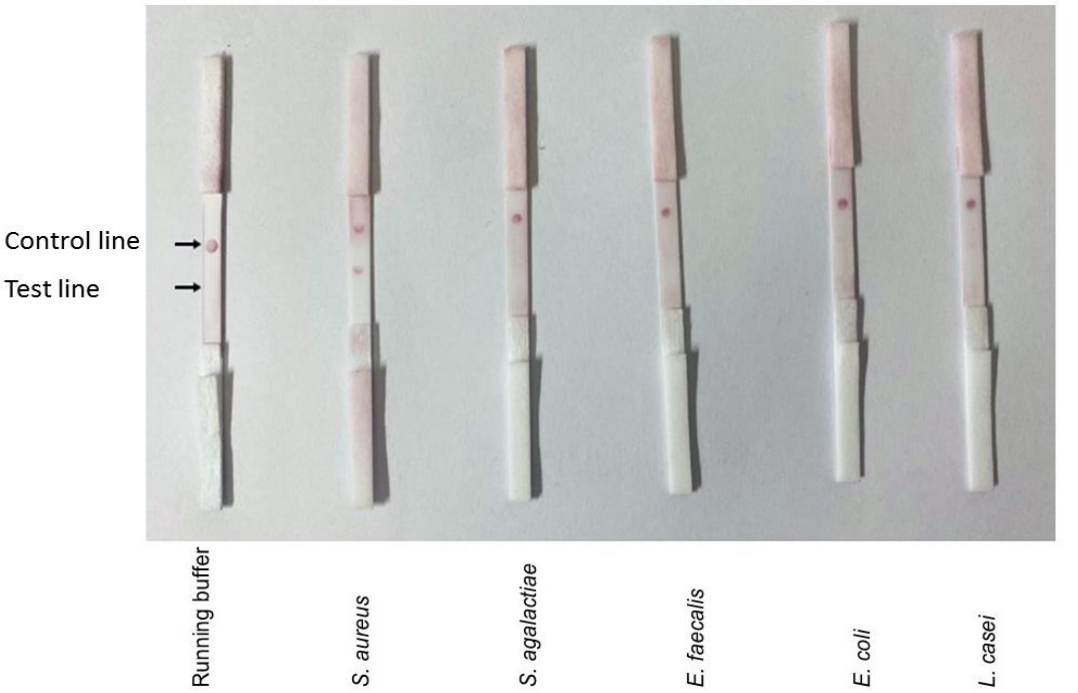


Figure 5. Specificity test of Immunochromatographic strip

## Conclusion

The immunochromatographic strip was developed to detect *S. aureus* AU17, the major cause of contagious mastitis in cows. Investigation of the mouse anti-*S. aureus* monoclonal antibodies properties, using indirect ELISA technique, showed that the monoclonal antibodies could attach to the pathogen when the concentration was at least $10^9$ CFU/mL. The antibody had 100 % specificity with *S. aureus* AU17. Furthermore, non-specific binding was not found with other bacteria (*E. faecalis*, *S. agalactiae* SA35 and *E. coli* TISTR117). This immunochromatographic strip provided an accurate detection of *S. aureus* and can be used as a prototype for further development of the test kit for field testing of mastitis pathogens.

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