การพัฒนาอิมมูโนโครมาโทกราฟีเพื่อการตรวจวัด *Salmonella* spp. ที่ไวและรวดเร็ว

นางสาวภัทรชยา ปรีชาเกษตรกิจ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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DEVELOPMENT OF IMMUNOCHROMATOGRAPHY FOR SENSITIVE AND RAPID DETECTION OF *Salmonella* spp.

Miss Pattarachaya Preechakasedkit

A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	DEVELOPMENT OF IMMUNOCHROMATOGRAPHY FOR SENSITIVE AND RAPID DETECTION OF *Salmonella* spp.
By	Miss Pattarachaya Preechakasedkit
Field of Study	Biotechnology
Advisor	Associate Professor Orawon Chailapakul, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

……Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr.rer.nat)

THESIS COMMITTEE

……Chairman
(Assistant Professor Warinthorn Chavasiri, Ph.D.)

……Thesis Advisor
(Associate Professor Orawon Chailapakul, Ph.D.)

……Examiner
(Associate Professor Nattaya Ngamrojnavanich, Ph.D.)

……Examiner
(Kittinan Komolpis, Ph.D.)

……External Examiner
(Weena Siangproh, Ph.D.)

ภัทรชยา ปรีชาเกษตรกิจ: การพัฒนาอิมมูโนโครมาโทกราฟีเพื่อการตรวจวัด *Salmonella* spp. ที่ไวและรวดเร็ว. (DEVELOPMENT OF IMMUNOCHROMATOGRAPHY FOR SENSITIVE AND RAPID DETECTION OF *Salmonella* spp.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร. อรวรรณ ชัยลภากุล, 84 หน้า.

ในงานวิจัยนี้ได้พัฒนาการตรวจวัดซัลโมเนลลาไทฟีอย่างรวดเร็วด้วยเทคนิคอิมมูโนแบบดอทบลอทร่วมกับการตรวจวิเคราะห์เชิงแสง และอิมมูโนโครมาโทกราฟี ในการตรวจวัดอาศัยปฏิกิริยาจับจำเพาะระหว่างแอนติเจนของซัลโมเนลลาไทฟี O901 และโพลีโคลนอลแรบบิทแอนติบอดีสำหรับโพลีแซคคาไรด์ของซัลโมเนลลาไทฟี O901 โดยมีโพลีโคลนอลแรบบิทแอนติบอดีที่ยึดติดกับทองอนุภาคนาโนเมตรเป็นสารติดฉลาก การตรวจวัดซัลโมเนลลาไทฟีในหน่วยมิลลิกรัมต่อมิลลิลิตรใช้เทคนิคอิมมูโนแบบดอทบลอทร่วมกับการตรวจวิเคราะห์เชิงแสง ซึ่งพบว่าขีดจำกัดของการตรวจวัดอยู่ที่ความเข้มข้น 0.14 มิลลิกรัมต่อมิลลิลิตร และช่วงความเป็นเส้นตรงระหว่างความเข้มสีและความเข้มข้นของซัลโมเนลลาไทฟีให้ค่าสัมประสิทธิ์สูงที่ 0.9986 ส่วนการตรวจวัดซัลโมเนลลาไทฟีในหน่วยจำนวนโคโลนีต่อมิลลิลิตรใช้เทคนิคอิมมูโนแบบดอทบลอทด้วยหลักการเกิดปฏิกิริยาแบบตรง และอิมมูโนโครมาโทกราฟีด้วยหลักการแบบแซนวิช ซึ่งแผ่นทดสอบแบบอิมมูโนโครมาโทกราฟีมีความกว้าง 0.5 เซนติเมตร และความยาว 8.3 เซนติเมตร โดยใช้โพลีโคลนอลแรบบิทแอนติบอดีสำหรับโพลีแซคคาไรด์ของซัลโมเนลลาไทฟี O901 และโกทแอนติแรบบิทแอนติบอดีในการสร้างจุดทดสอบและจุดควบคุมชุดทดสอบบนไนโตรเซลลูโลสเมมเบรนตามลำดับ ผลการทดสอบพบว่า ถ้าพบจุดสีแดงจำนวน 2 จุดบนแผ่นเมมเบรนจะให้ผลเป็นบวก และถ้าพบเฉพาะจุดควบคุมชุดทดสอบจะให้ผลเป็นลบ จากการวิเคราะห์พบว่าขีดจำกัดของการตรวจวัดด้วยเทคนิคอิมมูโนแบบดอทบลอท และอิมมูโนโครมาโทกราฟีอยู่ที่ 8.88×10^6 จำนวนโคโลนีต่อมิลลิลิตรภายในเวลา 1 ชั่วโมง 50 นาที และ 1.14×10^5 จำนวนโคโลนีต่อมิลลิลิตรภายในเวลา 15 นาทีตามลำดับ ซึ่งผลการทดสอบสามารถอ่านได้ด้วยตาเปล่า การเปรียบเทียบผลการตรวจวัดพบว่าอิมมูโนโครมาโทกราฟีให้ขีดจำกัดที่ต่ำกว่า และใช้เวลาน้อยกว่าเทคนิคอิมมูโนแบบดอทบลอท นอกจากนี้อิมมูโนโครมาโทกราฟีถูกนำมาประยุกต์ใช้ในการตรวจวิเคราะห์ซัลโมเนลลาไทฟีในตัวอย่างซีรัมได้อย่างมีประสิทธิภาพ ดังนั้นอิมมูโนโครมาโทกราฟีจึงเป็นเทคนิคที่มีความรวดเร็วใช้งานง่ายและราคาไม่แพงสำหรับการตรวจวัดซัลโมเนลลาไทฟี

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

5072409323 : MAJOR BIOTECHNOLOGY
KEYWORDS : *Salmonella* spp./ IMMUNOCHROMATOGRAPHY / DOT BLOT IMMUNOASSAY / OPTICAL DECTION / GOLD NANOPARTICLES

PATTARACHAYA PREECHAKASEDKIT : DEVELOPMENT OF IMMUNOCHROMATOGRAPHY FOR SENSITIVE AND RAPID DETECTION OF *Salmonella* spp.). THESIS ADVISOR : ASSOC. PROF. ORAWON CHAILAPAKUL, Ph.D., 84 pp.

In this work, the rapid detection of *Salmonella typhi* (*S. typhi*) using dot blot immunoassay coupled with optical detection, and immunochromatography was developed. *S. typhi* was detected using a specific binding reaction between antigens of *S. typhi* O901 and polyclonal rabbit antibody for polysaccharides of *S. typhi* O901. Polyclonal rabbit antibody-gold nanoparticles conjugate was used as the label. The detection of *S. typhi* (mg/mL) was performed by dot blot immunoassay coupled with optical detection. The limit of detection (LOD) was found to be 0.14 mg/mL. Linear region between mean intensity and the concentration of *S. typhi* was observed with a good coefficient of 0.9986. The detection of *S. typhi* (cfu/mL) was performed by dot blot immunoassay (direct immunoassay) and immunochromatography (sandwich immunochromatrography). The size of the test strip for sandwich immunochromatography was 0.5 cm in width and 8.3 cm in length. Polyclonal rabbit antibody for polysaccharides of *S. typhi* O901 and goat anti-rabbit IgG were applied on nitrocellulose membrane to create a test dot and a control dot respectively. Positive results presented two claret-colored dots on nitrocellulose membrane. For negative results, only one control dot appeared on the membrane. The limit of detection was 8.88×10^6 cfu/mL within 1 h and 50 min for dot blot immunoassay, and 1.14×10^5 cfu/mL within 15 min for immunochromatography, which could be visually evaluated by the naked eye. From the result, it could be concluded that immunochromatography provided lower detection limit and reaction time than dot blot immunoassay. In addition, immunochromatography was applied to detect *S. typhi* in human serum effectively. Therefore, immunochromatography is rapid, simple and low-cost method for the detection of *S. typhi*.

Field of Study : Biotechnology Student's Signature .....

Academic Year : 2010 Advisor's Signature .....

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LIST OF ABBREVIATIONS

PCR	polymerase chain reaction
ELISA	enzyme-linked immunosorbent assay
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
Ab	antibody
Ag	antigen
K	equilibrium constant
TEM	transmission electron microscopy
UV-vis	UV-visible
LPS	lipopolysaccharide
SPE	screen-printed electrode
AFB1	aflatoxin B1
BSA	bovine serum albumin
IgG	immunoglobulin G
IgM	immunoglobulin M
^{o}C	degree Celsius
cfu	colony forming unit
QDs	quantum dot
HRP	horseradish peroxidase
PBS	phosphate buffer saline
PEG	polyethylene glycol
NCM	nitrocellulose membrane
h	hour
min	minute

M	molar
mM	millimolar
g	gram
ng	nanogram
pg	picogram
mL	milliliter
µL	microliter
nm	nanometer
cm	centimeter
rpm	round per minute
LOD	limit of detection
R^2	coefficient

CHAPTER I

INTRODUCTION

1.1 Introduction

Currently, Typhoid fever is an important problem for public health in both undeveloped and developing countries. It is a life-threatening illness caused by the bacterium *Salmonella typhi* (*S. typhi*), a gram-negative rod-shaped and motile bacterium. It is found in humans only, with estimated global incidences of 22 million cases and 200,000 deaths per year [1-2]. Prior to 1988, it was estimated to be 16 million with over 600,000 deaths per year [3]. This clearly displays an increase in morbidity with a decrease in mortality. The transmission of *S. typhi* has several pathways, for example, by drinking water or eating food that is contaminated with these bacteria. From the risk of these bacteria, so the traditional methods for *S. typhi* detection are proposed such as polymerase chain reaction (PCR) [4-5], pulsed-field gel electrophoresis [6]. Though the above methods are effective for diagnosis, they are time consuming analysis, need expensive instruments and require highly qualified skill.

Immunoassay is one of the most popular alternatives for diagnosis, which is based on the specific binding reaction of the antibody to the target antigen [7-8]. A number of immunoassay methods including enzyme-linked immunosorbent assay (ELISA) [9], amperometric immunosensors [10], dot-blot ELISA [11] and metalloimmunoassay [12], have been used for the detection and diagnosis of *S. typhi*. Although these methods provide effective and accurate detection, rapid and low-cost methods have been required. Dot blot immunoassay and immunochromatography are alternative methods for *S. typhi* detection.

Dot blot immunoassay is a technique using a specific binding reaction of the antibody to its antigen on nitrocellulose membrane. The advantages of this method are low-cost, rapid and simple. Using this method, the results can be assessed with the naked eyes, but it cannot be used in quantitative analysis. Therefore, the dot blot assay coupled with optical detection was proposed for increasing capability. Optical

detection is a colorimetric assay using a scanner and a digital camera for image capturing. The image can be transmitted electronically and digitally to an off-site laboratory, which the data can be analyzed by a specialist, and the results of the analysis returned (ideally in real-time) to the person administering the test [13].

Immunochromatography combines chromatography with immunoassay which is a new diagnostic technique. For immunochromatography, the reaction between antibody and antigen occurs on nitrocellulose membrane using capillary flow. The advantages of this method include their user-friendly format, rapid operation and immediate results, long-term stability, easy to use and a relatively low cost [14-15].

The most popular labels used in immunoassay include enzyme labels such as alkaline phosphatase [16-18], horseradish peroxidase [19-21], and metal labels such as gold nanoparticles [22-25]. The use of an enzyme label requires an additional step for the detection itself using an unstable substrate. Therefore, gold nanoparticles are chosen as the label in this work. Gold nanoparticles have been used in different immunoassay techniques over the past several years, because of their easily controllable size distribution, long-term stability, and friendly biocompatibility with antibodies, antigen, proteins, DNA, and RNA [26]. Gold particles of any exactly defined size can be manufactured reproducibly under the appropriate manufacturing conditions. Different sizes of gold particles can be used for different applications. Gold is essentially inert and forms almost perfectly spherical particles when suitably manufactured. Proteins can bind to the surfaces of gold particles with enormous strength when correctly coupled. Thus, the complex of protein and gold particles provides a high degree of long-term stability.

In this work, dot blot immunoassay and immunochromatography were developed for the rapid detection of *S. typhi*. Gold nanoparticles were used as a label during immunoreactions events, and polyclonal rabbit antibody to *S.typhi* O901 bound with gold nanoparticles to form antibody-gold nanoparticles conjugate. Dot blot immunoassay coupled with optical detection was performed by the principle of direct immunoassay and AdobePhotoshop was used to investigate the mean intensity of each dot. Immunochromatography was performed using the principle of sandwich immunoassay.

1.2 Objective of the research

The two main goals of this work are as follows:

1. To develop dot blot immunoassay coupled with optical detection for the low-cost determination of *S. typhi*.
2. To develop immunochromatography with rapidity and low cost for the detection of *S. typhi* in human serum.

1.3 Scope of the research

To achieve the research objective, the following scope was set:

(i) Research and study the information of dot blot immunoassay, immunochromatography, gold nanoparticles, *S. typhi* and optical detection.

(ii) Prepare and characterize gold nanoparticles by UV-visible spectrophotometer and transmission electron microscopy.

(iii) Study the optimal concentration of antibody-gold nanoparticles for *S.typhi* detection by UV-visible spectrophotometer.

(iv) Prepare and optimize dot blot immunoassay based on optical detection for *S. typhi* detection.

(v) Prepare and optimize immunochromatography using IgG from murine serum as a model of the test strip.

(vi) Prepare and optimize sandwich immnochromatography for *S. typhi* detection.

(vii) Finally, all the results are discussed.

CHAPTER II

THEORY AND LITERATURE SURVEY

2.1 Immunoassay [27-28]

Immunoassay is a biochemical test that measures the concentration of substance in the solutions. Serum and urine are the analytes that are most frequently assayed using immunoassay. Immunoassay as an analytical technique is based on the binding reaction between the antibody (Ab) and the target antigen (Ag). The antibody combines specifically with the corresponding antigen that is similar to the binding of an enzyme to its substrate and involves hydrophobic and electrostatic interaction. The stability of the bonding between the antigen and the antibody depends on the complementary shape of the antigen and the binding site of the antibody. Because of the relative weakness of the forces that hold the antibody and the antigen together, these combinations are reversible and the complex will dissociate, dependent upon the strength of the binding:

$$Ag + Ab \rightleftharpoons AgAb$$

Where the binding is strong, the equilibrium will lie to the right. The equilibrium will lie to the left when the binding is weak. The strength of the binding of the antibody to the antigen is referred to as its affinity and is defined by the equilibrium constant *K*:

$$K = [AgAb]/[Ag][Ab] \qquad \text{(Equation 2.1)}$$

Two general systems of immunoassay can be basically differentiated. The competitive immunoassay is based on the competition between labeled and unlabeled antigens for the antigen binding sites. The second is the sandwich immunoassay that is also very popular.

2.1.1 Competitive immunoassay

The competitive immunoassay is based on the competition of two antigen populations for the same free binding sites of antibody population in a defined volume of solution. One of the two antigen populations consists of antigens with labeled, while the other is similar antigens without labeled. Therefore, the competitive immunoassay requires labeled antigens, unlabeled antigens, antibody or antiserum that specifically binds to its antigen (Figure 2.1).

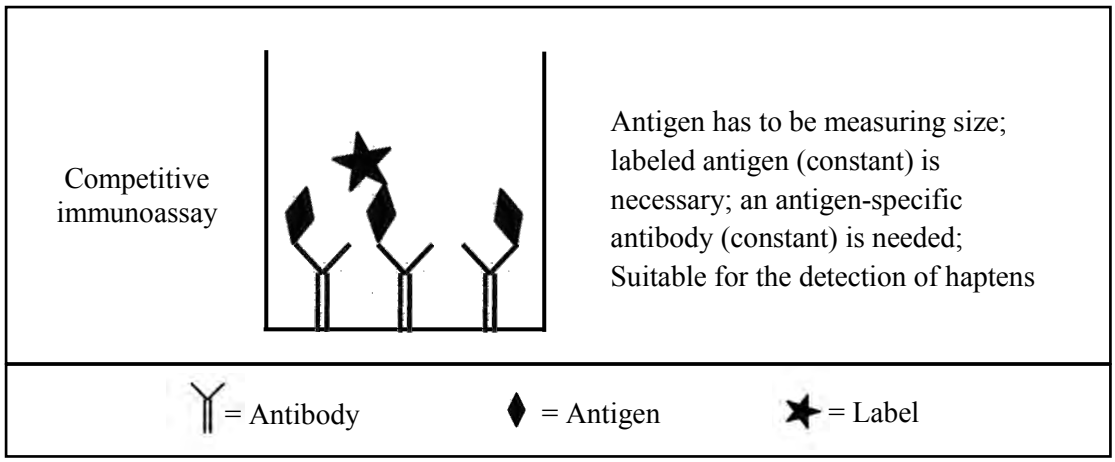

Figure 2.1 Schematic of competitive immunoassay [27]

The quantity of unlabeled antigens in the solution can be determined when the concentration of the antibody, the concentration of labeled antigen and the fluid volumes in all starting solutions are steady. Briefly, antibody is coated onto the surface. The antibody is then incubated with the unlabeled antigen. After the reaction is allowed to reach equilibrium, the labeled antigen is added. The labeled antigen binds with the antibody wherever its binding sites are not already occupied by unlabeled antigen. Therefore, a high concentration of unlabeled antigens in the sample means a weak label signal in the bound fraction.

Figure 2.2 shows a typical standard curve of a competitive immunoassay. The concentration of unlabeled antigen increases, the label signal in the bound fractions decreases.

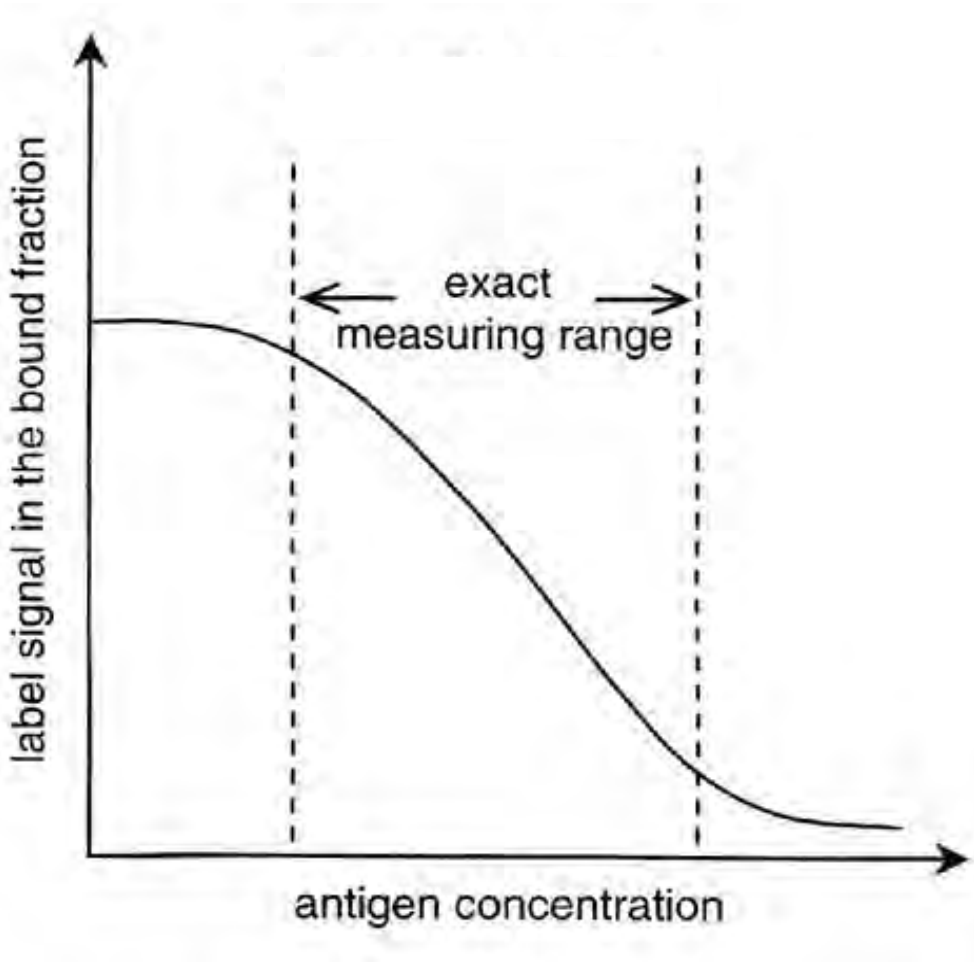

Figure 2.2 Typical standard curve of competitive immunoassay [27]

2.1.2 Sandwich immunoassay

A sandwich immunoassay measures the amount of antigens between two layers of antibodies. The antigen must contain at least two antigenic sites, capable of binding to the antibody, because at least two antibodies act in the sandwich (figure 2.3). For this reason, sandwich immunoassays are restricted to the quantitative of multivalent antigens such as proteins or polysaccharides. Sandwich immunoassays for quantitative of antigens are especially valuable when the concentration of antigens is low and/or they are contained in high concentrations of contaminating protein.

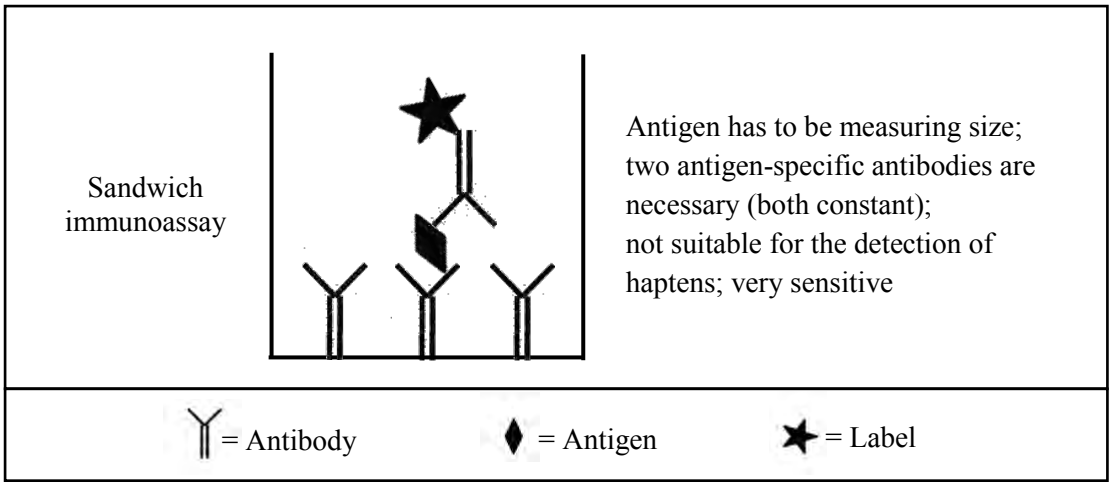

Figure 2.3 Schematic of sandwich immunoassay [27]

For sandwich immunoassay, one antibody is coated on the surface of material such as polystyrene plate or nitrocellulose membrane. Antigen is then added and allowed to capture with antibody. Unbound substances can be removed by washing solution. In contrast to the competitive immunoassay, the antibody does not work in a limiting manner because it is applied in excess. The labeled antibody is then allowed to bind with the antigen to complete sandwich immunoassay. A typical standard curve of sandwich immunoassay is shown in figure 2.4. However, in contrast to the competitive immunoassay, a weak label signal is received with a low antigen concentration and a strong signal is received with a high antigen concentration with the sandwich immunoassay.

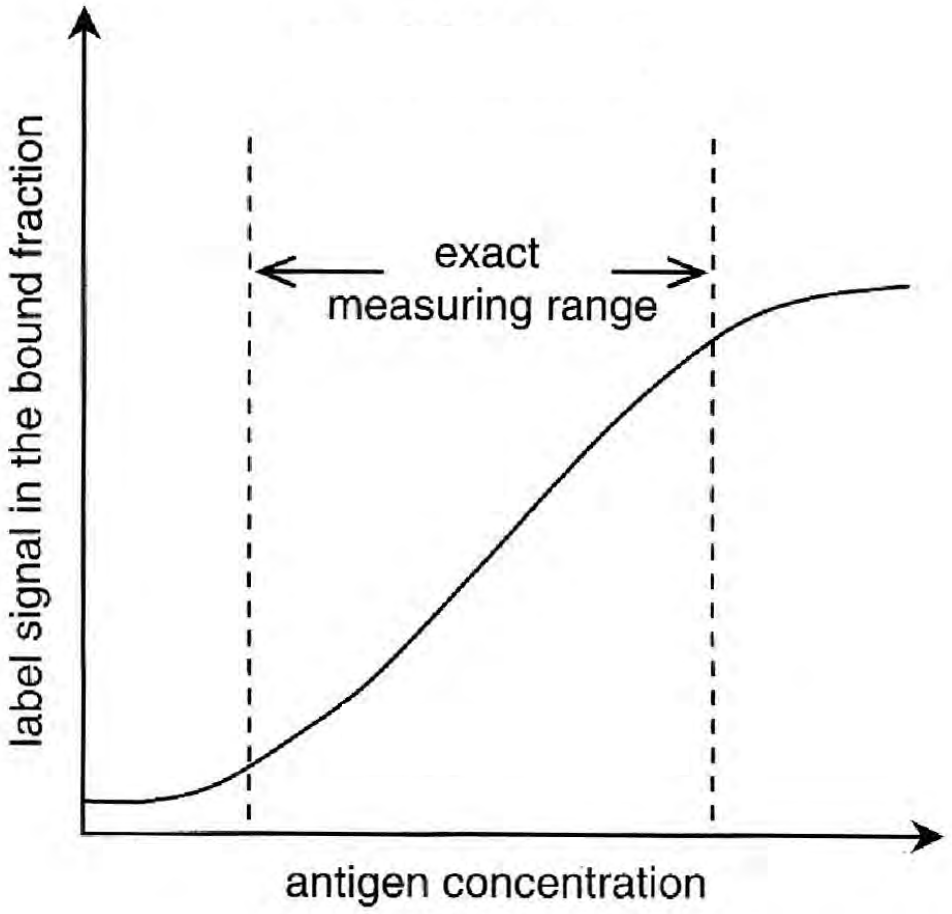

Figure 2.4 Typical standard cure of sandwich immunoassay [27]

2.2 Dot blot immunoassay [28-30]

Dot blot immunoassay is one technique of immunoassay. Antibodies may also be used to determine the presence or identity of soluble antigens by a process known generally as dot blot immunoassay. In this technique, samples (0.5-5 μL) are applied on the nitrocellulose membrane in the form of dots and allowed to dry. Membrane is then blocked, incubated with antibody and developed by one of the detection systems. The types of immunoassay for dot blot can be divided into direct immunoassay and indirect immunoassay.

2.2.1 Direct immunoassay

The direct immunoassay uses the method of directly labeling the antibody itself. The target antigen is coated on the surface and the labeled antibody is then allowed to bind with the antigen (Figure 2.5). Direct immunoassay is relatively quick and avoids potential problems of cross-reactivity of the secondary antibody with components in the antigen sample.

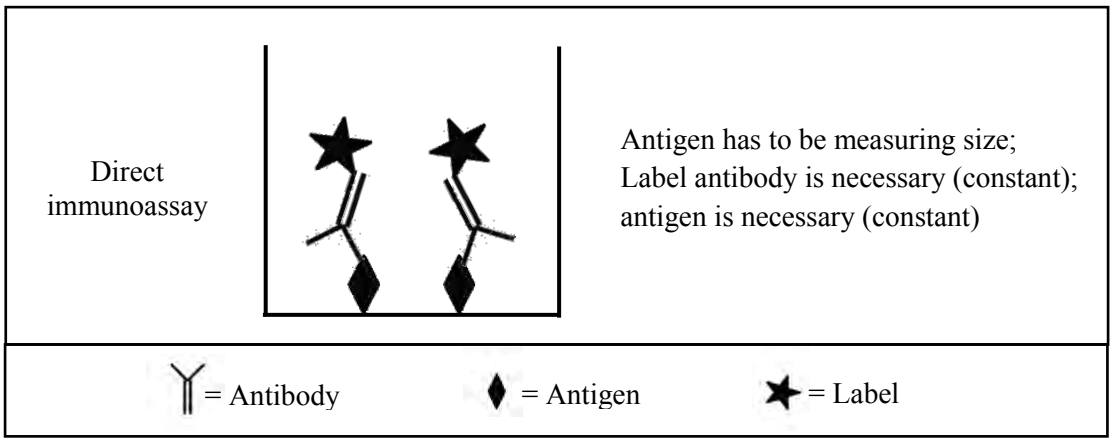

Figure 2.5 Schematic of direct immunoassay [27]

2.2.2 Indirect immunoassay

The indirect immunoassay uses two labeled secondary antibodies for detecting. First, the primary antibody is incubated with antigen. This is followed by adding the labeled secondary antibody that recognizes the primary antibody (Figure 2.6). The indirect immunoassay is high sensitivity because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody and can use commercially labeled secondary antibody that has a widely variety. The limitation of indirect immunoassay is time-consuming because an extra incubation step is required in the procedure.

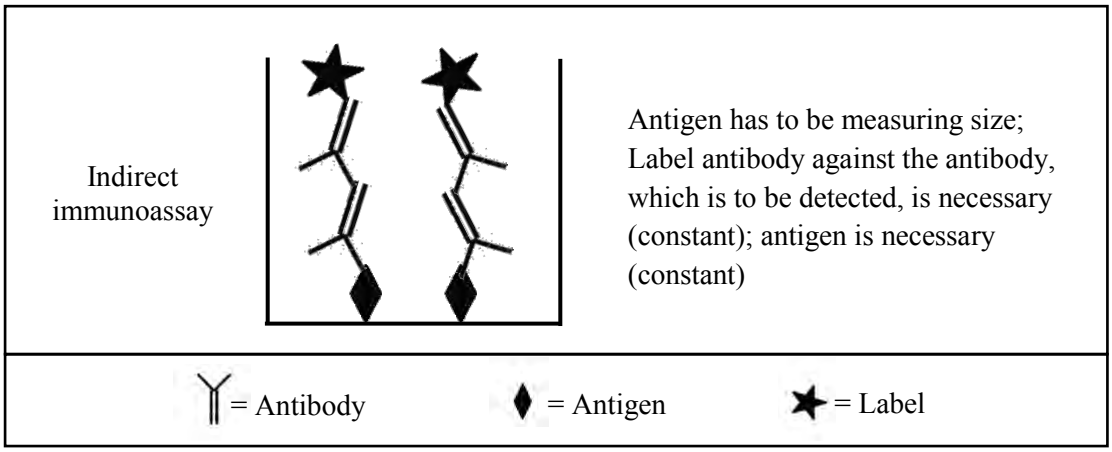

Figure 2.6 Schematic of indirect immunoassay [27]

2.3 Immunochromatography [31-32]

Immunochromatography, which is also called lateral flow immunoassay, combines immunoassay and chromatography to offer a new analytical tool for protein and clinical diagnosis [14]. It has been widely used as an in-field and point-of-care diagnosis tool to detect and identify infectious diseases, hormones and allergens [33-35]. The configuration of the test strip using immunochromatography is shown in Figure 2.7. The test strip is made up of four components including a sample pad, a conjugate pad, an absorbent pad and an analytical membrane that contains a test line and a control line.

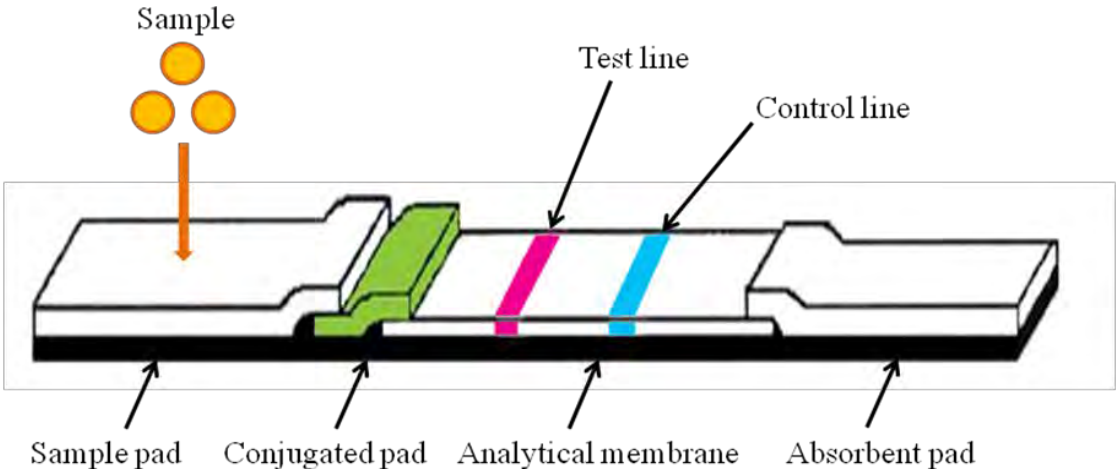

Figure 2.7 Configuration of immunochromatography

The sample pad is placed at the beginning of the test strip. Their major test is to absorb the sample and provide a uniform flow of the sample fluid from the sample pad via the conjugate pad onto the analytical membrane. Moreover, the sample pad acts as a filtration device by removing unwanted particles. Most sample pads are made from paper filter and glass fiber.

The conjugate pad performs multiple tasks. It is the most important being the delivery of the labeled antibody that is deposited into the pad material typically in conjugation with some combination of blocking reagents and/or surfactants. When the sample flows from the sample pad into the conjugate pas, the labeled antibody should be released consistently, quickly and then float with sample. Materials that are commonly used to make conjugate pads include glass fiber, cellulose and propylene.

The analytical membrane is the most important part of the test strip. Several different strip test membranes are available, varying in capillary flow rate, thickness and surface quality. The parameters that effect capillary flow rate are membrane's pore size, pore size distribution and porosity. Nitrocellulose is probably the most commonly used for strip test membrane because it can bind protein electrostatically. The test line is the part that shows the results of the assay. The control line is the antibody that captures with the labeled antibody in order to confirm the operation of the test strip correctly.

The absorbent pad is placed at the end of the test strip to act as a pump or reservoir that controls the reagent's or sample's flow and encourages complete movement of the sample through the membrane. Most absorbent pads are made from cellulose.

When describing the principle of the strip test, there are two principles for immunochromatrohraphy including the sandwich immunochromatography to detect high-molecular-mass components, usually proteins, and the competitive immunochromatography to detect low-molecular-mass analytes such as drug residues, antibiotic and hormone.

2.3.1 Sandwich immunochromatography

In sandwich immunochromatography for detecting high-molecular-mass components such as proteins (Figure 2.8), the labeled is coated with specific antibodies to the protein of interest. The sample is applied to the sample pad (Figure 2.8A) and then flow through the sample pad to the conjugate pad. When the particular protein presents in the sample, it will react with the labeled antibody in the conjugate pad (Figure 2.8B). The formed protein-labeled antibody complexes are mobile and are able to move freely to the analytical membrane. At the test line, the complexes will be captured by immobilized antibody specific to protein (Figure 2.8C). Therefore, the presence of the protein of interest in the sample will result in a colored test line. The color intensity of the test line is proportional to the concentration of the analyte in the sample. When the concentration of the interested protein is lower than the lowest detection concentration or when the interested protein is absent, no test line will be visible (Figure 2.8E). At the control line, the complexes and the labeled will be captured by the immobilized unspecific antibody that can bind with the antibody specific to protein (Figure 2.8D). This principle is widely used for detecting virus and bacteria [36-38], and in pregnancy tests [39].

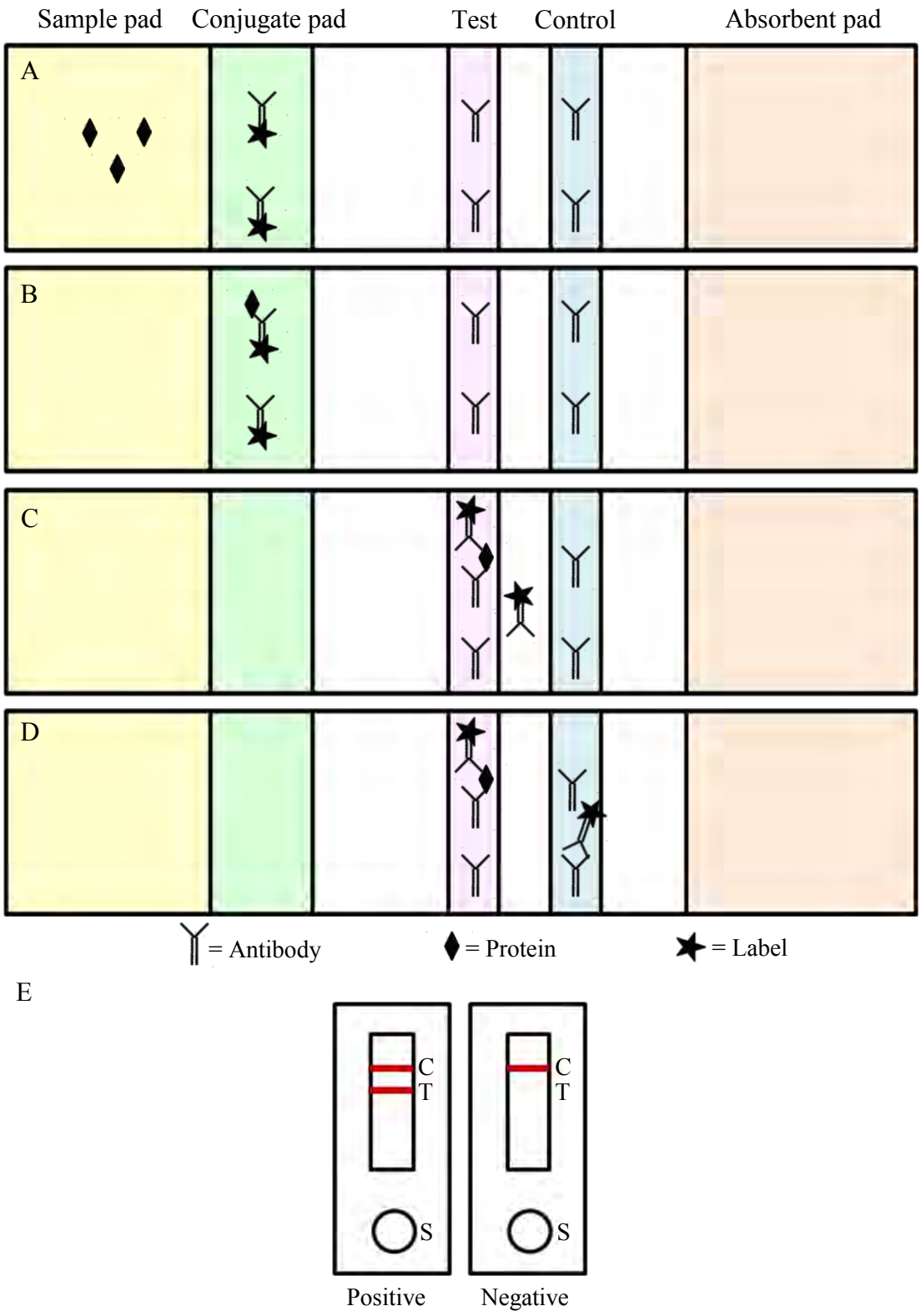

Figure 2.8 The principle of sandwich immunochromatography; (A) Sample is applied, (B) Sample binds with labeled antibody, (C) the complex is captured by specific antibody, (D) label or labeled antibody is captured by an unspecific antibody, and (E) Positive and negative results of the test strips

2.3.2 Competitive immunochromatography

In competitive immunochromatography for detecting low-molecular-mass components (Figure 2.9), labeled is coated with specific antibodies to the analyte of interest. The test line consists of the same analyte as used in the sample. The sample is applied in sample pad (Figure 2.9A) and then captures with the labeled antibody in the conjugate pad (figure 2.9 B). The formed analyte-labeled antibody complexes are mobile and are able to move freely to the analytical membrane. At the test line, the complexes will not be captured by the immobilized analyte (Figure 2.9C). Therefore, the absence of the analyte in the sample will result in a colored test line because labeled antibody will be captured by the immobilized analyte, whereas an increase in the amount of analyte will result in a decrease of signal in the test zone. At a certain concentration of analyte in the sample, the test line will be no longer visible (Figure 2.9E). The lower detection limit is defined as the amount of analyte in the sample that just causes total invisibility of the test line. At the control line, the complexes and the labeled will be captured by the immobilized unspecific antibody that can bind with the antibody specific to the analyte (Figure 2.9D). This principle is widely used for detecting drugs [22], hormones [34], toxins [40], and pesticides [41].

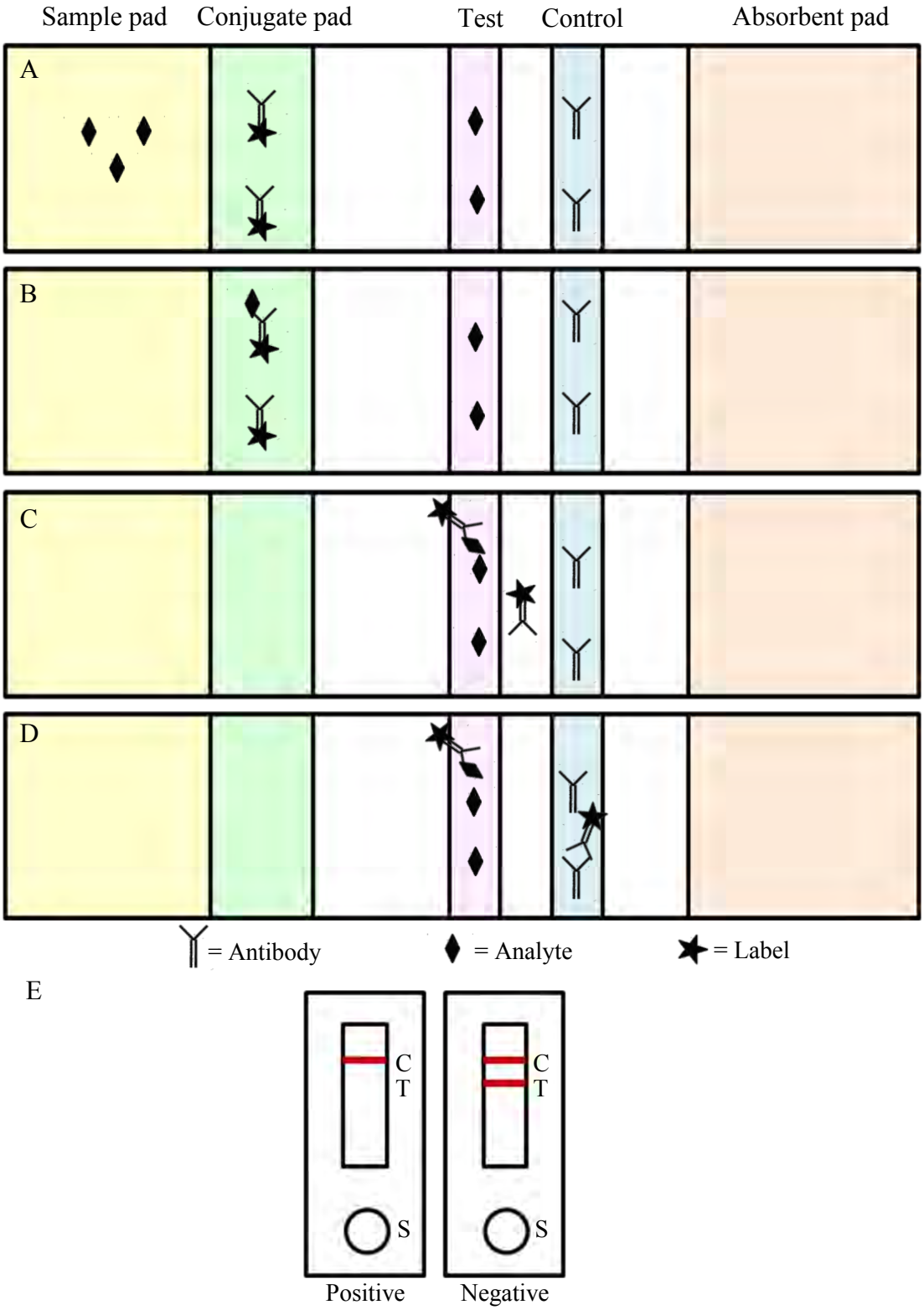

Figure 2.9 The principle of competitive immunochromatography; (A) Sample is applied, (B) Sample binds with labeled antibody, (C) the complex is not captured by analyte at test line, (D) label or labeled antibody is captured by an unspecific antibody, and (E) Positive and negative results of test strips

2.3.3 Problem and troubleshooting

In developing a test strip, reagents, materials, and techniques are brought together in order to create a sensitive, specific, rapid, inexpensive, and easy to perform assay for the detection. Moreover, the test should be applicable for a wide variety of sample matrices such as whole blood, plasma, serum, urine, and others. All these aspects in developing a test strip rely on one another for their efficacy. When one of these items is often changed, it can lead to completely unexpected or undesired results. The possible that causes of a poorly performing test strip are numerous. The most common difficulties encountered in creating a test strip are listed in Table 2.1 [31], which consists of the troubleshooting list.

Table 2.1 The most common difficulties encountered in creating a test strip and the possible solutions.

Problem	**Possible solutions**
Nonspecific binding	Evaluate absorption Add antibody Add surfactants Antibody fragmentation Change pH Add buffers
General nonspecific reactions	Add a blocking reagent Change the blocking reagent Add or change surfactant Smaller particles production Change pH Change buffer Decrease reagent/strength Change conjugation procedure
Lack of sensitivity	Increase quantity capture material Increase quantity of conjugate Increase strength of the conjugate Increase particle size

	Decrease or change blocking reagent Decrease flow rate Decrease rate of conjugate release Use higher affinity antibody/strong antigen Repurify reagents to up activity Add activity enhancer Check for analyte absorption Increase capacity of the absorbent pad
Lack of specificity	Repurify the reagents Try new reagents Check pH of sample
Migration problems	Try smaller particles Try other type of particles Change production protocols Change or increase blocking reagents Add a surfactant Block membrane directly Look for leeching of reagents Check the viscosity of the sample
Stability issues	Change the conjugate pad material Change preparation of conjugate pad Look for moisture problems Try new membrane materials Add preservatives to membrane block Add preservatives to capture protein Look for leeching effects Look for degradation of materials on sample pad
No control line visible	Repeat with new device and ass fewer drops Check the control capture reagent Check the labeled antibody Check pH of sample
Faint lines	Check dispensing procedure Check the capture reagent

	Check the labeled antibody Check membrane blocking, drying and washing Check pH of the sample
No sample front visible	Add more sample Check the sample pad
Labeled antibody are not completely released from the conjugate pad	Change conjugate pad material Increase sample volume Add or increase sugar concentration on conjugate pad

2.4 Label for immunoassay [27-28]

Immunoassays require a pure sample of either antibody or antigen for labeling with an appropriate molecule. The most popular labels used in immunoassay include enzyme labels such as alkaline phosphatase [16-18], horseradish peroxidase [19-21] and metal labels such as gold nanoparticles [22-25].

2.4.1 Enzyme label

Enzymes are often used for the detection of antigen-antibody interactions. Detection and quantification of the bindings in these cases result indirectly from enzyme activity, which can be easily determined by means of the conversion of colored or fluorescent substrates. In order to be suitable as a label, an enzyme should possess the following characteristics. It should be cleaned, available in large quantities, high specific activity for verifiable substrates, conjugational as easily as possible and without loss of activity, and its antibody-enzyme conjugates should be able to be stored without loss of activity for as long a time as possible. Enzymes are frequently used in immunological procedures such as alkaline phosphatase from calf intestine and horseradish peroxidase. The use of enzyme label requires substrate for detecting itself. Substrates such as phenyl phosphate, 1-naphthyl phosphate, 3-indoxyl phosphate and 2-phospho-phenyl phosphate have been employed [42].

2.4.2 Metal label

Metal label that is mostly used in immunoassay includes gold nanoparticles. Gold nanoparticles can be prepared by both chemical and physical methods. Normally, gold derivatives such as chloroauric acid are reduced and controlled to grow particles with a nanometer scale in the chemical method. The Turkevich-Frens method [43] is the most representative and popularly used procedure to synthesize the gold nanoparticles with sizes between 10 and 60 nm in diameter by adjusting the ratio of reducing agents (trisodium citrate) and gold (III) derivatives in boiling water. This method is often used even now because the loose shell of citrates on the particle surfaces is easily replaced by other desired ligands with valuable function. The example of gold nanoparticles at the different particle sizes from transmission electron microscopy (TEM) is shown in figure 2.10 [24, 44].

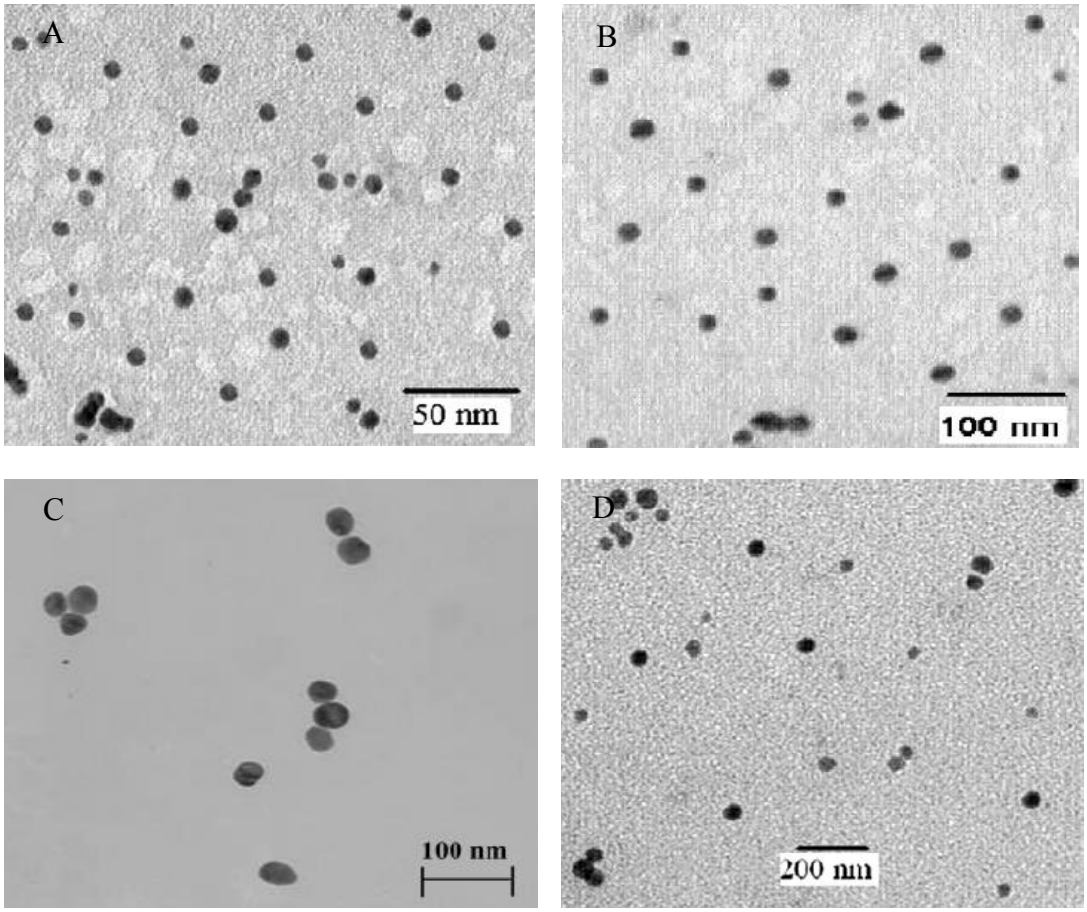

Figure 2.10 Particle sizes of gold nanoparticles; (A) 10 nm, (B) 20 nm, (C) 40 nm, and (D) 60 nm

The advantages of gold nanoparticles as label are easily controllable size distribution, long-term stability, and friendly biocompatibility with antibodies, antigen, proteins, DNA, and RNA [26].

For further developing gold nanoparticles based assays, attaching the molecular recognition motifs (functional groups) of interest to the gold nanoparticles has to be readily achieved and most importantly. The functional gold nanoparticles (gold nanoparticle probes) should not cross-talk with each other or with anything else present in the system under investigation. To improve the selectivity and accuracy, various stabilizer/ligands and modified methods have been developed to enhance the stability and dispersity of gold nanoparticles because keeping monodispersity of gold nanoparticles in the reaction medium is the key issue in practical applications. Electrostatic interaction, covalent coupling (Au-S covalent, etc.), and specific recognition (antibody-antigen, biotin-avidin, DNA hybridization, etc.) are three kinds of widely used (Figure 2.11) [45].

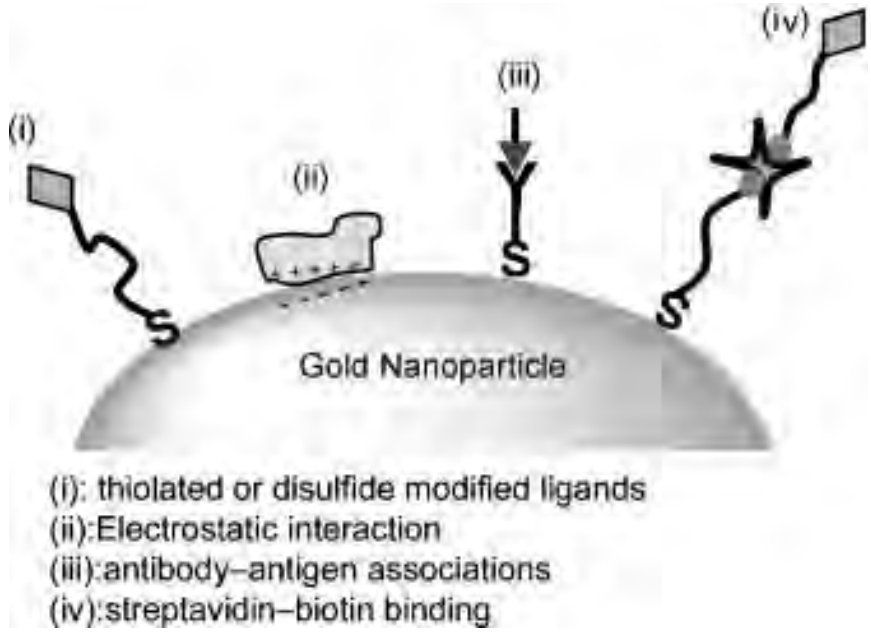

Figure 2.11 Schematic representation of formation of gold nanoparticle probes

The application of gold nanoparticles has several pathways including heavy metallic cations determination, small molecules detection, DNA detection, protein analysis, cellular analysis, drug delivery, analytical label and others (figure 2.12) [43, 46-47].

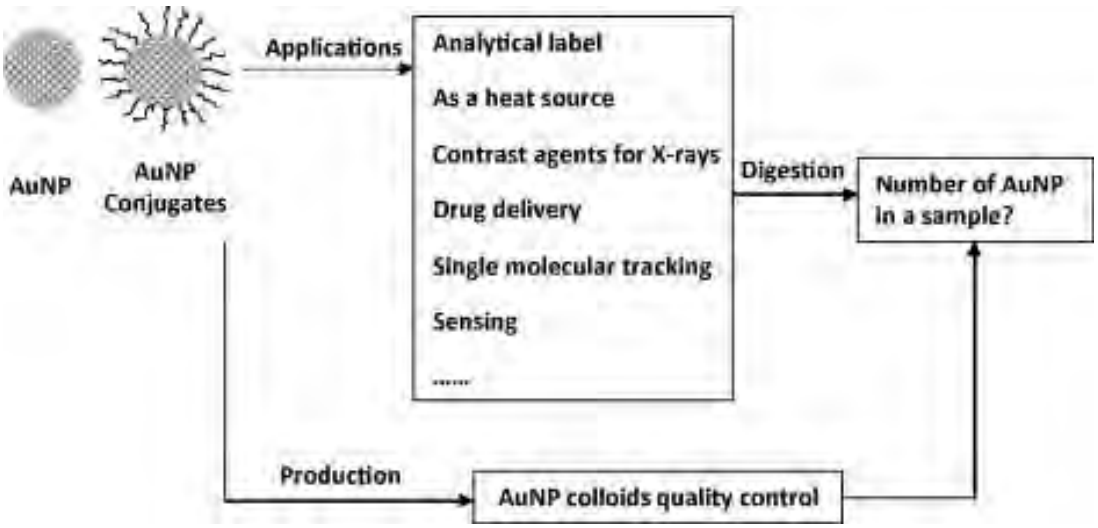

Figure 2.12 Application of gold nanoparticles

2.5 Optical detection [13]

Optical detection is a colorimetric assay using a scanner and a digital camera for image capturing. The image can be transmitted electronically and digitally to an off-site laboratory, which the data can be analyzed by a specialist, and the results of the analysis returned (ideally in real-time) to the person administering the test. The general strategy for performing inexpensive bioassays in remote locations and for exchanging the results of the tests with offsite technicians is shown in Figure 2.13.

Figure 2.13 General strategy for performing inexpensive bioassays in remote locations and for exchanging the results of the tests with offsite technicians [13]

The quantitative analysis using optical detection can be performed according to the procedure in Figure 2.14. Firstly, the colorimetric results are captured by camera or scanner. The pictures are then converted into grayscale or CMYK color for increasing precision in the analysis and the test zone is selected. Finally, the mean intensity is recorded. This procedure for processing the data can be automated using general software. The advantages of this method are easy use, rapidity, inexpensive instrument and off-site laboratory.

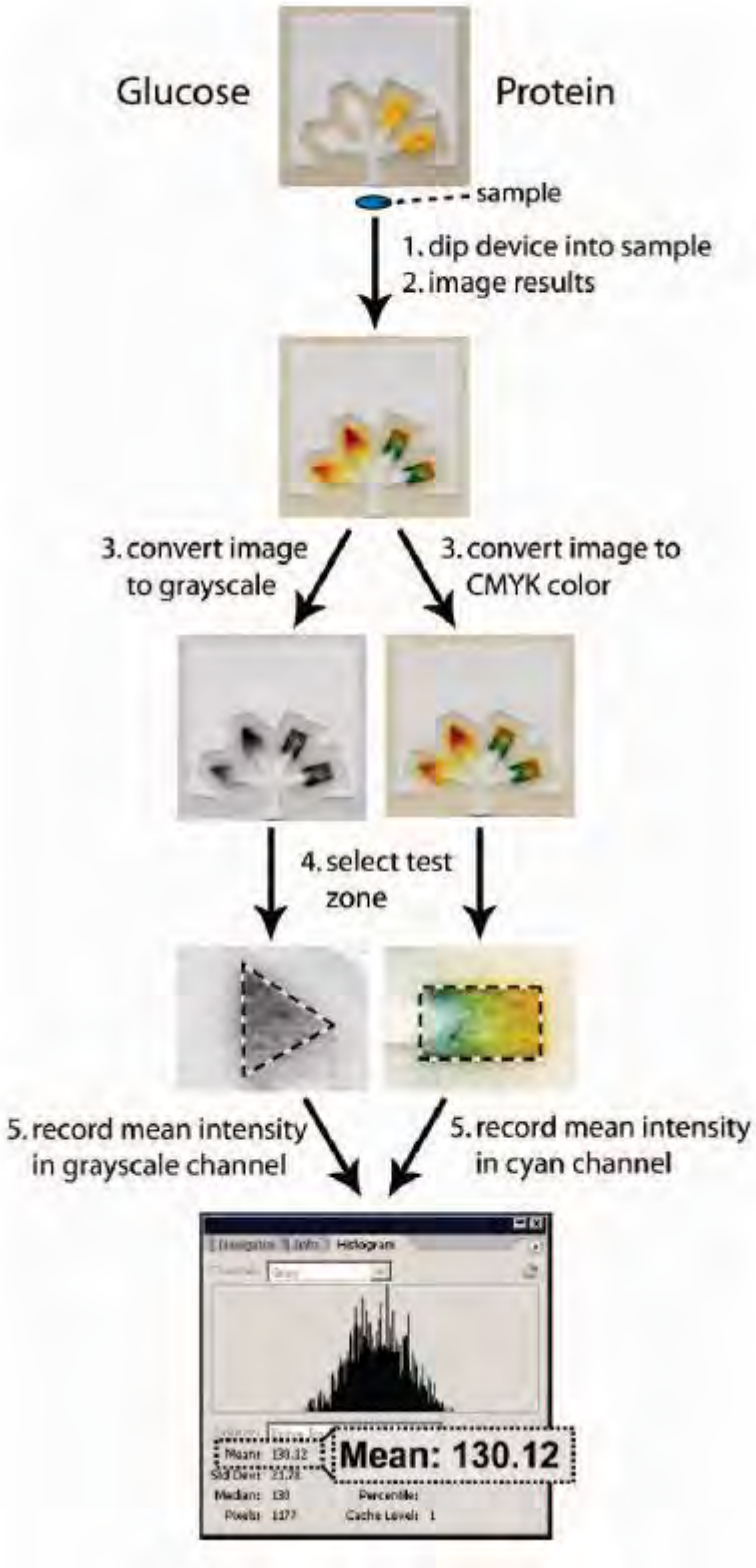

Figure 2.14 Procedure for quantifying using image editing software (Adobe Photoshop) [13]

2.6 *Salmonella* spp. [48-50]

Salmonella spp. is a member of the Enterobacteriaceae. It is gram-negative, rod shape and non-spore bacteria which is anaerobic and generally motile with peritrichous flagella (Figure 2.15).

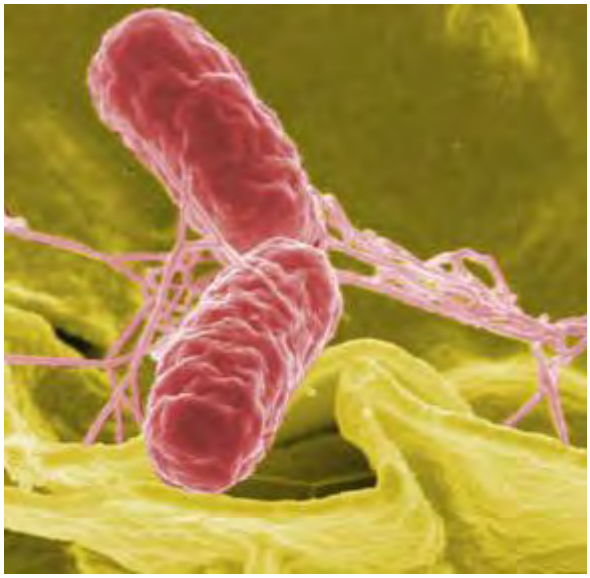

Figure 2.15 Characteristics of *Salmonella* spp.

Growth of *Salmonella* spp. has been recorded from temperatures above 5 ^{o}C up to 47 ^{o}C with an optimum at 37 ^{o}C. The pH for optimal growth is around neutrality, with values between 4.0 and 9.0. The species of *Salmonella* spp. include *S. paratyphi* A, B and C, *S. schottmueller*, *S. typhimurium*, *S. hirschfeldii*, *S. choleraesuis*, *S. oranienburg*, *S. montevideo*, *S. Newport*, *S. typhi*, *S. enteritidis*, *S. gallinarum*, *S. anatum* and others.

All species and strains of *Salmonella* may be considered to be pathogenic for man, and the disease syndromes divide themselves into several distinct clinical types. Typhoid fever, cause by *S. typhi*, is the most severe of all diseases cause by this genus and a classic example of enteric fever. Enteric fever includes typhoid fever and paratyphoid fever. The paratyphoid fevers are caused by *S. paratyphi* A, *S. paratyphi* B, and others. The paratyphoid syndrome tends to be milder than typhoid. In the latter, the period of incubation is longer and a higher body temperature is produced. Thus, the organisms may be isolated from the blood and urine, and the mortality rate

is higher. Blood cultures are often positive in the paratyphoid syndrome. The etiologic agents of the typhoid and paratyphoid syndromes are specifically pathogenic for man.

The third disease entity caused by salmonellae is gastroenteritis or salmonellosis. This syndrome differs from the enteric fevers in having an incubation period as short as 8 h, generally negative blood cultures, and a lack of host specificity among the numerous species capable of causing this disease. While some of the gastroenteritis or food-poisoning strains can be identified on the basis of biochemical and cultural characteristics, by far the largest numbers are identified on the basis of antigenic analysis.

From above, the typhoid fever caused by *S. typhi* is focused because it is the most severe of all diseases caused by salmonellae. Typhoid fever continues to be a significant global problem. There are estimated global incidences of 22 million cases and 200,000 deaths per year [1-2]. Prior to 1988, it was estimated to be 16 million with over 600,000 deaths per year [3]. This clearly displays an increase in morbidity with a decrease in mortality. The majority of those affected are 3-19 years of age, with case fatality rates ranging from 0-5%. The transmission of these bacteria has several pathways such as oral transmission via food or beverages handled by an individual who chronically sheds the bacteria through stool or urine, hand-to-mouth transmission after using a contaminated toilet and neglecting hand hygiene, and oral transmission via sewage-contaminated water or shellfish (especially in the developing world) [51].

The symptoms of typhoid fever have two phases. For the first phase, the patient's temperature rises gradually to 40 ºC, and the general condition becomes very poor with bouts of sweating, no appetite, coughing and headache. Skin symptoms and constipation may be the clearest symptoms. Children often retch and have diarrhea. The first phase lasts a week and towards the end the patient presents increasing listlessness and clouding of consciousness.

For the second phase, symptoms of intestinal infection are manifested and the fever remains very high and the pulse becomes weak and rapid in the second to third weeks of the disease. In the third week, the constipation is replaced by severe pea-

soup-like diarrhea and the faeces may also contain blood. It's not until the fourth or fifth week that the fever drops and the general condition slowly improves [52].

Typhoid fever can be prevented by scrupulous personal, food and water hygiene and vaccination. A single dose of Vi polysaccharide antigen vaccine can protect typhoid fever for 3 years.

2.7 Literature surveys

2.7.1 *Salmonella typhi*

Chaicumpa *et al.* (1995) reported the detection of *S. typhi* using monoclonal antibody-based dot-blot ELISA [11]. The assay was performed on nitrocellulose membrane. The *S.typhi* lipopolysaccharides (LPS) were used which the LPS was varied from 0.12-1,000 ng/μL and the *S.typhi* was varied from 1-10^7 cells/μL. Firstly, 3 μL of *S.typhi* LPS was also dotted onto nitrocellulose membrane. After blocking with blocking reagent, the membrane was submerged in monoclomal antibody. The membrane was then placed in a solution of rabbit anti-mouse immunoglobulin-alkaline phosphatase conjugate. Finally, the membrane was placed in a substrate solution. The label and substrate of this method were alkaline phosphatase and 5-bromo-4-chloro-3-indolyl phosphate, respectively. This method completed within 1 h and 30 min. For positive results, a colored dot appeared on nitrocellulose membrane. When the dot-blot ELISA was performed on serially diluted *S.typhi* whole cells and LPS, it was found that positive results were obtained when *S.typhi* were presented 10^5 cfu/mL (100 cells/1 μL).

In 2005, Massi *et al.* quantified the gene copies from *S. typhi* in the blood of patients suspected of having typhoid fever using TaqMan-based real-time PCR (TaqMan assay) to target the *S. typhi* flagellin gene in genomic DNAs isolated from blood samples [5]. 55 bloods samples were tested. From the blood culture test, 8 bloods samples with a positive blood culture had *S. typhi* in load ranging from 1.01×10^3 to 4.35×10^4 copies/mL blood, and 47 bloods samples with negative blood culture. A blood culture test was positive only when *S. typhi* loads were greater than 10^3 copies/mL blood. For the TaqMan assay, 40 (85.1%) of the 47 negative blood from blood culture test were positive and the loads ranged from 3.9×10^0 to 9.9×10^2

copies/mL blood. Therefore, TaqMan assay may be useful for assessing *S. typhi* loads, especially in cases of suspected typhoid fever with negative results from the standard blood culture test.

In 2005, Rao *et al.* developed amperometric immunosensor for the detection of antibodies of *S. typhi* in patient serum [10] which involved usage of screen-printed electrode (SPE) and recombinant flagellin fusion protein. An indirect ELISA was used to detect antibodies of *S. typhi* in patient serum. The SPEs were made using polystyrene and graphite. These electrodes were tested for their ability to detect 1-naphthol, which was the product formed due to the hydrolysis of the substrate 1-naphthyl phosphate by the enzyme alkaline phosphatase. The immunosensor was performed by using amperometric method. Both the pooled healthy human serum samples and patient sera were subjected to the widal agglutination test and the amperometric method. A 100% correlation was found between the widal test and the amperometric method. The time taken for the detection by electrochemical methods is 1 h and 15 min, while the time taken by the widal test is 18 h.

In 2008, Kumar *et al.* detected *S. typhi* from food and water samples by sandwich ELISA and optimized enrichment procedures for use with the developed ELISA to increase sensitivity of the assay [9]. Sandwich ELISA was developed using polyclonal antibody as capture antibody and monoclonal antibody as detection antibody. To design the efficient culture strategies for use with the sandwich ELISA, different pre-enrichment and enrichment broths were evaluated. The sandwich ELISA had the detection limit of 10^4-10^5 cfu/mL. Enrichment-ELISA, when tested in artificially inoculated food samples, could detect 10^2 cfu/mL of *S. typhi* within 10 h.

In 2008, Dungchai *et al.* developed metalloimmunoassay based on a copper-enhanced gold label for the determination of *S. typhi* using electrochemical amplification [12]. Monoclonal antibodies for *S. typhi* were first immobilized on a polystyrene microwell and then captured by *S. typhi*. After immunoreactions occurred, a polyclonal antibody-colloidal gold conjugate was added. Next, a copper-enhancer containing ascorbic acid and copper (II) sulfate was added. The ascorbic reduced the copper (II) ions to copper (0), which was subsequently deposited onto the gold nanoparticle tags. After the copper was dissolved in nitric acid, the released

copper ions were detected by anodic stripping voltammetry. The anodic stripping peak current was linearly dependent in the range of 1.30×10^2 to 2.6×10^3 cfu/mL of *S. typhi* in logarithmic plot. The detection limit was found to be 98.9 cfu/mL. This method completed within 6 h and 30 min.

This year (2010), Ngan *et al.* developed a novel multiplex PCR for the detection *S. typhi* and *S. paratyphi* [4]. The differentiation of *S. typhi* and *S. paratyphi* was performed by targeting the intergenic region (SSPAI) for *S. paratyphi* and fimbrial subunit protein (*stgA*) for *S. typhi*. The detection limit of spiked blood samples for *S. typhi* and *S. paratyphi* was approximately 1×10^5 cfu/mL and 2×10^5 cfu/mL, respectively. After 8 h enrichment spiked blood samples, the sensitivity of *S. typhi* and *S. paratyphi* increased to 4.5×10^4 cfu/mL and 5.5×10^4 cfu/mL, respectively.

2.7.2 Immunochromatography

Lyubavina *et al.* (2005) reported an express morphine assay in aqueous samples by immunochromatography using monoclonal antibodies labeled with colloidal gold [22]. The competitive immunochromatography was used to detect morphine hydrochloride. An average particle size of colloidal gold was approximately 20 nm. Morphine ovalbumin (polyvalent antigen) and antimouse antibody were applied on the test line and the control line, respectively. For positive results, no band of test line occurred. The detection limit for morphine in aqueous sample was 10 ng/mL, and the analysis time was 5 min.

In 2005, Xiulan *et al.* detected aflatoxin B1 (AFB1) using competitive immunochromatography [40]. The gold nanoparticle was used as label with average particle size of 10 nm. AFB1-BSA antigen and goat antirabbit antibody were used as the test line and the control line, respectively. For positive results, no band of test line occurred. The detection limit was found to be 2.5 ng/mL of aflatoxin B1 standard solution. Analysis was completed in less than 10 min.

In 2006, Shim *et al.* developed immunochromatography strip test for detection of atrazine in water samples [23]. The competitive immunochromatography was used and colloidal gold particles of 40 nm were selected. Atrazine-BSA and goat anti-mouse IgG were used as the test line and the control line, respectively. For positive results, no band of test line occurred. The visual detection limit was 3 ng/mL and this test required only 10 min to get results and one step of sample to perform the assay.

In 2007, Huang detected *Staphylococcus aureus* using gold nanoparticles-based immunochromatographic assay [33]. Staphylococcal protein A, a cell wall protein of *Staphylococcus aureus*, was detected. The assay was constructed in the form of sandwich by using anti-protein A IgG to immobilize on the test zone and conjugate with gold nanoparticles. The average size of gold nanoparticles was 20 nm. For positive results, two colored bands of test line and control line appeared. Analysis was completed within 10 min and the detection limit was 25 ng/mL of protein A. This method was used to detect *Staphylococcus aureus* in foods and the sensitivity was found to be 100%.

In 2007, Liu *et al.* developed immunochromatographic strip coupled electrochemical detection to determine IgG [14]. The quantum dot (QDs, Cds@Zns) was used as the label which bound to anti IgG and 0.1 M hydeochloric acid (HCl) was dropped into the test line to release cadmium ion (Cd^{2+}) from the captured QD labels. The screen printed electrode was placed underneath the detection zone. The sandwich immunoassay and square-wave voltammetric measurements were performed. The linearity occurred in the range of 0.1-10 ng/mL. The limit of detection was estimated to be 30 pg/mL is associated with 7 min immunoreactions time and the detection limit was improved to 10 pg/mL using 20 min immunoreactions time.

In 2007, Inoue *et al.* reported a competitive immunochromatographic assay for the determination of testosterone based on electrochemical detection [34]. Immunochromatographic assay was combined with electrochemical detection using an electrode chip in order to determine testosterone. The electrode chip consisted of a gold working electrode, a counter electrode and a pseudo-reference electrode. Competitive immunoreactions were initiated by applying a solution containing

testosterone and HRP-labeled testosterone over the membrane. Membrane was then placed in a solution containing ferroenenethanol (FeOH) and hyrogenperoxide (H_2O_2) in the well of the electrode chip, and the enzyme reaction was detected by amperometry. Labeled HRP captured on the membrane catalyzed the oxidation of FeOH to the oxidized form $FeOH^+$, which was reduced electrochemically by the electrode chip. The electrochemical response of the reduction current decreased with the increasing concentration of testosterone over the range 1-625 ng/mL.

In 2008, Mao *et al.* developed multiplex electrochemical immunoassay using gold nanoparticles probes and immunochromatographic strips [35]. Rabbit IgG and human IgM were used as model targets. The gold nanoparticles based sandwich immunoreactions were performed and the captured gold nanoparticle labels on the test line were determined by stripping voltammetric measurement of the dissolved gold ions (III) with a carbon electrode. A hydrogen bromide-bromine ($HBr\text{-}Br_2$) solution was used to dissolve the captured gold nanoparticles labels. The detection limits were 1.0 and 1.5 ng/mL for rabbit IgG and human IgM, respectively. Linear range was 2.5-250 ng/mL and the total assay time was around 25 min.

CHAPTER III

EXPERIMENTAL

3.1 Chemical and reagents

3.1.1 Hydrogen tetrachloroaurate (III) trihydrate (Sigma-Aldrich)

3.1.2 Sodium citrate tribasic dehydrate (Riedel de Haen)

3.1.3 Bovine serum albumin (Sigma-Aldrich)

3.1.4 Sodium chloride (Mallinck rodt)

3.1.5 Potassium chloride (Ajax finechem Pty Ltd)

3.1.6 Disodium hydrogen phosphate (Merck)

3.1.7 Potassium dihydrogen orthophosphate (BDH)

3.1.8 Tween 20 (Sigma-Aldrich)

3.1.9 Sucrose (Sigma)

3.1.10 Gelatin (Sigma-Aldrich)

3.1.11 Polyethylene glycol 400 (Fluka)

3.1.12 Sodium hydroxide (Merck)

3.1.13 Hydrochloric acid fuming 37% (Merck)

3.1.14 *Salmonella typhi* O901 (Thammasat University)

3.1.15 Polyclonal rabbit antibody for *S.typhi* O901 (Siriraj Hospital)

3.1.16 Goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.)

3.1.17 IgG from murine serum (Sigma)

3.1.18 Anti-mouse IgG (Sigma)

3.1.19 Normal human serum (Jackson ImmunoResearch Laboratories, Inc.)

3.1.20 A standard buffer solution pH 4 and pH 7 (Metrohm)

3.2 Instruments and equipments

3.2.1 UV-visible spectrophotometer CARY 50 (Varian, USA)

3.2.2 Transmission electron microscopy JEM-2100 (JEOL Ltd., Japan)

3.2.3 Hot plate stirrer HS-115 (HL instrument, USA)

3.2.4 Milli Q Water System (Millipore, USA, R ≥ 18.2 MΩcm)

3.2.5 Stop watch (Mini Timer, Japan)

3.2.6 Vortex mixer VTX-3000L (Mixer Uzusio LMS. Co. LTD., Japan)

3.2.7 pH meter (Metrohm 744 pH meter, Metrohm, Switzerland)

3.2.8 Centrifuge Allegra 64 R (Beckman Coulter, Inc., USA)

3.2.9 Scanner 600 dpi (Canon, Thailand)

3.2.10 Digital Camera (Sony, Thailand)

3.2.11 Notebook with AdobePhotoshop (Sony, Thailand)

3.2.12 Analytical balance (Mettler AT 200, Mettler, Switzerland)

3.2.13 Autopipette and tips (Eppendorf, Germany)

3.2.14 Standard 17 (17 mm × 50 m, Whatman, UK)

3.2.15 Nitrocellulose membrane AE 100 (25 × 300 mm, Whatman, UK)

3.2.16 Cellulose Chromatography paper (0.18 mm, 25 × 68 cm Whatman, UK)

3.2.17 Nitrocellulose membrane HF180 (20 × 30 cm, Millipore, USA)

3.2.18 Cellulose absorbent pad (2 × 30 cm, Millipore, USA)

3.2.19 Magnetic stirring bars (10 × 6 mm, SCS ICS, Switzerland)

3.2.20 Polystyrene 96-well microtiter plates (Shanghai, China)

3.2.21 Cuvette quartz cell

3.2.22 Beaker 10, 25, 50, 100 and 250 mL

3.2.23 Volumetric flask 10, 25 and 100 mL

3.3 Preparation of solution

3.3.1 1% $HAuCl_4$ solution (100 mL)

1% $HAuCl_4$ solution was prepared using 1 g of 99.9 % hydrogen tetrachloroaurate (III) trihydrate dissolved in 100 mL of Milli Q water.

3.3.2 1% Sodium citrate solution (100 mL)

1% Sodium citrate solution was prepared using 1 g of 99.5% sodium citrate tribasic dehydrate dissolved in 100 mL of Milli Q water.

3.3.3 0.01 M PBS solution, pH 7.4 (100 mL)

0.01 M PBS solution was prepared using 137 mM sodium chloride, 2.7 mM potassium chloride, 2 mM potassium dihydrogen orthophosphate, and 10 mM disodium hydrogen phosphate dissolved in 100 mL of Milli Q water and then adjusted to pH 7.4 by sodium hydroxide and hydrochloric acid.

3.3.4 1, 3, 5 and 10% BSA solution, pH 7.4 (100 mL)

1, 3, 5 and 10% BSA solution was prepared using 1, 3, 5 and 10 g of bovine serum albumin dissolved in 100 mL of PBS solution, respectively.

3.3.5 10% NaCl solution (100 mL)

10% NaCl solution was prepared using 10 g of sodium chloride dissolved in 100 mL of Milli Q water.

3.3.6 1% BSA and 1% sucrose solution, pH 7.4 (100 mL)

1% BSA and 1% sucrose solution was prepared using 1 g of bovine serum albumin and 1 g of sucrose dissolved in 100 mL of PBS solution.

3.3.7 0.05% Tween in 10% BSA solution (100 mL)

0.05% Tween in 10% BSA solution was prepared using 0.05 mL of Tween 20 dissolved in 100 mL of 10% BSA solution.

3.3.8 10% geletin solution (100 mL)

10% gelatin was prepared using 10 g of gelatin dissolved in 100 mL of Milli Q water.

3.3.9 0.1% geletin in 10% BSA solution (100 mL)

0.1% gelatin in 10% BSA solution was prepared using 1 mL of 10% gelatin dissolved in 99 mL of 10% BSA solution.

3.3.10 0.5, 1, 2 and 3% PEG 400 in 10% BSA solution (100 mL)

0.5, 1, 2 and 3% PEG 400 in 10% BSA solution were prepared using 0.5, 1, 2 and 3 mL of PEG 400 dissolved in 99.5, 99, 98 and 97 mL of 10% BSA solution, respectively.

3.4 Preparation of polyclonal rabbit antibody-gold nanoparticles conjugate

3.4.1 Synthesis of gold nanoparicles

The gold nanoparticles were prepared by mixing 1 mL of 1% $HAuCl_4$ solution and 100 mL of ultrapure water and boil under stirring. Then 2.5 mL of 1% sodium citrate was added into the solution under continuous heating and stirring for 15 min until the color of the solution changed to claret-colored as shown in Figure 3.1. The gold nanoparticles solution was left to cool at room temperature under stirring and was later stored in dark bottles at 4 ^{o}C. The solution of gold nanoparticles was characterized by UV–visible spectrophotometer and transmission electron microscopy.

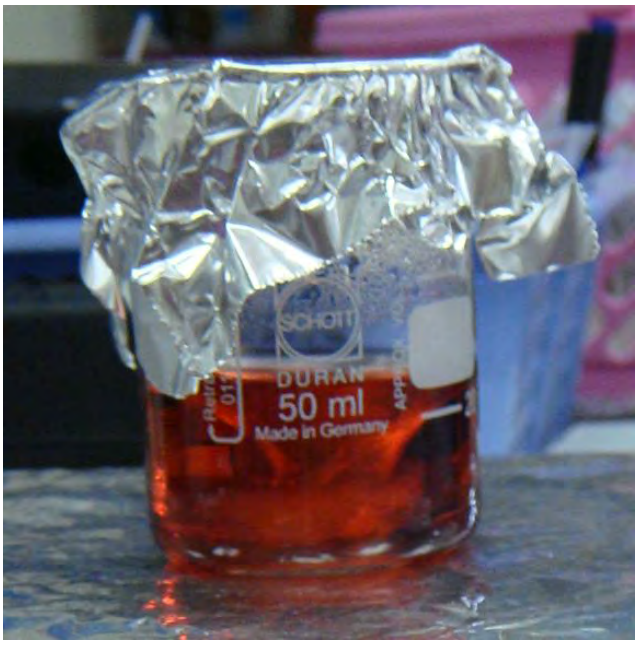

Figure 3.1 The gold nanoparticles produced by sodium citrate reduction of solutions of $HAuCl_4$

3.4.2 Formation of polyclonal rabbit antibody-gold nanoparticles conjugate

Polyclonal rabbit antibody-gold nanoparticles conjugate was prepared according to the method in ref. [12] with slightly modification. The amount of polyclonal rabbit antibody for polysaccharides of *S. typhi* O901 was optimized from 0 to 1 mg/mL. The solutions were prepared from 15 mg/mL stock solution of polyclonal rabbit antibody. An appropriate volume of stock solution was added to 2 mL of gold nanoparticles solution followed by incubation under continuous stirring at room temperature for 1 h. Then 100 µL of 3% BSA in PBS solution was added to minimize nonspecific reaction and incubation under continuous stirring at room temperature for 1 h. The polyclonal rabbit antibody-gold nanoparticles conjugate was centrifuged at 15,000 rpm for 10 min, and the soft sediment was washed and suspended in 3% BSA in PBS solution (pH 7.4). The polyclonal rabbit antibody-gold nanoparticles conjugate was stored at 4 ^{o}C. The solution of conjugate was characterized by UV–vis spectrophotometer.

3.5 Dot blot immunoassay coupled with optical detection for the determination of *S. typhi*

The schematic of dot blot immunoassay was shown in Figure 3.2. The dot blot immunoassay was performed onto nitrocellulose membrane. Briefly, 1 µL of *S. typhi* was applied in dots onto the nitrocellulose membrane and dried for 30 min. The membrane was blocked with a blocking reagent (3% BSA) for 1 h. After that, the membrane was soaked in the polyclonal rabbit antibody-gold nanoparticles conjugate for 20 min and the membrane was then washed by Milli Q water. The specific binding reaction between the antigen and the polyclonal rabbit antibody-gold nanoparticles conjugate caused the claret-colored dot on nitrocellulose membrane.

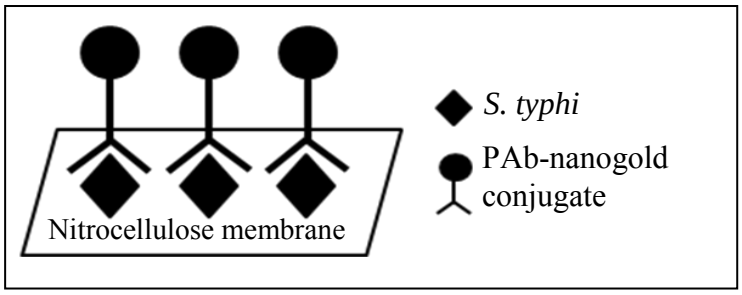

Figure 3.2 The schematic of dot blot immunoassay for *S. typhi* detection

For optical detection, the membrane was captured by a scanner and the image was converted into grayscale. Adobe Photoshop was then used to investigate the mean intensity from the histogram and the relationship between the mean intensity and the concentration of *S.typhi* was plotted. The procedure of optical detection for quantifying the concentration of *S. typhi* using Adobe Photoshop program was shown in figure 3.3.

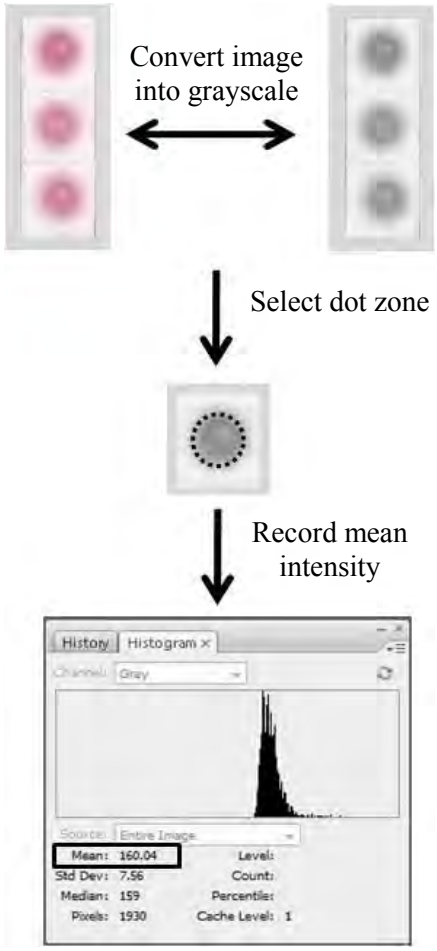

Figure 3.3 The procedure of optical detection for quantifying the concentration of *S. typhi* using Adobe Photoshop program

3.6 Immunochromatography using IgG from murine serum as model of the test strip

3.6.1 Configuration of immunochromatography using IgG from murine serum as the model of test strip

3.6.1.1 Direct immunochromatography

The direct immunochromatographic strip consisted of three pads (sample, conjugate, and absorbent pads) and one nitrocellulose membrane (Figure 3.4). Anti-mouse IgG-gold nanoparticles conjugate was applied to the glass fiber membrane to be used as the conjugate pad and the conjugate pad was then dried at 4 ^{o}C for 1 h. To prepare test dot, IgG from murine serum was dotted on the nitrocellulose membrane and dried at room temperature for 1 h. All of the pads and the membrane were attached on polyethylene sheet. A running buffer was applied to the sample pad and allowed to migrate up the membrane. The negative test resulted in no-colored dot. The positive test presented claret-colored dots on nitrocellulose membrane.

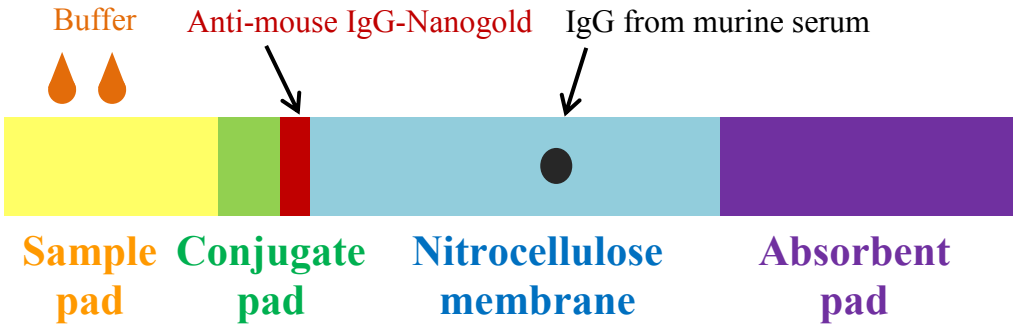

Figure 3.4 The configuration of direct immunochromatography using IgG from murine serum as the model of the test strip

3.6.1.2 Sandwich immunochromatography

The sandwich immunochromatographic strip consisted of three pads (sample, conjugate, and absorbent pads) and one nitrocellulose membrane (Figure 3.5). Anti-mouse IgG-gold nanoparticles conjugate was applied to the glass fiber membrane to be used as the conjugate pad and the conjugate pad was then dried at 4 ^{o}C for 1 h. For preparation of the test dot, Anti-mouse IgG antibody was dotted on the nitrocellulose membrane and dried at room temperature for 1 h. All of the pads and the membrane were attached on polyethylene sheet. IgG from murine serum was applied to the sample pad and allowed to migrate up the membrane. The negative test resulted in no-colored dot. The positive test presented claret-colored dots on the nitrocellulose membrane.

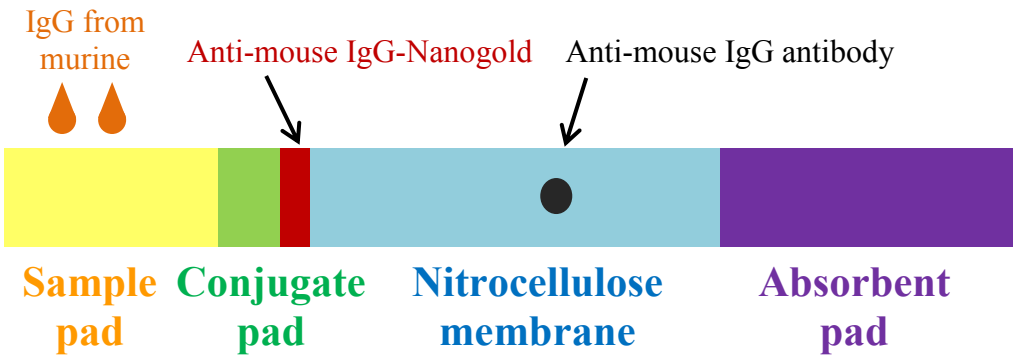

Figure 3.5 The configuration of sandwich immunochromatography using IgG from murine serum as the model of the test strip

3.6.2 The effect of anti-mouse IgG concentration for the preparation of anti-mouse IgG-gold nanoparticles conjugate

The optimization of anti-mouse IgG by colorimetric assay and UV-vis spectrophotometer was performed according to the method in ref. [23, 53] with slightly modification. Briefly, 2 mL of gold nanoparticles was distributed into tubes and 1 mg/mL of anti-mouse IgG solution (0-60 μL) was added to each tube. Then, 200 μL of 10% NaCl was added to each tube and immediately mixed for 1 min. A minimum amount of anti-mouse IgG was evaluated by observing color changed from red to blue. If a tube contained a minimum amount of the antibody, the color of the mixed solutions was not changed. Finally, the mixed solutions were characterized by UV–vis spectrophotometer.

3.6.3 The effect of time for incubating anti-mouse IgG and gold nanoparticles and final volume of anti-mouse IgG-gold nanoparticles conjugate

The anti-mouse IgG-gold nanoparticles conjugate was performed by mixing 2 mL of gold nanoparticles and 20 μL of 1 mg/mL of anti-mouse IgG. The time for incubating gold nanoparticles and anti-mouse IgG was performed from 0 min to overnight. The solution was then blocked with 100 μL of 10% BSA for 1 h. After that, the solution was centrifuged at 15,000 rpm for 10 min. The soft sediment was resuspended in 1 mL of 10% BSA and was centrifuged again. Finally, the soft sediment was resuspended in 100 μL and 200 μL of 10% BSA. A minimum time for incubating was evaluated by direct immunochromatography.

The direct immunochromatographic strip was performed by applying 3 μL of anti-mouse IgG-gold nanoparticles conjugate to the glass fiber membrane to be used as the conjugate pad and 1 μL of IgG from murine serum on the nitrocellulose membrane. The sample pad and the absorbent pad were used without treatment. 120 μL of running buffer (10% BSA) [54] was applied to the sample pad and allowed to migrate up the membrane. The negative test resulted in no-colored dot. The positive test presented claret-colored dots on the nitrocellulose membrane.

3.6.4 The effect of conditions for treating nitrocellulose membrane using direct immunochromatrography

The direct immunochromatographic strip was performed by applying 3 µL of anti-mouse IgG-gold nanoparticles conjugate to the glass fiber membrane to be used as the conjugate pad. The sample pad and the absorbent pad were used without treatment. The nitrocellulose membrane was treated under 7 conditions (Table 3.1). All treatments were dotted with 1 µL of IgG from murine serum. 120 µL of running buffer (10% BSA) was applied to the sample pad and allowed to migrate up the membrane. The negative test resulted in no-colored dot. The positive test presented claret-colored dots on the nitrocellulose membrane.

Table 3.1 The condition for treating the nitrocellulose membrane

Conditions	Nitrocellulose membrane (NCM)
1	NCM was used without treatment.
2	NCM was incubated in PBS for 10 min before dotting
3	NCM was incubated in 1% BSA for 10 min before dotting
4	NCM was incubated in 1% BSA and 1% sucrose for 10 min before dotting
5	NCM was incubated in PBS for 10 min after dotting
6	NCM was incubated in 1% BSA for 10 min after dotting
7	NCM was incubated in 1% BSA and 1% sucrose for 10 min after dotting

3.6.5 The effect of conditions for treating nitrocellulose membrane using sandwich immunochromatography

The sandwich immunochromatographic strip was performed by applying 3 μL of anti-mouse IgG-gold nanoparticles conjugate to the glass fiber membrane to be used as the conjugate pad. The sample pad and the absorbent pad were used without treatment. The nitrocellulose membrane was treated with condition of 1, 5, 6 and 7 (see Table 3.1). All treatments were dotted with 1 μL of anti-mouse IgG. 120 μL of IgG from murine serum in 10% BSA was applied to the sample pad and allowed to migrate up the membrane. The negative test resulted in no-colored dot. The positive test presented claret-colored dots on the nitrocellulose membrane.

3.6.6 The effect of IgG volume on nitrocellulose membrane

The sandwich immunochromatographic strip was performed by applying 3 μL of anti-mouse IgG-gold nanoparticles conjugate to the glass fiber membrane to be used as the conjugate pad. The sample pad and the absorbent pad were used without treatment. The anti-mouse IgG (0-3 μL) was dotted on nitrocellulose membrane. 120 μL of IgG from murine serum in 10% BSA was applied to the sample pad and allowed to migrate up the membrane. The negative test resulted in no-colored dot. The positive test presented claret-colored dots on the nitrocellulose membrane.

3.7 Immunochromatography for the detection of *S.typhi*

3.7.1 Synthesis of polyclonal rabbit antibody-gold nanoparticles conjugate for immunochromatography

An appropriate volume of 0.5 mg/mL of polyclonal rabbit antibody was added to 2 mL of gold nanoparticles solution, followed by incubation under stirring at room temperature for 1 h. Then 100 µL of 10% BSA was added and incubated under stirring at room temperature for 1 h. The polyclonal rabbit antibody-gold nanoparticles conjugate was centrifuged at 15,000 rpm for 10 min, and the soft sediment was washed and suspended in 10% BSA. The solution was again centrifuged and the soft sediment was suspended in 100 µL of 10% BSA. The polyclonal rabbit antibody-gold nanoparticles conjugate was stored at 4 ^{o}C.

3.7.2 Configuration and procedure of sandwich immunochromatography for the detection of *S.typhi*

The sandwich immunochromatographic strip consisted of three pads (sample, conjugate, and absorbent pads) and one nitrocellulose membrane (Figure 3.6). Polyclonal rabbit antibody-gold nanoparticles conjugate was applied to the glass fiber membrane to be used as the conjugate pad. Polyclonal rabbit antibody for polysaccharides of *S. typhi* O901 and goat anti-rabbit IgG were dotted on the nitrocellulose membrane to create a test dot and a control dot respectively.

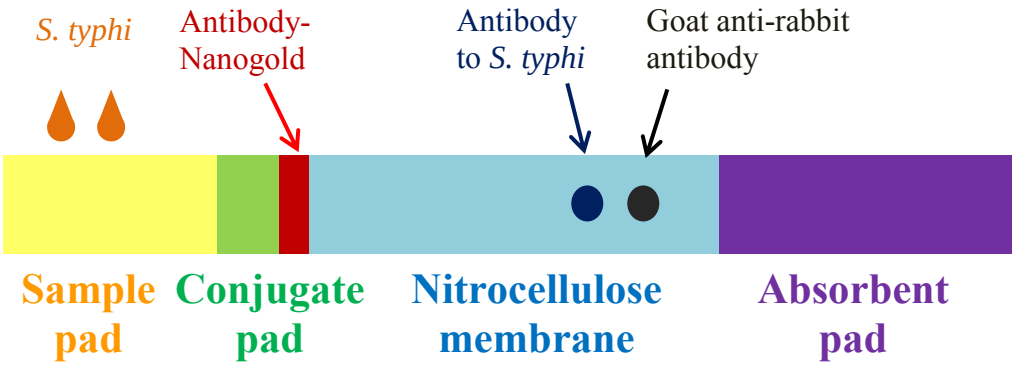

Figure 3.6 The configuration of sandwich immunochromatography for the detection of *S. typhi*

For preparing the test strip, the glass fiber membrane, the nitrocellulose membrane and the cellulose absorbent pad was initially cut off into widths of 0.5 cm and the length of each strip was later optimized. Polyclonal rabbit antibody-gold nanoparticles conjugate was applied to the top of the glass fiber membrane to be used as the conjugate pad (Figure 3.7) and the conjugate pad was then dried at 4 ^{o}C for 1 h.

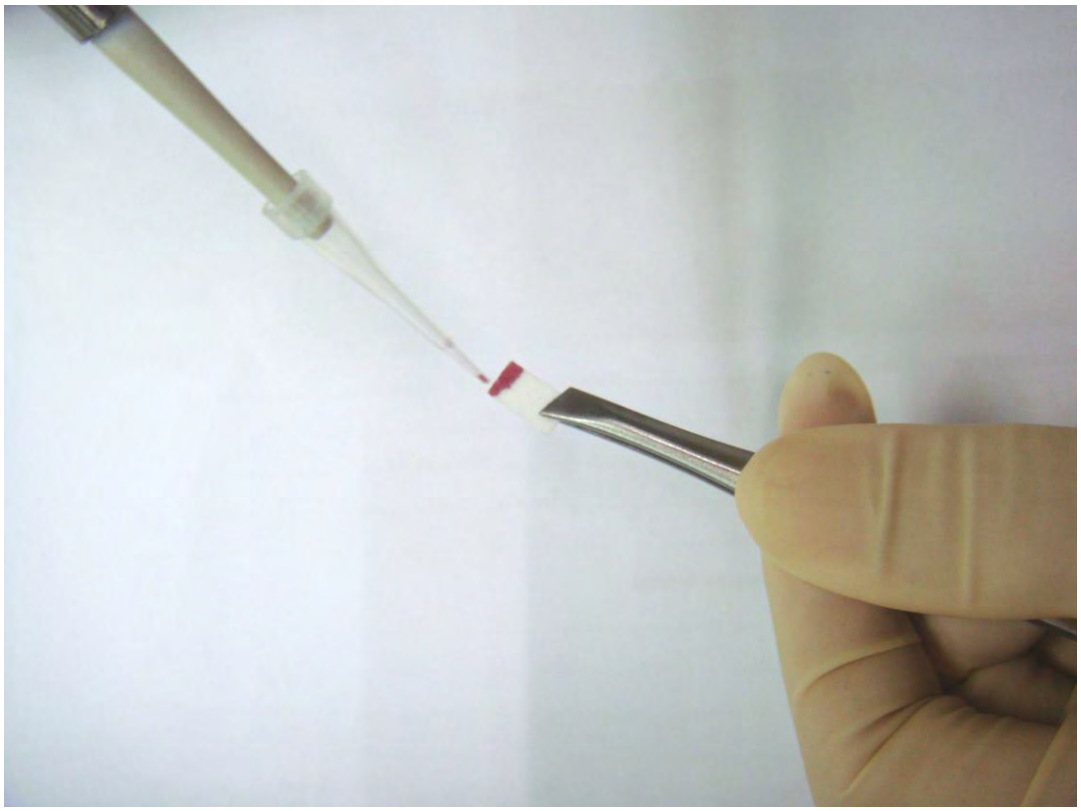

Figure 3.7 The performance of conjugate pad

After that, polyclonal rabbit antibody for polysaccharides of *S. typhi* O901 and goat anti-rabbit IgG were dotted on the nitrocellulose membrane to create a test zone and a control zone respectively (Figure 3.8). The distance between the control dot and the top of the nitrocellulose membrane was 0.7 cm and the distance between the control dot and the test dot was 0.3 cm. Nitrocellulose membrane was dried at room temperature for 1 h. The glass fiber membrane and the cellulose absorbent pad were used as the sample pad and the absorbent pad without treatment.

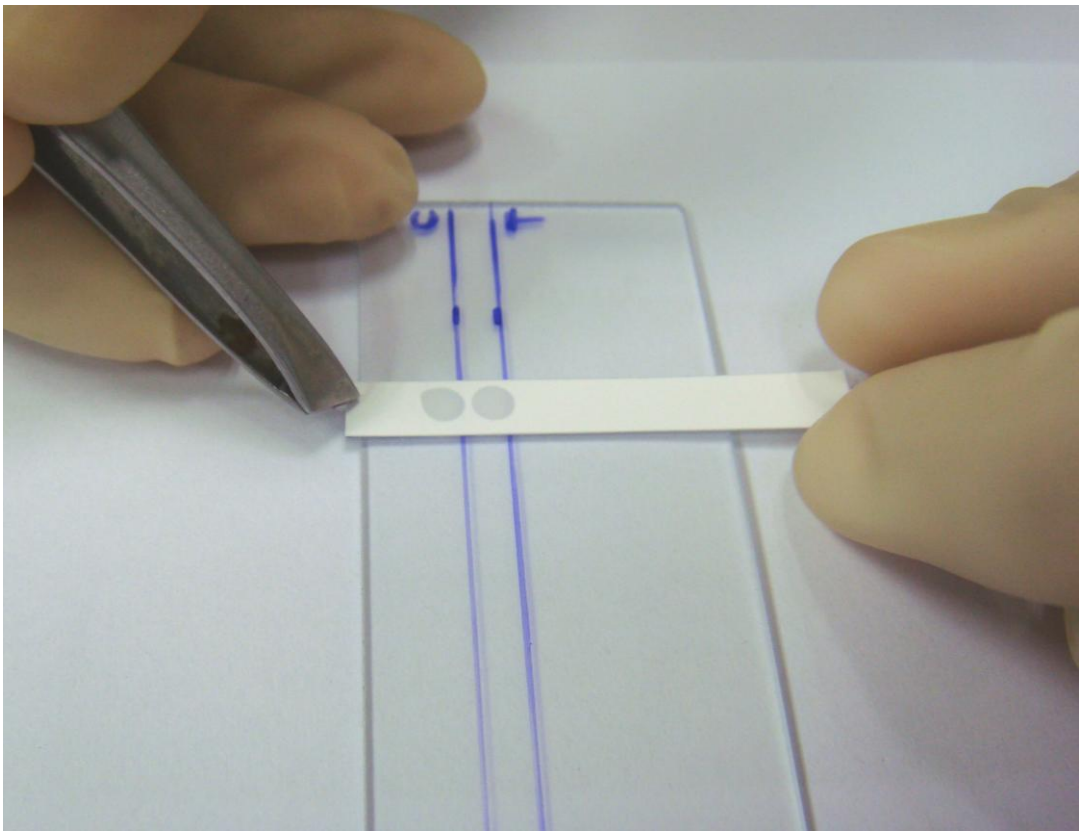

Figure 3.8 The performance of test zone and control zone on nitrocellulose membrane

To create the test strip (Figure 3.9), the nitrocellulose membrane with the test dot and the control dot was firstly attached on polyethylene sheet. After that, the conjugate pad was attached on the bottom of the nitrocellulose membrane. The sample pad was then attached on the bottom of the conjugate pad. Finally, the absorbent pad was attached on the top of the nitrocellulose membrane.

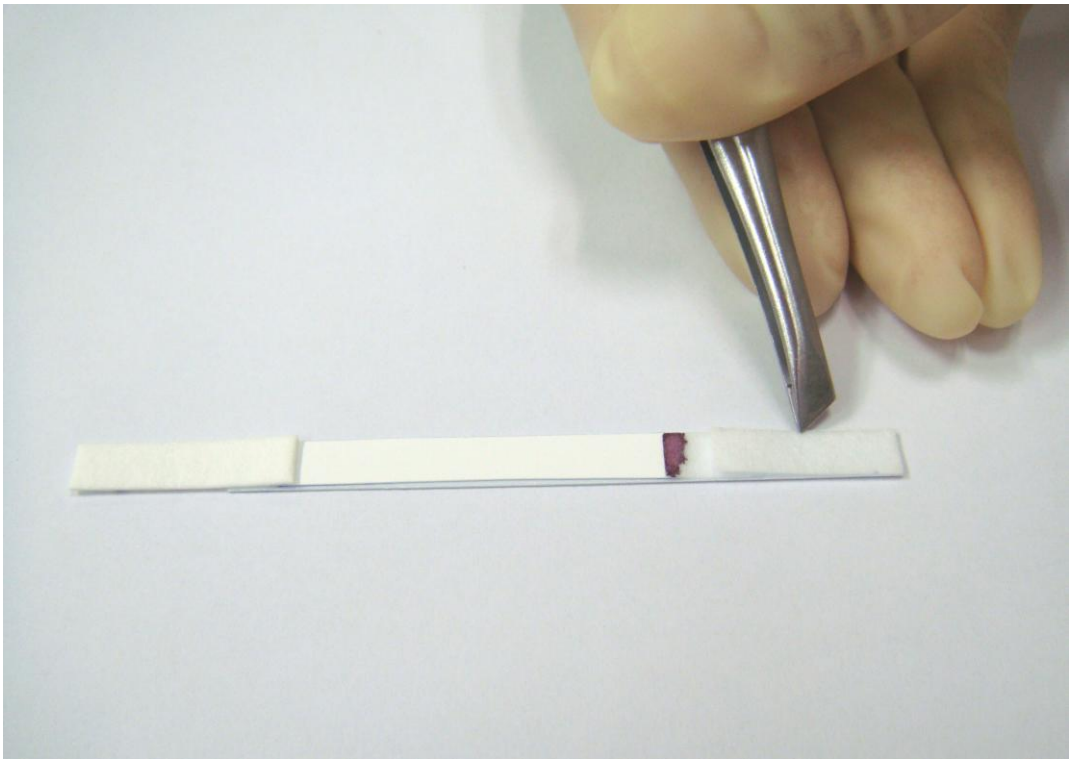

Figure 3.9 The test strip for the detection of *S. typhi*

For the detection of the sample, *S. typhi* with appropriate volume was applied on the center of the sample pad and allowed to migrate up the membrane (Figure 3.10). For the negative results, only one control dot appeared on the nitrocellulose membrane. The positive results presented two claret-colored dots on the nitrocellulose membrane (Figure 3.11).

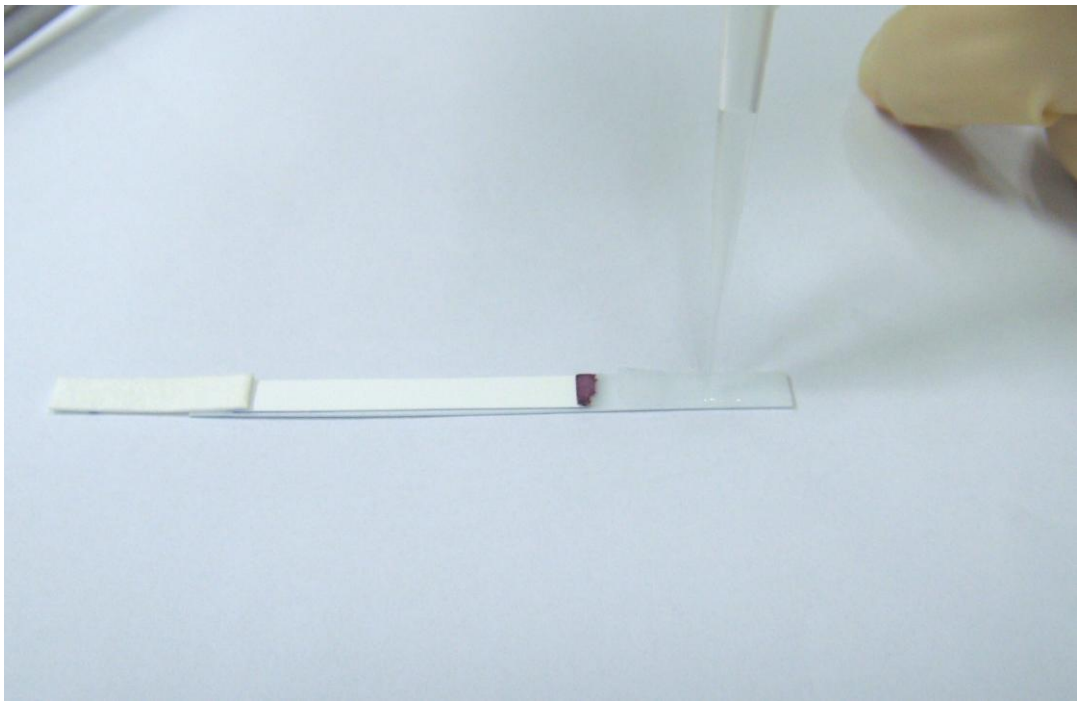

Figure 3.10 Applying *S. typhi* sample on the test strips

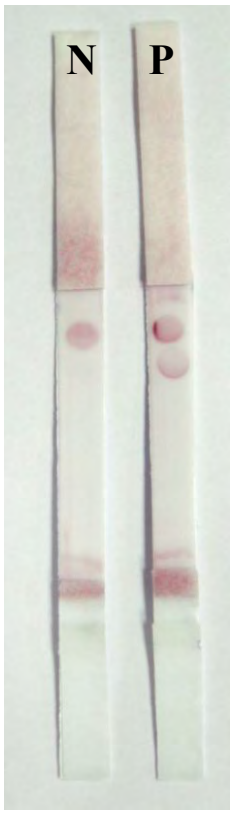

Figure 3.11 Negative result (N) and positive result (P) of the test strip using sandwich immunochromatography

3.7.3 The effect of the length of the nitrocellulose membrane and the sample volume for applying on test strip

The length of the nitrocellulose membrane was studied at 2.5, 3.0 and 3.5 cm using sandwich immunochromatography. To find optimal volume of the sample, coloring solution was applied to the test strip with the volume of 100-120 µL for 2.5 cm of nitrocellulose membrane, 120-140 µL for 3.0 cm of nitrocellulose membrane and 140-160 µL for 3.5 cm of nitrocellulose membrane.

For the effect of the length of the nitrocellulose membrane, the movement of coloring solution on the test strip was recorded. For the effect of sample volume, color intensity of test dot at different lengths of nitrocellulose membrane was recorded.

3.7.4 The effect of the running buffer and the additive

The running buffer was studied using PBS, 1% BSA, 5% BSA and 10% BSA to dilute the concentration of *S. typhi*. The color intensity of the test dot at different running buffers was recorded. The optimal running buffer was 10% BSA which provided the best results. The additive was then studied by adding 0.05% Tween 20, 0.1% gelatin and 1% PEG 400 in 10% BSA to compare with 10% BSA without additive. For the effect of the additive, 10% BSA without additive and 1% PEG 400 in 10% BSA provided good results. Therefore, 0.5%, 1%, 2% and 3% PEG 400 in 10% BSA were later studied to compare with 10% BSA without additive. Finally, the color intensity of test dot at the different conditions was recorded.

3.7.5 The effect of the volume of polyclonal rabbit antibody-gold nanoparticles conjugate

The volume of conjugate solution was studied by applying 3, 6 and 9 µL of polyclonal rabbit antibody-gold nanoparticles conjugate on glass fiber membrane. The time of reaction and color intensity of the test dot at different volumes of conjugate solution were recorded.

3.7.6 The effect of concentration of *S. typhi*

The concentration of *S. typhi* was studied between 1.14×10^3 cfu/mL and 1.14×10^8 cfu/mL. The solutions were prepared from 1.14×10^9 cfu/mL stock solution of *S. typhi*. An appropriate volume of *S. typhi* at different concentrations was applied to the test strip and the reaction was completed with 15 min. The limit of detection (LOD) was visually evaluated by the naked eye. For the negative results, only one control dot appeared on nitrocellulose membrane. The positive results presented two claret-colored dots on the nitrocellulose membrane.

3.7.7 The application of immunochromatography for the detection of *S. typhi* in human serum

The sandwich immunochromatography was also applied to detect *S. typhi* in human serum. The different concentrations of *S. typhi* between 1.14×10^5 cfu/mL and 1.14×10^8 cfu/mL were spiked to normal human serum. The result was evaluated by the naked eye within 15 min.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Characterization of gold nanoparticles

Gold nanoparticles were synthesized by chemical condensation using reduction of hydrogen tetrachloroaurate (III) ($HAuCl_4$). Hydrogen tetrachloroaurate (III) was reduced to neutral gold atoms by the sodium citrate. The solutions became supersaturated and the gold gradually started to precipitate in the form of gold nanoparticles. The color of the solution changed from yellow of hydrogen tetrachloroaurate (III) to claret-colored of gold nanoparticles. To characterize the hydrogen tetrachloroaurate (III) and the gold nanoparticles, UV-vis spectra of hydrogen tetrachloroaurate (III) and gold nanoparticles were recorded (Figure 4.1). The maximum absorbance of the hydrogen tetrachloroaurate (III) and the gold nanoparticles occurred at the wavelength of 285 nm and 520 nm respectively, which was similar to other reports [40]. From TEM images, the average particles size of gold nanoparticles was found to be 15 nm (Figure 4.2).

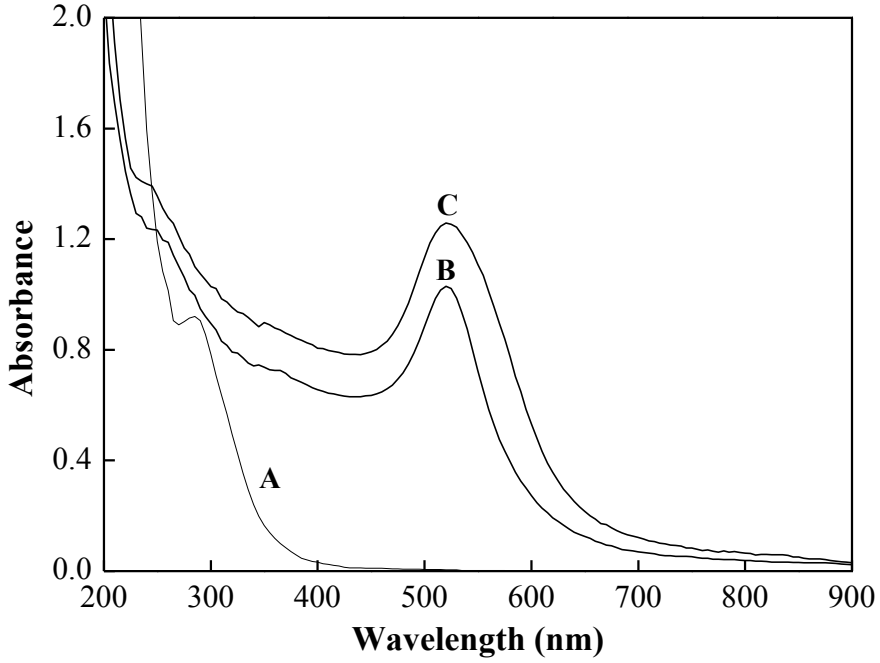

Figure 4.1 UV-vis spectra of the $HAuCl_4$ (A), gold nanoparticles (B) and the polyclonal rabbit antibody-gold nanoparticles conjugate (C)

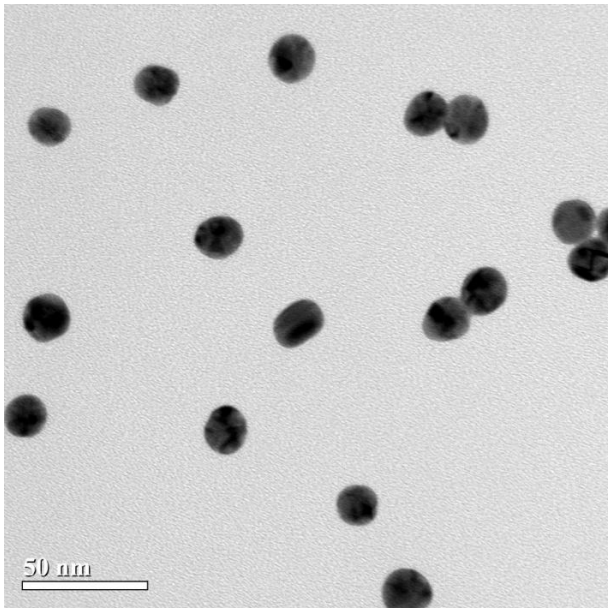

Figure 4.2 TEM images of gold nanoparticle sizes produced by sodium citrate reduction of solutions of $HAuCl_4$

4.2 Formation of polyclonal rabbit antibody-gold nanoparticles

4.2.1. Characterization of polyclonal rabbit antibody-gold nanoparticles

For conjugation, polyclonal rabbit antibody was adsorbed on the gold nanoparticles surfaces. The UV-vis spectra of polyclonal rabbit antibody-gold nanoparticles gave the maximum absorbance at the wavelength of 520 nm (Figure 4.1). There were no significant variation in the absorption spectrum between polyclonal rabbit antibody-gold nanoparticles and gold nanoparticles. This could be explained that gold still presented nanosized after binding with polyclonal rabbit antibody [12].

4.2.2 Optimization of polyclonal rabbit antibody-gold nanoparticles by UV-Vis spectrophotometer

The effect of the amount of binding polyclonal rabbit antibody on the surface of gold nanoparticles was studied in the concentration of polyclonal rabbit antibody from 0 to 1 mg/mL. From the plot between absorbance at 520 nm and antibody concentrations (Figure 4.3), the absorbance increased until 0.5 mg/mL of polyclonal rabbit antibody and then the absorbance decreased. Therefore, the minimal polyclonal rabbit antibody to stabilized gold nanoparticles was approximately at 0.5 mg/mL. Moreover, it was also effective in preventing aggregation [12].

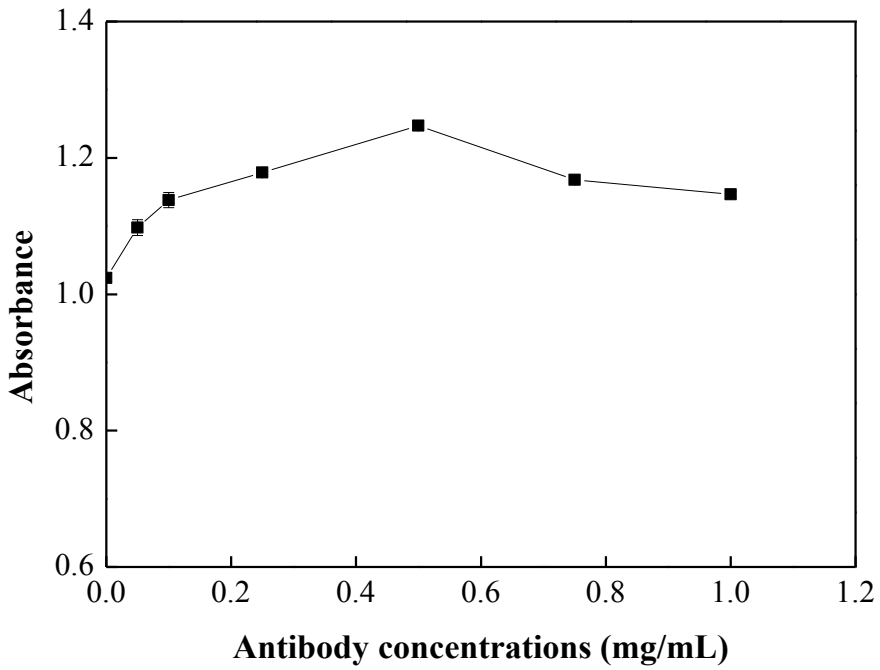

Figure 4.3 The effect of the amount of binding polyclonal rabbit antibody on the surface of gold nanoparticles measuring by UV-vis spectrophotometer

4.3 Dot blot immunoassay coupled with optical detection

4.3.1 Dot blot immunoassay

The detection of *S. typhi* (mg/mL) by dot blot assay using polyclonal rabbit antibody-gold nanoparticles conjugate as label was shown in Figure 4.4 which was studied in the concentration between 0.02 mg/mL and 4.50 mg/mL with 2-fold serial dilution. The weak claret-colored dot in the result was shown at 0.14 mg/mL of *S. typhi*. It means, the lowest concentration was observed at least 0.14 mg/mL. Therefore, the limit of detection was 0.14 mg/mL of *S. typhi* which could be visually evaluated by the naked eye within 1 h and 50 min. The resulting membrane was captured by scanner and optical detection was later performed.

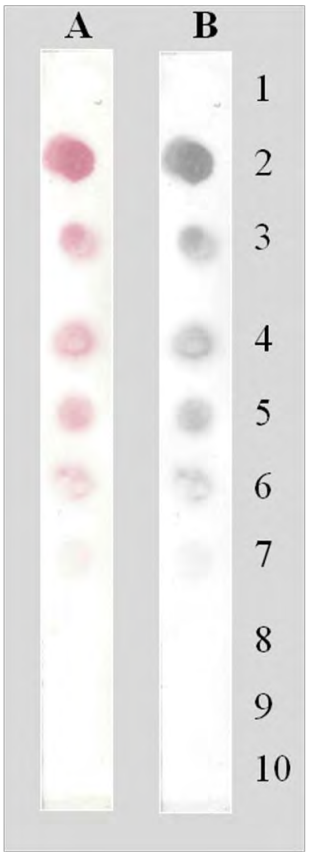

Figure 4.4 The image of dot blot assay of *S. typhi* in mg/mL; A) RBG color, B) gray scale, 1: PBS (control; 0 mg/mL), 2: 4.50 mg/mL, 3: 2.25 mg/mL, 4: 1.12 mg/mL, 5: 0.56 mg/mL, 6: 0.28 mg/mL, 7: 0.14 mg/mL, 8: 0.07 mg/mL, 9: 0.04 mg/mL and 10: 0.02 mg/mL ($n \geq 3$)

The detection of *S. typhi* (cfu/mL) by dot blot assay using polyclonal rabbit antibody-gold nanoparticles conjugate as label was shown in Figure 4.5. The concentration of *S. typhi* was studied between 4.44×10^6 cfu/mL and 1.14×10^9 cfu/mL with 2-fold serial dilution. The weak claret-colored dot in the result was shown at 8.88×10^6 cfu/mL of *S. typhi*. Therefore, the limit of detection was found to be 8.88×10^6 cfu/mL of *S. typhi* which could be visually evaluated by the naked eye.

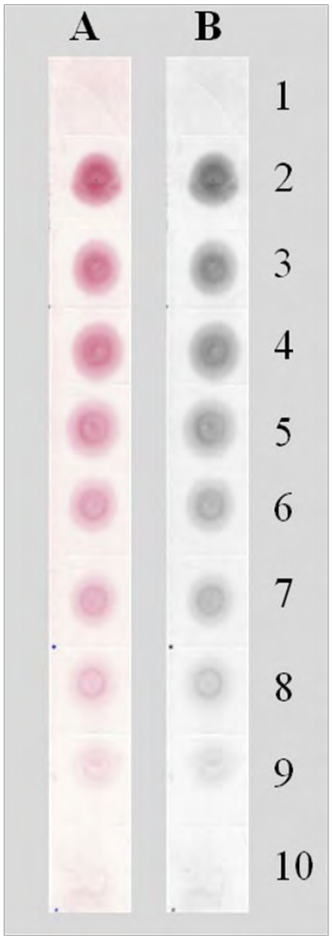

Figure 4.5 The image of dot blot assay of *S. typhi* in cfu/mL; A: RBG color, B: gray scale, 1: PBS (control), 2: 1.14×10^9 cfu/mL, 3: 5.79×10^8 cfu/mL, 4: 2.84×10^8 cfu/mL, 5: 1.42×10^8 cfu/mL, 6: 7.11×10^7 cfu/mL, 7: 3.55×10^7 cfu/mL, 8: 1.78×10^7 cfu/mL, 9: 8.88×10^6 cfu/mL and 10: 4.44×10^6 cfu/mL ($n \geq 3$)

4.3.2 Optical detection

For optical detection, images of the results were converted into gray scale. The mean intensity for each data point was obtained from the histogram in AdobePhotoshop. Analytical calibration between mean intensity and the concentration of *S. typhi* (mg/mL) was plotted in Figure 4.6. Linear region was observed with a coefficient of 0.9986, intercept of 0.0226 and slope of 18.234 for *S. typhi* concentration between 0 and 0.56 mg/mL (Figure 4.7).

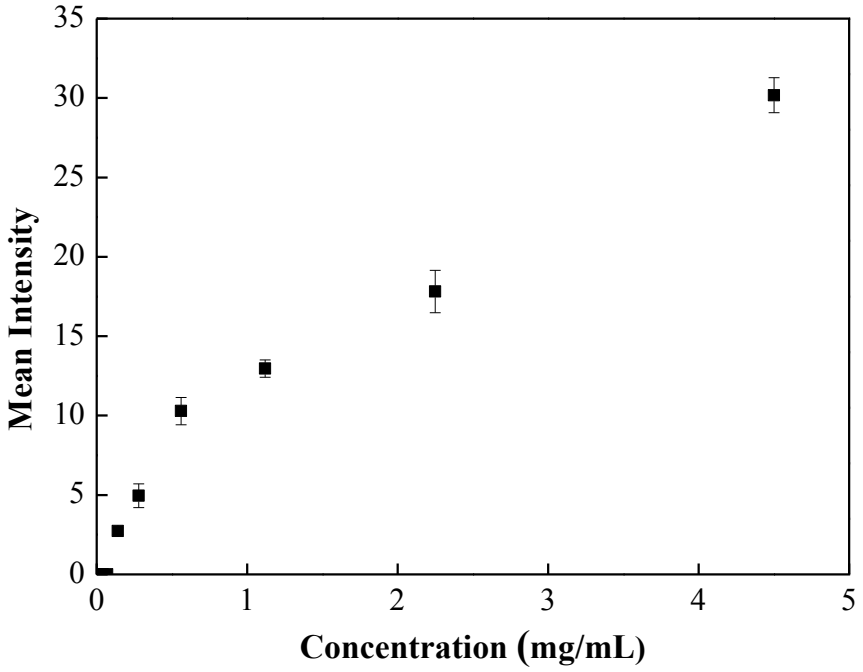

Figure 4.6 The analytical calibration between mean intensity and *S. typhi* at the concentration from 0 mg/mL to 4.50 mg/mL (n=3)

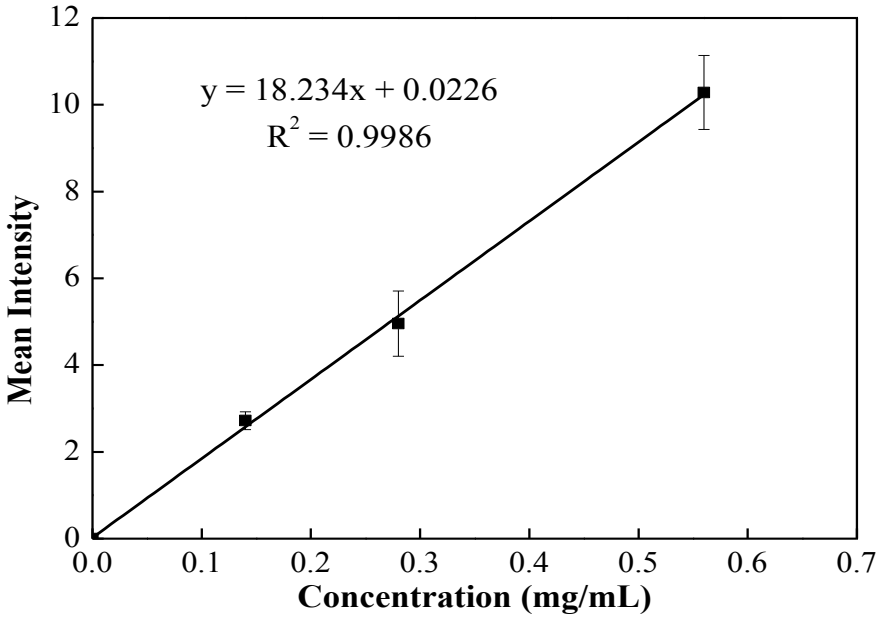

Figure 4.7 Linear region of *S. typhi* detection at the concentration between 0 mg/mL and 0.56 mg/mL (*n=3, slope=18.234, intercept =0.0226, R^2=0.9986*)

For the concentration in the unit of cfu/mL, analytical calibration between mean intensity and the concentration of *S. typhi* (cfu/mL) was plotted as shown in Figure 4.8. From analytical calibration between mean intensity and the concentration of *S. typhi* (cfu/mL), linear region could not be observed. So the plot between mean intensity and the concentration of *S. typhi* (cfu/mL) in the form of log was performed, which was shown in Figure 4.9. From the plot between mean intensity and log of the concentration of *S. typhi* (cfu/mL), linear region was observed with the coefficient of 0.9927, the intercept of 24.3170 and the slope of 26.6016 for *S. typhi* concentrations between 8.88×10^6 and 1.14×10^9 cfu/mL.

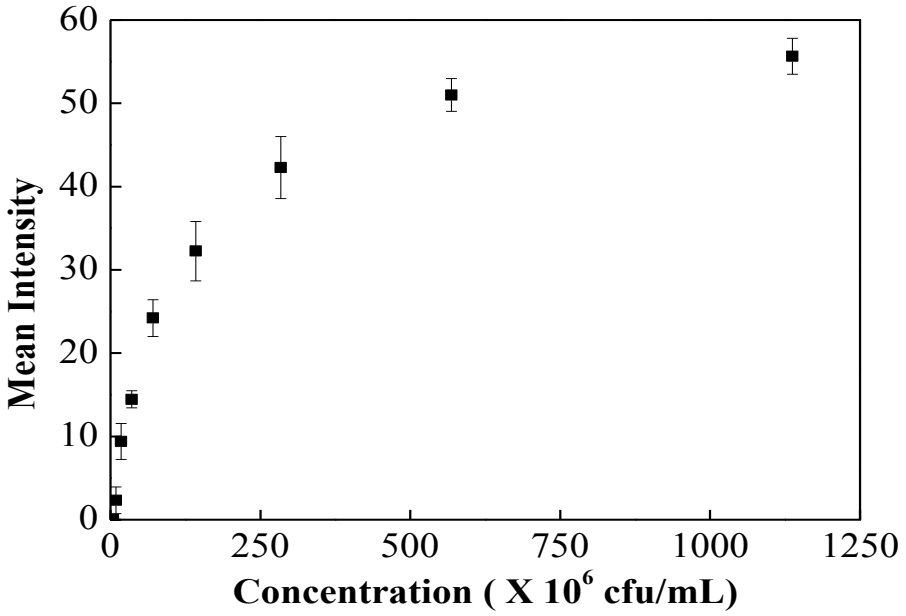

Figure 4.8 The analytical calibration between mean intensity and *S. typhi* at the concentration from 0 cfu/mL to 1.14×10^9 cfu/mL (n=3)

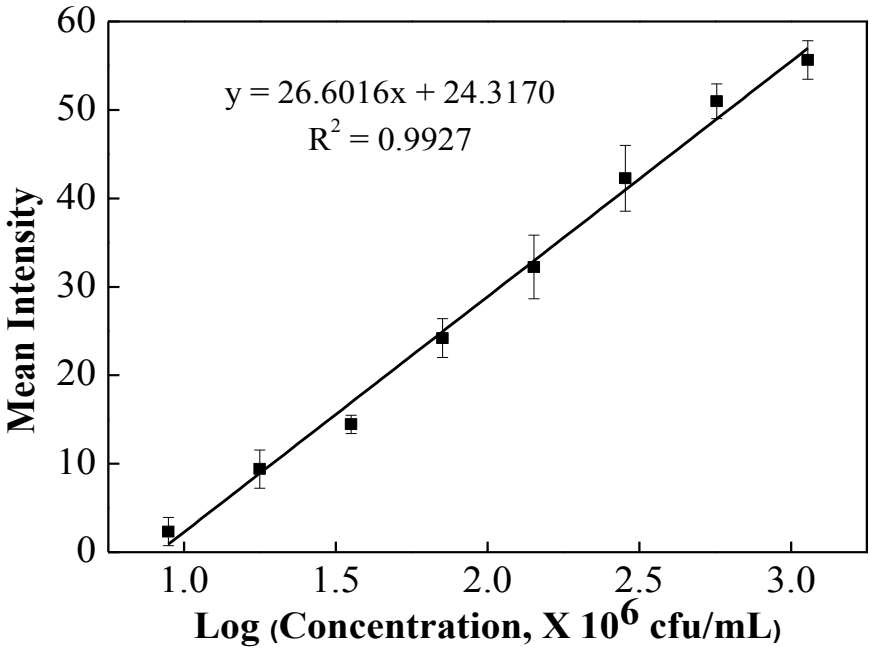

Figure 4.9 Linear region of *S. typhi* detection at the concentration between 8.88×10^6 and 1.14×10^9 cfu/mL (*n=3, slope=26.6016, intercept =24.3170, R^2=0.9927)*

4.4 Immunochromatography using IgG from murine serum as a model of the test strip

4.4.1 The effect of anti-mouse IgG concentration for the preparation of anti-mouse IgG-gold nanoparticles conjugate

For optimization of anti-mouse IgG using colorimetric assay, the minimum amount of anti-mouse IgG was determined by adding NaCl to gold nanoparticles containing different amounts of anti-mouse IgG. After reacting with NaCl, a gold nanoparticles solution containing an appropriate volume of anti-mouse IgG could be kept up red color (Figure 4.10, Table 4.1). A change in the color of solution was observed since small nanoparticles of gold were red and large nanoparticles were blue. When the amount of antibodies was not enough to bind to the surface of gold nanoparticles, NaCl allowed gold nanoparticles to aggregate and color of solution changed from red to blue [55]. In this work, 20 µL of anti-mouse IgG was confirmed to be the minimum amount for the stabilization of 2000 µL of gold nanoparticles. Therefore, the minimal anti-mouse IgG to stabilized gold was approximately at 0.009 mg/mL.

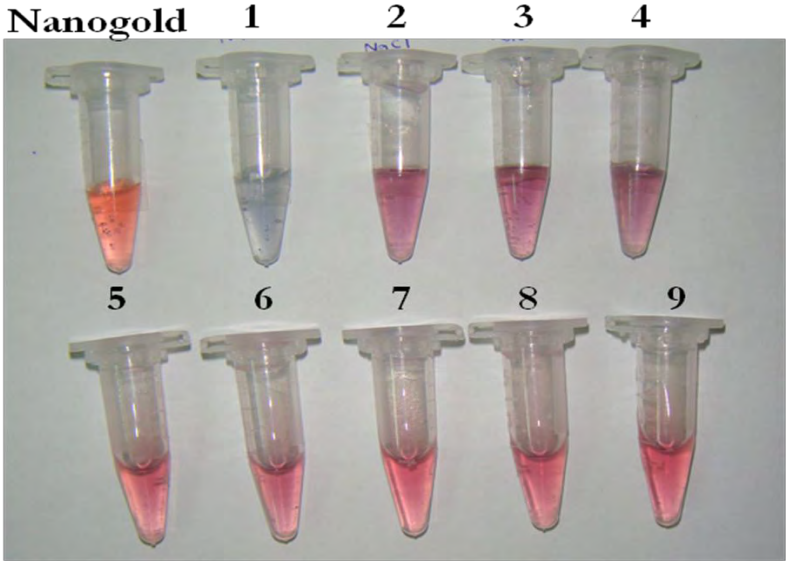

Figure 4.10 The colorimetric assay of gold nanoparticles (2,000 µL) conjugated with the different amount of anti-mouse IgG; 1: 0 µL, 2: 2.5 µL, 3: 5 µL, 4: 10 µL, 5: 20 µL, 6: 30 µL, 7: 40 µL, 8: 50 µL and 9: 60 µL

Table 4.1 The colorimetric assay of gold nanoparticles conjugated with the different amount of anti-mouse IgG

Fraction	Nanogold (µL)	Anti-mouse IgG (µL)	Concentration of anti-mouse IgG (µL)	Colors
1	2000	0	0	Blue
2	2000	2.5	0.001	Violet
3	2000	5	0.002	Violet
4	2000	10	0.005	Violet
5	2000	20	0.009	Red
6	2000	30	0.013	Red
7	2000	40	0.018	Red
8	2000	50	0.022	Red
9	2000	60	0.027	Red

For UV-vis spectrophotometer, the minimum amount of anti-mouse IgG was determined by adding NaCl to gold nanoparticles containing different amounts of anti-mouse IgG. After reaction with NaCl, a gold nanoparticles solution containing minimum anti-mouse IgG was optimized and characterized by UV-vis spectrophotometer. The absorbance at 520 nm which was the maximum absorbance of each tube was recorded (Table 4.2). From the results, the absorbance increased until 20 µL of anti-mouse IgG and then the absorbance was stable. The minimal anti-mouse IgG to stabilize gold nanoparticles was approximately 20 µL in 2000 µL of gold nanoparticels. Therefore, the minimal anti-mouse IgG to stabilized gold was approximately at 0.009 mg/mL which was similar to colorimetric assay.

Table 4.2 The absorbance of gold nanoparticles conjugated with the different amount of anti-mouse IgG at the wavelength of 520 nm

Fraction	Nanogold (µL)	Anti-mouse IgG (µL)	Concentration of anti-mouse IgG (µL)	Absorbance at 520 nm
1	2000	0	0	0.7512
2	2000	2.5	0.001	0.9908
3	2000	5	0.002	1.0149
4	2000	10	0.005	1.0159
5	2000	20	0.009	1.0325
6	2000	40	0.018	1.0296
7	2000	60	0.027	1.0308

4.4.2 The effect of time for incubating anti-mouse IgG and gold nanoparticles and final volume of anti-mouse IgG-gold nanoparticles conjugate

The time for incubating anti-mouse IgG and gold nanoparticles was optimized using direct immunochromatography and the results were shown in Figure 4.11. The size of test strip was 0.5 cm in width and 7.3 cm in length. The final volume of conjugate was studied at 100 µL and 200 µL because the volume less than 100 µL was not enough to suspend soft sediment of conjugate. The results were found that the 100 µL of conjugate provided better results than 200 µL because 100 µL gave more intensity in claret-red dot than the one of 200 µL. Therefore, the final volume of 100 µL was chosen as optimal value for preparing conjugate. The time of 30 min for incubating provided more intensity of claret-colored dot than 0 and 5 min and the color intensity of 60 min, 120 min and overnight was similar to 30 min. Therefore, 30 min was selected as the optimal time for incubating anti-mouse IgG and gold nanoparticles.

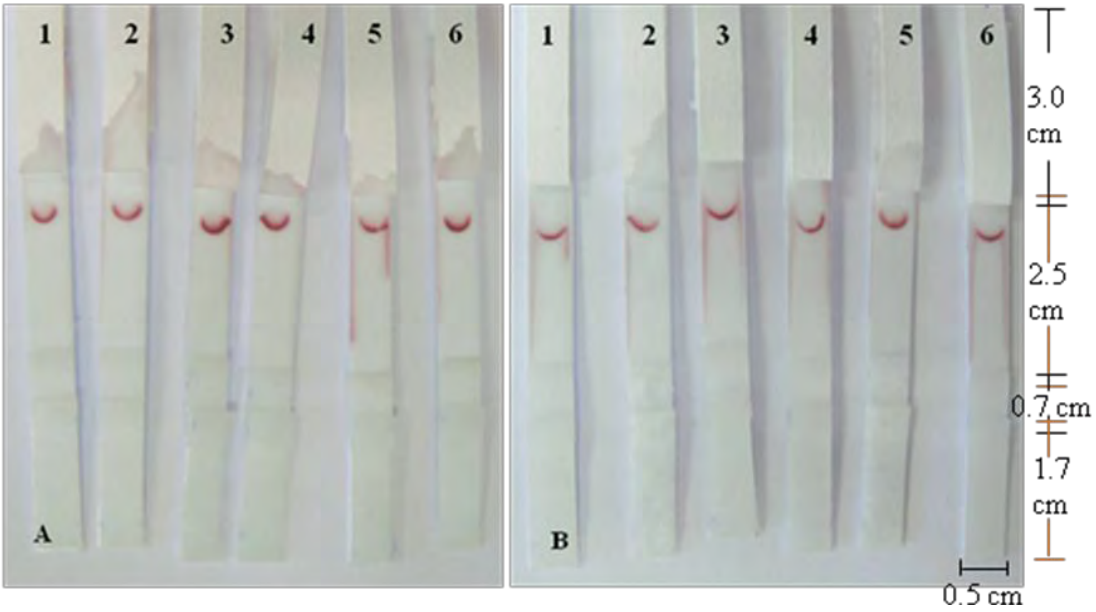

Figure 4.11 The effect of time for incubating anti-mouse IgG and gold nanoparticles with the final volume of conjugate of 100 µL (A) and 200 µL (B); 1: 0 min, 2: 5 min, 3: 30 min, 4: 60 min, 5: 120 min and 6: overnight

4.4.3 The effect of conditions for treating nitrocellulose membrane using direct immunochromatrography

The optimization of conditions for nitrocellulose membrane using direct immunochromatography was studied under 7 conditions including untreated, treated with PBS before dotting, treated with 1% BSA before dotting, treated with 1% BSA and 1% sucrose before dotting, treated with PBS after dotting, treated with 1% BSA after dotting and treated with 1% BSA and 1% sucrose after dotting. The results were shown in Figure 4.12. The size of test strip was 0.5 cm in width and 7.3 cm in length. The untreated membrane provided more rapid result and treated membrane after dotting gave clear color with more intensity in claret-colored dot. The treated membrane before dotting provided lower intensity of claret-colored dot than the others (untreated, treated after dotting). It could be explained that the running buffer moved through the membrane slowly because the membrane was blocked with treating solution. When the membrane was blocked, IgG from murine serum less adsorbed on the nitrocellulose membrane. Hence, the untreated membrane and treated membrane after dotting was selected to perform sandwich immunochromatography.

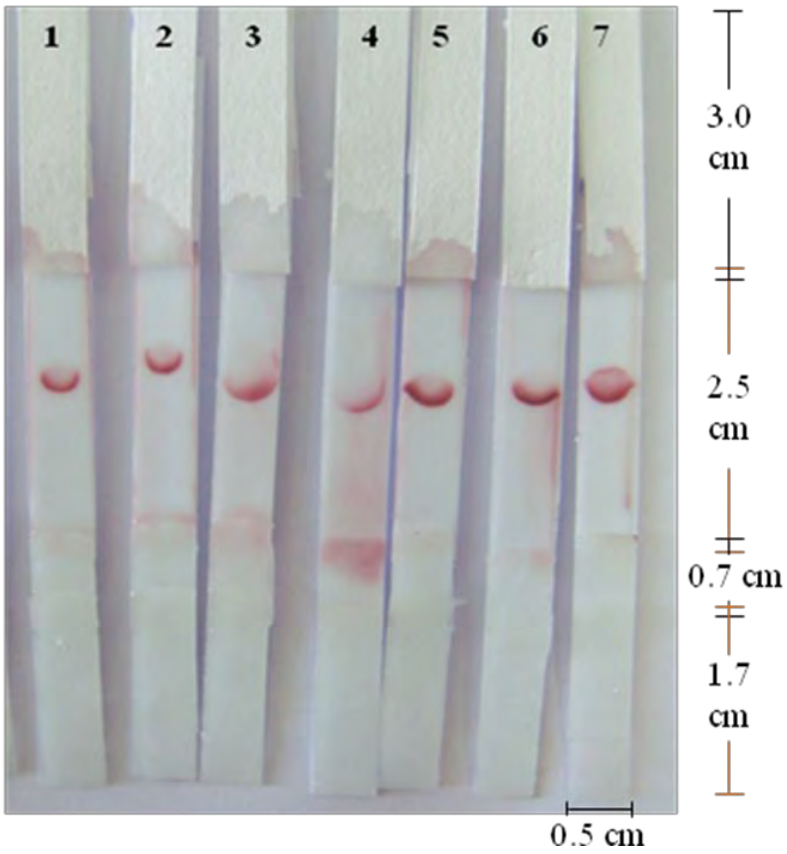

Figure 4.12 The effect of conditions for treating of nitrocellulose membrane using direct immunochromatography; 1: untreated, 2: treated with PBS before dotting, 3: treated with 1% BSA before dotting, 4: treated with 1% BSA and 1% sucrose before dotting, 5: treated with PBS after dotting, 6: treated with 1% BSA after dotting, 7: treated with 1% BSA and 1% sucrose after dotting

4.4.4 The effect of conditions for treating nitrocellulose membrane using sandwich immunochromatrography

The optimization of conditions for nitrocellulose membrane using sandwich immunochromatography was studied with the untreated membrane and treated membrane after dotting. The results were shown in Figure 4.13. The size of test strip was 0.5 cm in width and 7.3 cm in length. The untreated membrane provided better results than the treated membrane after dotting because solutions could flow through the membrane rapidly and the results appeared on nitrocellulose membrane quickly and completely. Therefore, the untreated membrane was selected for sandwich immunochromatography.

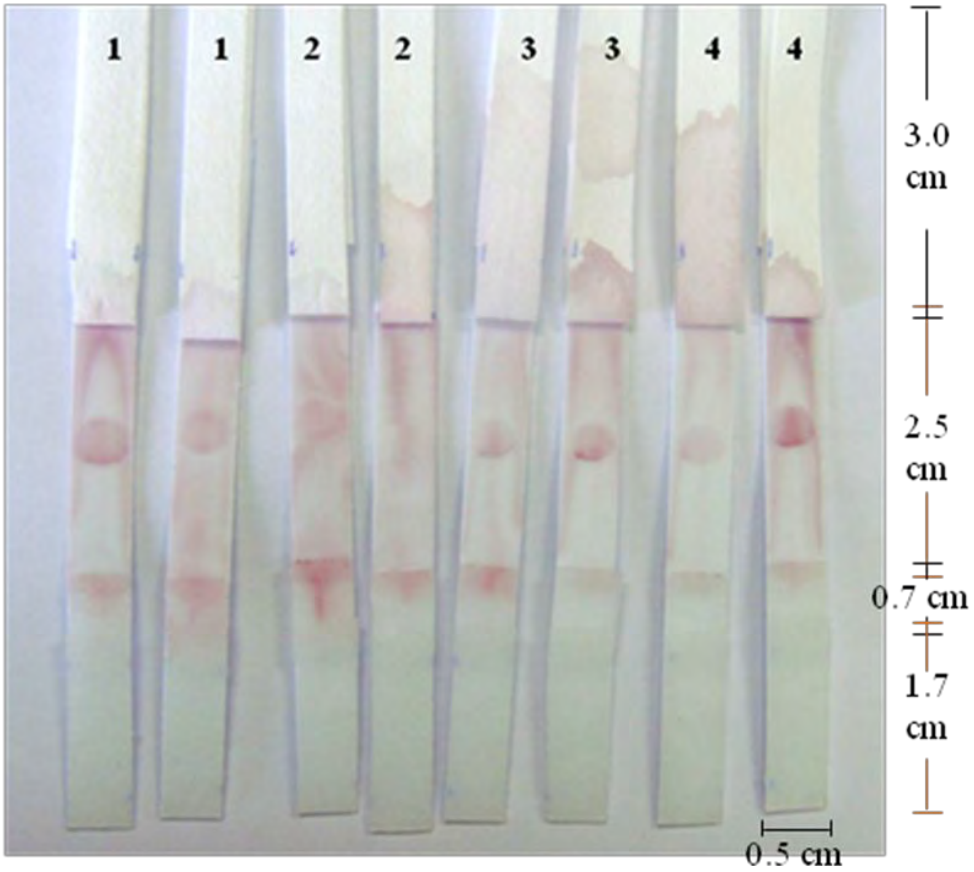

Figure 4.13 The effect of conditions for treating nitrocellulose membrane using sandwich immunochromatography; 1: untreated, 2: treated with PBS after dotting, 3: treated with 1% BSA after dotting, 4: treated with 1% BSA and 1% sucrose after dotting

4.4.5 The effect of IgG volume on nitrocellulose membrane

The IgG volume for dotting on nitrocellulose membrane was studied from 1 µL to 3 µL which the results were shown in Figure 4.14. The size of test strip was 0.5 cm in width and 7.3 cm in length. 1 µL of IgG provided better results than 2 and 3 µL of IgG because 2 and 3 µL were dotted using 1 µL for two and three times per each membrane on the same position on the nitrocellulose membrane. 2 and 3 µL, which were dotted for one time, could not be performed because they were more dispersed over an area of membrane. The results were found that the claret-colored dot of 2 and 3 µL dispersed on the nitrocellulose membrane and provided unsatisfactory results. Therefore, 1 µL of IgG was chosen as the optimal value for creating the test strip.

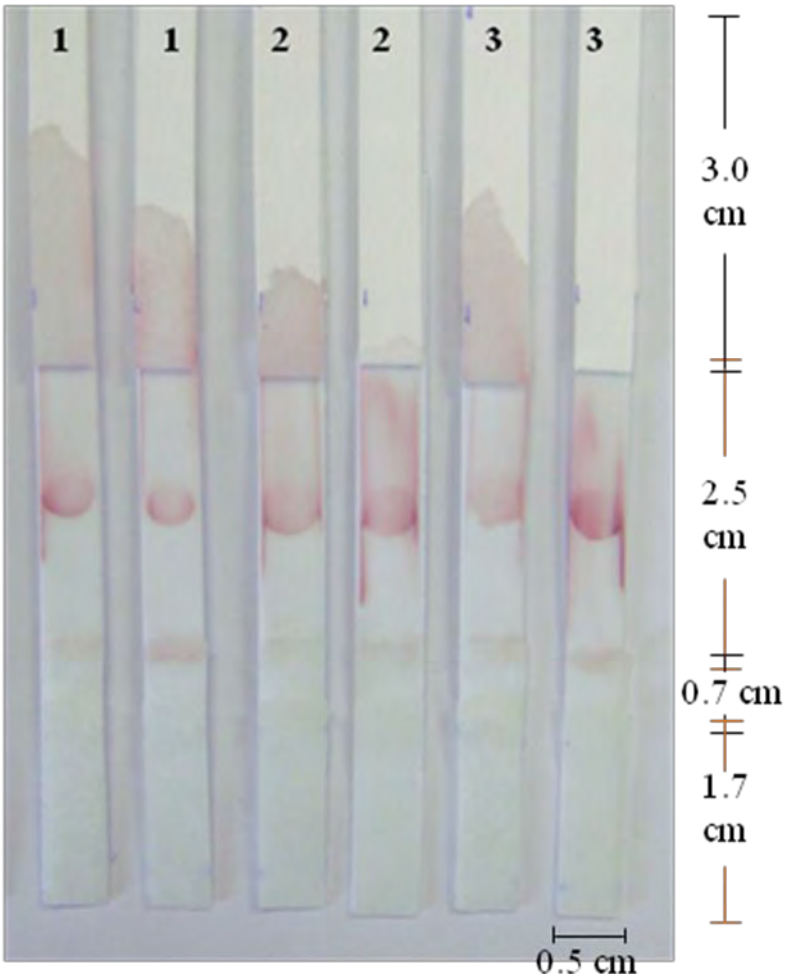

Figure 4.14 The effect of IgG volume using sandwich immunochromatography; 1: 1 µL, 2: 2 µL and 3: 3 µL

4.5 Immunochromatography for the detection of *S.typhi*

4.5.1 The effect of the length of the nitrocellulose membrane and the sample volume for applying on test strip

The length of the nitrocellulose membrane was studied at 2.5, 3.0 and 3.5 cm. The size of test strip was 0.5 cm in width and the different in length. To find the optimal volume of the sample, coloring solution was applied on the test strip with the volume of 100-120 µL for 2.5 cm of the nitrocellulose membrane as shown in Figure 4.15. The volume of 100 µL was the minimal volume that the sample could flow through the nitrocellulose membrane to the absorbent pad. Therefore, the volume of 100 µL was suitable for flow on the test strip. For the length of 3.0 cm and 3.5 cm, the results were shown in Figure 4.16 and 4.17 respectively. The optimal volume for 3.0 cm and 3.5 cm of the nitrocellulose membrane were 120 µL and 140 µL respectively.

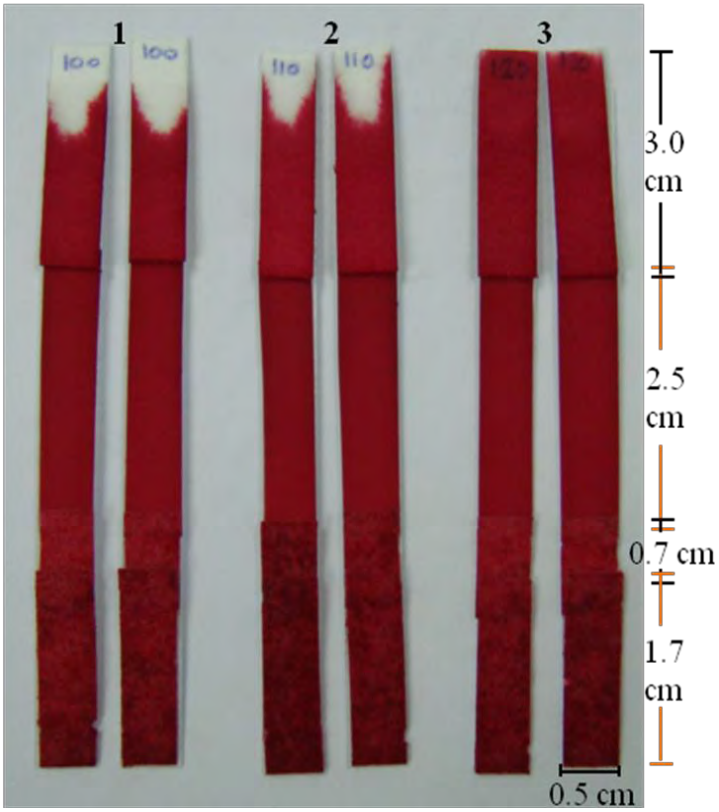

Figure 4.15 The effect of the sample volume at 2.5 cm of the nitrocellulose membrane; 1: 100 µL, 2: 110 µL and 3: 120 µL

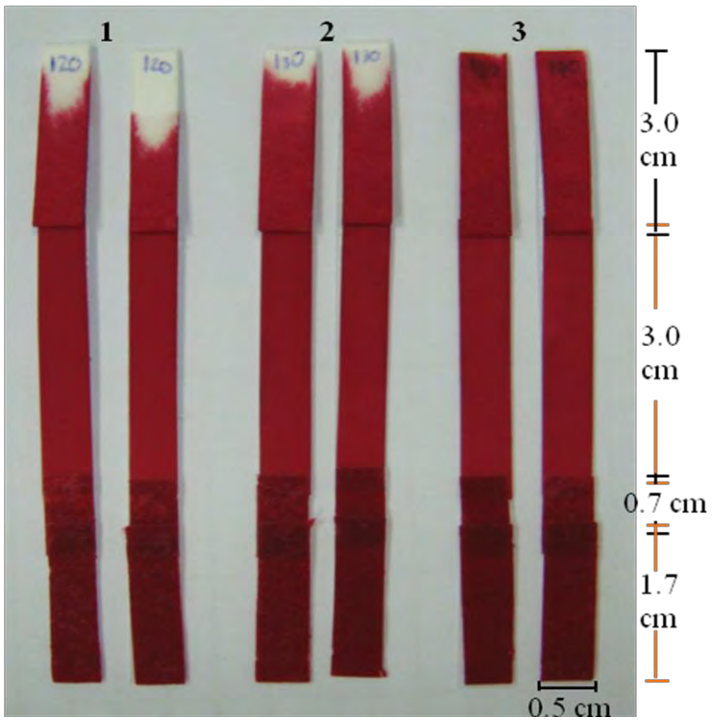

Figure 4.16 The effect of the sample volume at 3.0 cm of the nitrocellulose membrane; 1: 120 µL, 2: 130 µL and 3: 140 µL

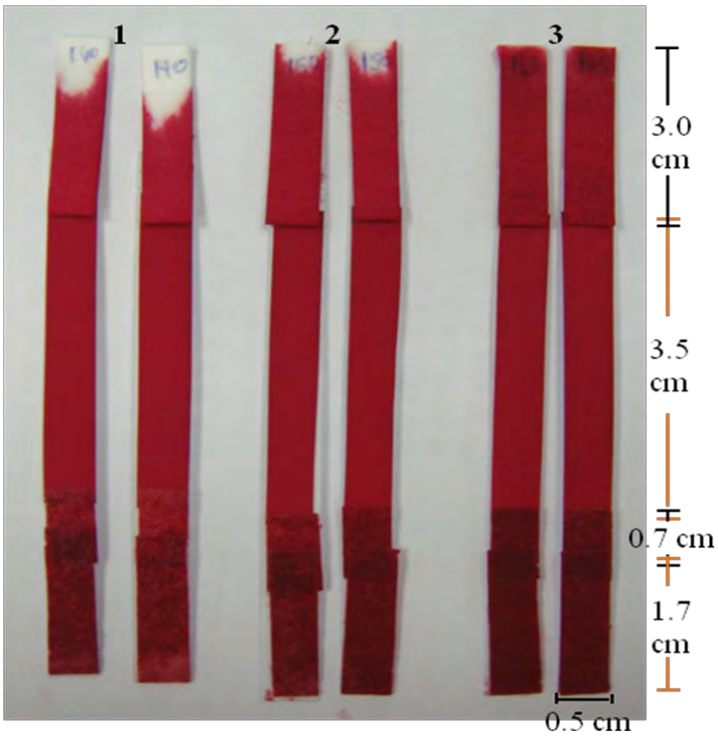

Figure 4.17 The effect of the sample volume at 3.5 cm of the nitrocellulose membrane; 1: 140 µL, 2: 150 µL and 3: 160 µL

The optimization of the length of nitrocellulose membrane was studied at 2.5, 3.0 and 3.5 cm which the results were shown in Figure 4.18. For negative results, only one control dot appeared on the nitrocellulose membrane. Positive results presented two claret-colored dots on the nitrocellulose membrane. For 2.5, 3.0 and 3.5 cm of the nitrocellulose membrane, the reaction completed within 5, 10 and 15 min respectively. The results showed that the color intensity of 3.5 cm provided the best result because there was more reaction time between *S. typhi* and polyclonal rabbit antibody-gold nanoparticles conjugate than the others. Furthermore, 4 cm of nitrocellulose membrane was also studied. Unfortunately, the reaction time for 4 cm was more than 30 min. Therefore, 3.5 cm was chosen as the optimal value.

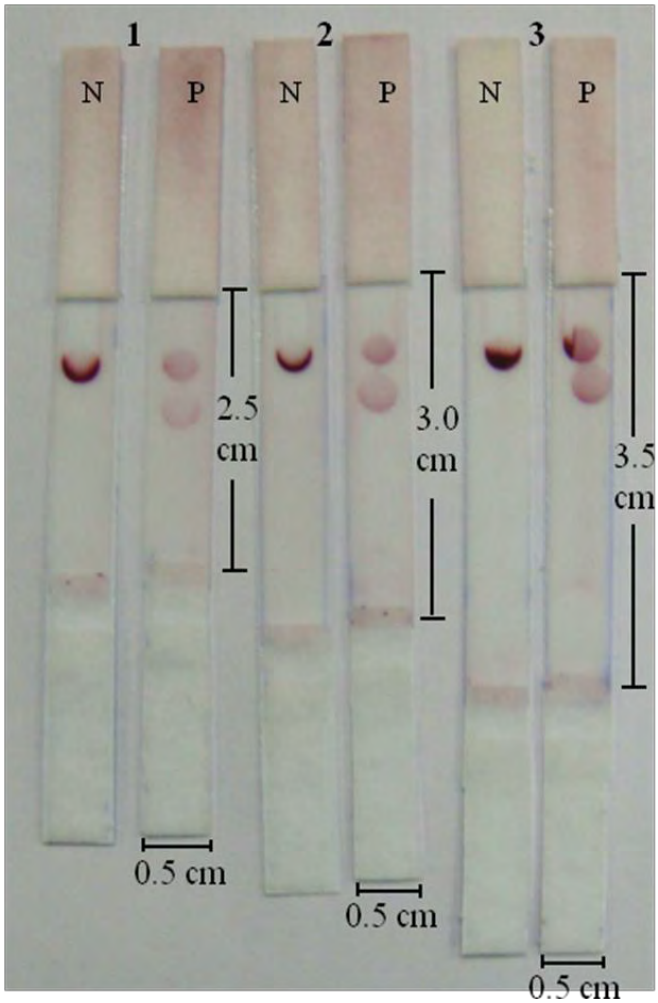

Figure 4.18 The effect of the length of the nitrocellulose membrane; N: negative result, P: positive result, 1: 2.5 cm, 2: 3.0 cm and 3: 3.5 cm

4.5.2 The effect of the running buffer and the additive

The effect of the running buffer was studied using PBS, 1% BSA, 3% BSA and 10% BSA which the results were shown in Figure 4.19. The size of test strip was 0.5 cm in width and 8.3 cm in length. The higher concentration than 10% BSA was not studied because it cannot dissolve in PBS. For PBS, no dot appeared on the nitrocellulose membrane because PBS could not block the membrane. Therefore, polyclonal rabbit antibody-gold nanoparticles conjugate could absorb on the surface of the nitrocellulose membrane before the solution flow to test zone and control zone.

1% BSA provided the flow of buffer slower than PBS because BSA was blocking reagent which could block the nitrocellulose membrane. Therefore, polyclonal rabbit antibody-gold nanoparticles conjugate had less absorption on the surface of the nitrocellulose membrane before the solution flow to test zone and control zone. However, the reaction time between *S. typhi* and polyclonal rabbit antibody-gold nanoparticles conjugate still was not enough. The complex of *S. typhi* and conjugate could not bind with polyclonal rabbit antibody on the membrane. Thus, only control dots appeared.

3% BSA and 10% BSA can slow down the flow of buffer, so two claret-colored dots appeared on membrane. The color intensity of 10% BSA provided the best result because there was more reaction time between *S. typhi* and polyclonal rabbit antibody-gold nanoparticles conjugate than 3% BSA. Therefore, 10% BSA was chosen as optimal value of running buffer.

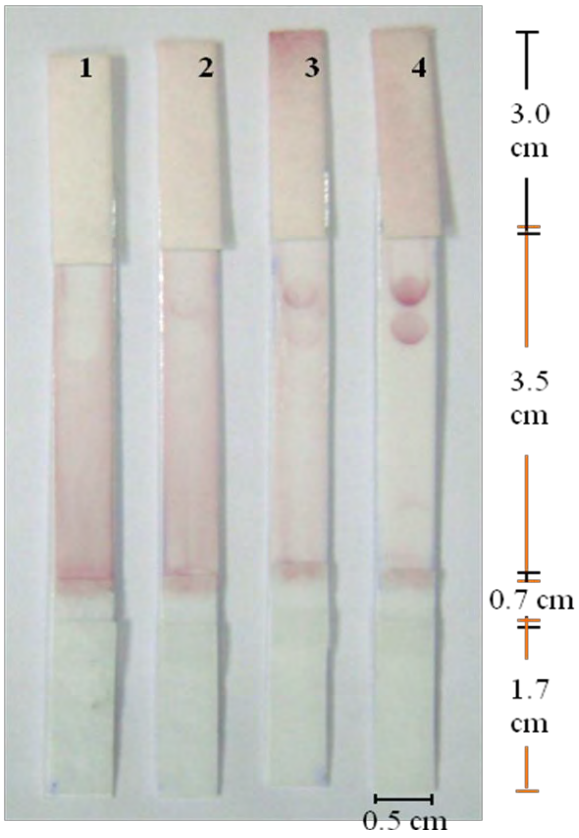

Figure 4.19 The effect of the running buffer; 1: PBS, 2: 1% BSA, 3: 5% BSA and 4: 10% BSA

An additive such as PEG or surfactant was applied to immunoassay to reduce the background in analysis [56]. In this work, the additive (Tween, gelatin and PEG) was applied in running buffer which might improve the effectiveness of results. The effect of the additive was studied by adding 0.05% Tween, 0.1% gelatin and 1% PEG 400 to 10% BSA. The running buffer with additive was compared to the running buffer without additive which the results were shown in Figure 4.20. The results showed that the color intensity of the test dot and the control dot of PEG 400 and no additive were better than Tween and gelatin. Therefore, PEG 400 and no additive were selected for the optimization of the running buffer later.

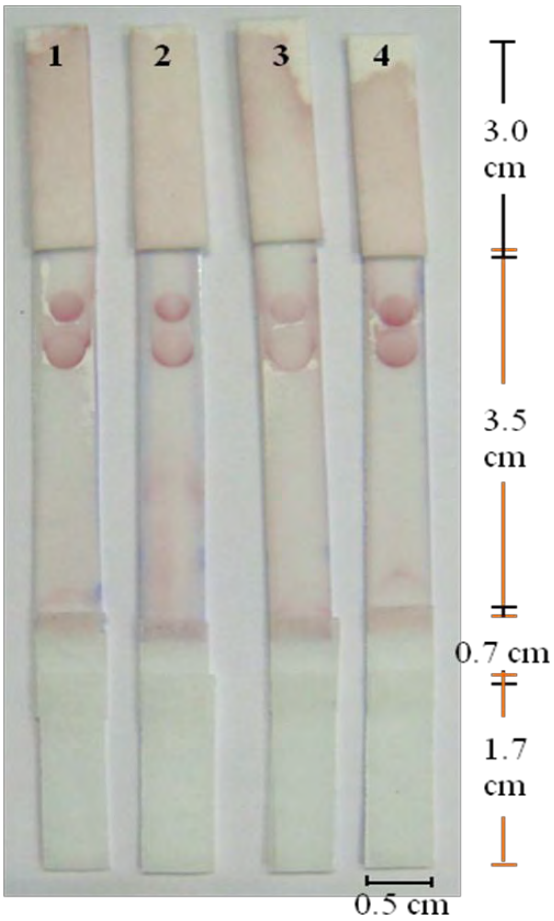

Figure 4.20 The effect of the additive in the running buffer; 1: 10% BSA, 2: 0.05% Tween in 10% BSA, 3: 0.1% gelatin in 10% BSA and 4: 1% PEG 400 in 10% BSA

The running buffer was later optimized using no additive and PEG 400. The concentration of PEG 400 in 10% BSA was studied at 0.5, 1, 2 and 3%. The results were shown in Figure 4.21. The color intensity of the test dots of no additive was similar to 0.5, 1, 2 and 3% PEG but the preparation of the solution without additive was more simple and the complete reaction of no additive was quicker than the solution with PEG 400. Therefore, 10% BSA without additive was selected as the running buffer.

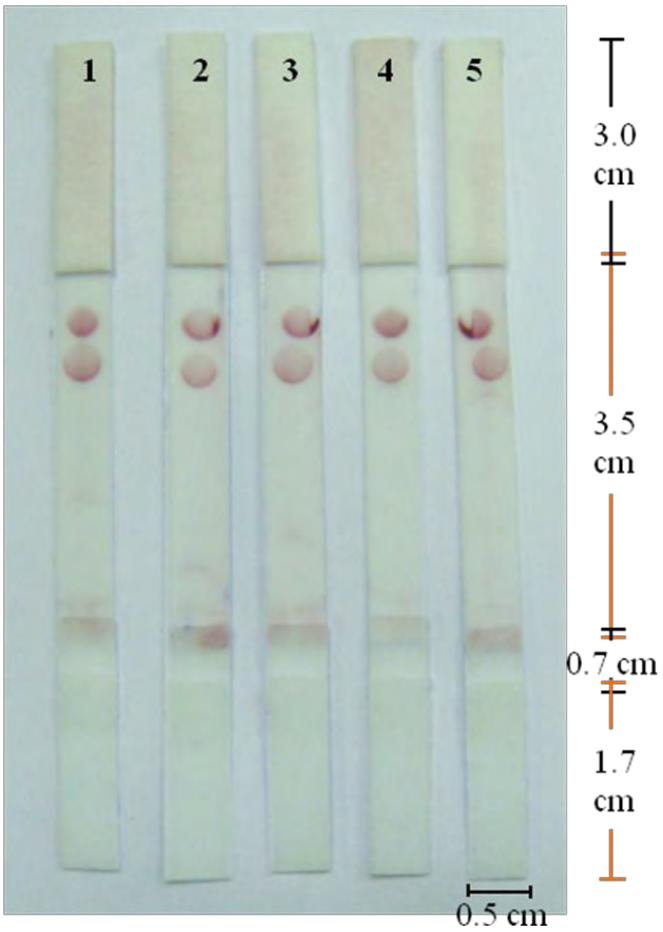

Figure 4.21 The effect of the additive in the running buffer; 1: 10% BSA, 2: 0.5% PEG 400 in 10% BSA, 3: 1% PEG 400 in 10% BSA, 4: 2% PEG 400 in 10% BSA and 5: 3% PEG 400 in 10% BSA

4.5.3 The effect of the volume of polyclonal rabbit antibody-gold nanoparticles conjugate

For the design of strip test, the maximum volume of solution absorbed in the conjugate pad was 9 µL so the volume of polyclonal rabbit antibody-gold nanoparticles conjugate for creating the conjugate pad was studied at 3, 6 and 9 µL. The results were shown in Figure 4.22. The size of test strip was 0.5 cm in width and 8.3 cm in length. The results found that the color intensity of 9 µL provided the best result because the volume of polyclonal rabbit antibody-gold nanoparticles conjugate was enough to react with *S. typhi* completely. Therefore, the volume of 9 µL was selected.

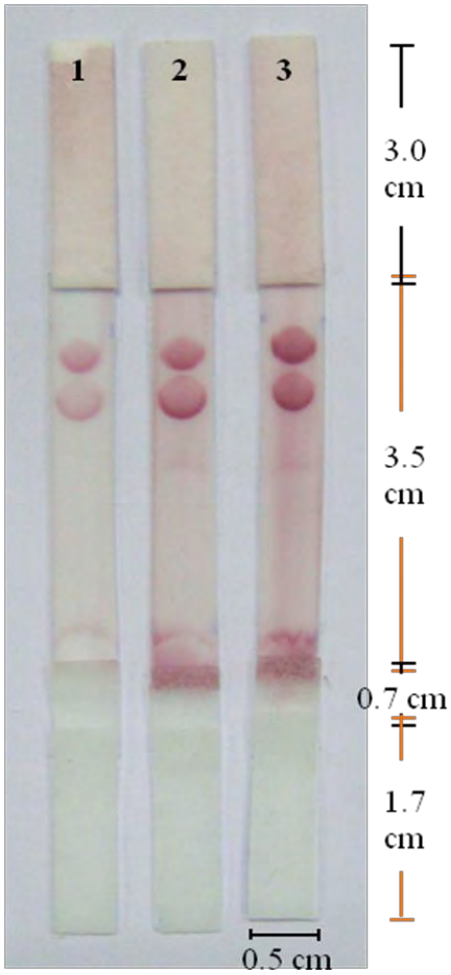

Figure 4.22 The effect of the volume of polyclonal rabbit antibody-gold nanoparticles conjugate for creating conjugate pad; 1: 3 µL, 2: 6 µL and 3: 9 µL

4.5.4 The effect of concentration of *S. typhi*

The concentration of *S. typhi* was studied between 1.14×10^3 cfu/mL and 1.14×10^8 cfu/mL which the results were shown in Figure 4.23. The size of test strip was 0.5 cm in width and 8.3 cm in length. The results showed that two claret-colored dots displayed until 1.14×10^5 cfu/mL of *S. typhi*. At the lower concentration than 1.14×10^5 cfu/mL, only one control dot appeared on nitrocellulose membrane. Therefore, the limit of detection (LOD) was found to be 1.14×10^5 cfu/mL of *S. typhi* which was evaluated by the naked eye within 15 min. The advantage of the detection of *S. typhi* using immunochromatography over dot blot assay (LOD: 8.88×10^6 cfu/mL, 1 h and 50 min) was rapid, simple, and low LOD. Thus, the immunochromatography provided attractive methods for the detection of *S. typhi*.

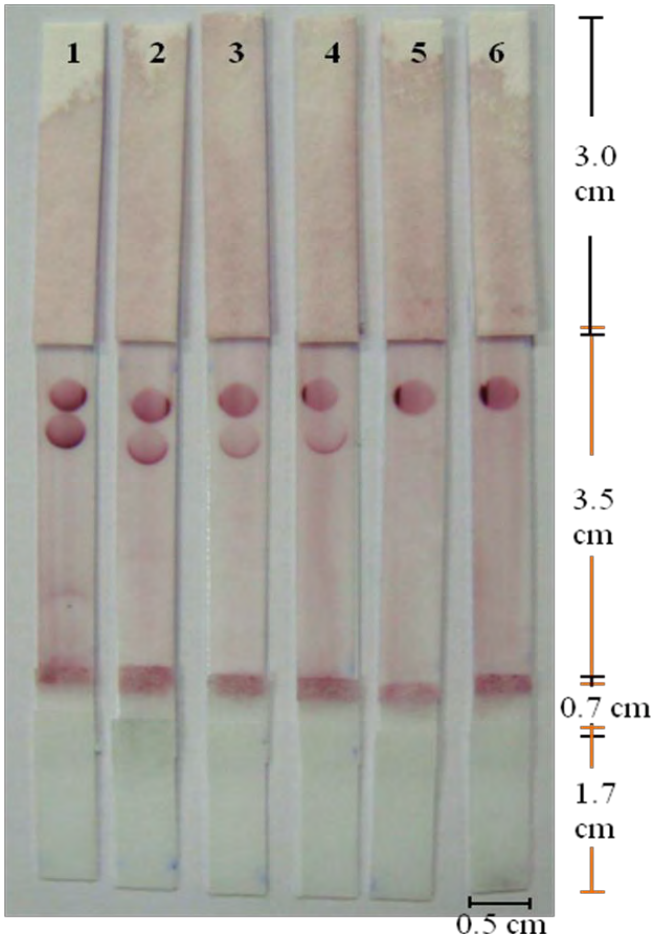

Figure 4.23 The effect of concentration of *S. typhi* in running buffer; 1: 1.14×10^8 cfu/mL, 2: 1.14×10^7 cfu/mL, 3: 1.14×10^6 cfu/mL, 4: 1.14×10^5 cfu/mL, 5: 1.14×10^4 cfu/mL and 6: 1.14×10^3 cfu/mL

4.5.5 The application of immunochromatography for the detection of *S. typhi* in human serum

To evaluate this method, the immunochromatography was applied to detect *S. typhi* in human serum. The different concentrations of *S. typhi* between 1.14 × 10^5 cfu/mL and 1.14 × 10^8 cfu/mL were spiked to normal human serum. The results were shown in Figure 4.24. The size of test strip was 0.5 cm in width and 8.3 cm in length. The results showed that two claret-colored dots appeared on all nitrocellulose membranes. Therefore, sandwich immunochromatography was effective for the detection of *S. typhi* in the serum sample.

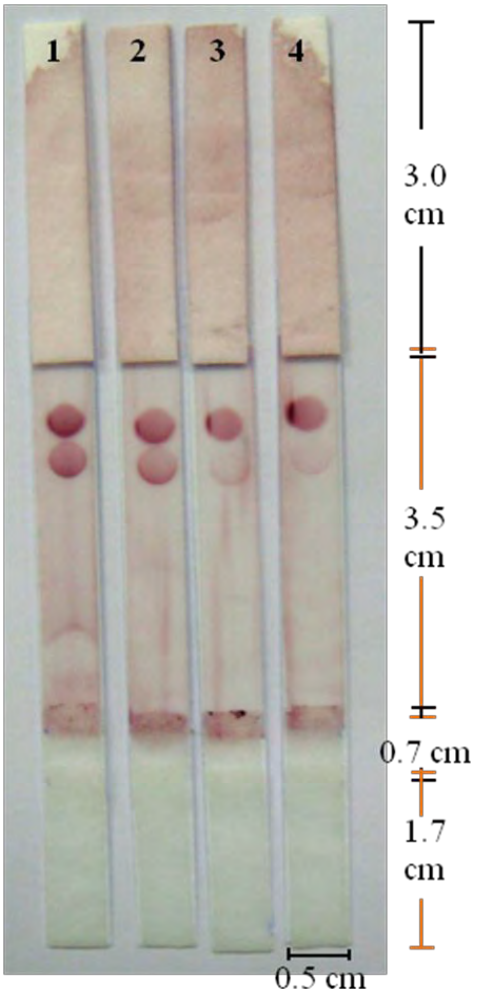

Figure 4.24 The detection of *S. typhi* in human serum; 1: 1.14 × 10^8 cfu/mL, 2: 1.14 × 10^7 cfu/mL, 3: 1.14 × 10^6 cfu/mL and 4: 1.14 × 10^5 cfu/mL

CHAPTER V

CONCLUSIONS

5.1 Conclusions

In this work, an alternative approach to the rapid detection of *S. typhi* using dot blot immunoassay coupled with optical detection, and immunochromatography was developed. *S. typhi* was detected using specific binding reaction between antigens of *S. typhi* O901 and polyclonal rabbit antibody for polysaccharides of *S. typhi* O901. Gold nanoparticles (average size of 15 nm) were used as label that bound with polyclonal rabbit antibody to form polyclonal rabbit antibody-gold nanoparticles conjugate. The minimal polyclonal rabbit antibody to stabilized gold nanoparticles was approximately at 0.5 mg/mL.

For dot blot immunoassay, the limit of detection of *S. typhi* was 0.14 mg/mL and 8.88×10^6 cfu/mL which could be visually evaluated by the naked eye within 1 h and 50 min. For optical detection, the resulting membrane was captured by a scanner and the image was converted into gray scale. The mean intensity for each data point was obtained from histogram in AdobePhotoshop and the relationship between mean intensity and the concentration of *S. typhi* (mg/mL) was plotted. Linear region was observed with a good coefficient of 0.9986. For the concentration in cfu/mL, optical detection could not be performed because the color of dot irregularly dispersed. Thus, the mean intensity could not be evaluated.

Immunochromatography was developed using IgG from murine serum as a model of the test strip. The optimal ratio of anti-mouse IgG and gold nanoparticles for the formation of conjugate was found to be 1:100. The optimization of conditions for nitrocellulose membrane was studied under 7 conditions which the untreated membrane provided the best results. The IgG volume for dotting on nitrocellulose membrane was 1 μL. The test strip of IgG using direct and sandwich immunochromatography was successfully performed. Then, immunochromatography for the detection of *S. typhi* was developed. The sandwich immunochromatographic strip consisted of three pads (sample, conjugate, and absorbent pads) and one

nitrocellulose membrane. 9 µL of polyclonal rabbit antibody-gold nanoparticles conjugate was applied to the glass fiber membrane to be used as the conjugate pad (0.5 × 0.7 cm). 1 µL of polyclonal rabbit antibody for polysaccharides of *S. typhi* O901 and goat anti-rabbit IgG were dotted on nitrocellulose membrane (0.5 × 3.5 cm) to create the test dot and the control dot respectively. The distance between the control dot and the top of the nitrocellulose membrane was 0.7 cm and the distance between the control dot and the test dot was 0.3 cm. The glass fiber membrane and the cellulose absorbent sample pad were used as the sample pad (0.5 × 1.7 cm) and the absorbent pad (0.5 × 3.0 cm) without treatment. The nitrocellulose membrane was firstly attached onto polyethylene sheet. After that, the conjugate pad was attached on the bottom of the nitrocellulose membrane. The sample pad was then attached on the bottom of the conjugate pad. Finally, the absorbent pad was attached on the top of the nitrocellulose membrane. The size of the test strip was found to be 0.5 cm in width and 8.3 cm in length. 140 µL of 10% BSA containing *S. typhi* was used as the running buffer. For the negative results, only one control dot appeared on nitrocellulose membrane. The positive results presented two claret-colored dots on nitrocellulose membrane. The limit of detection of this method was found to be 1.14×10^5 cfu/mL of *S. typhi* which the results could be evaluated by naked eye within 15 min. To compare the results, immunochromatography (LOD: 1.14×10^5 cfu/mL, 15 min) provided lower LOD and reaction time than dot blot immunoassay (LOD: 8.88×10^6 cfu/mL, 1 h and 50 min). From these results, it can be concluded that immunochromatography is very rapid and easy method for the detection of *S. typhi*. In addition, immunochromatography was applied to detect *S. typhi* in normal human serum effectively. Therefore, immunochromatography offers an attractive method for the detection of *S.typhi* in clinical diagnosis.

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VITA

NAME : Miss Pattarachaya Preechakasedkit

ADDRESS : 519 Moo 3, Sriprachan, Sriprachan, Suphanburi 72140

E-MAIL : may_thekop@hotmail.com

PERSONAL INFORMATIOM

Date of birth: April 26, 1985 **Race:** Thai

Nationality: Thai **Religion:** Buddhism

Domicile/Habitation: Suphanburi **Gender:** Female

EDUCATION

2007-2010 : Chulalongkorn University, Master of Science in Biotechnology

2002-2006 : Thammasat University, Bachelor of Science in Biotechnology

2000-2002 : Triamudom Suksa School, Major of Science-Mathematic

PROCEEDING

1. **Preechakasedkit, P.;** Siangproh, W.; Chaicumpa, W.; Tongtawe, P.; and Chailapakul, O. (21-23 January, 2010) *Salmonella typhi* detection using dot blot assay based on gold nanoparticles. Proceeding of PACCON2010 (Pure and Applied Chemistry International Conference), Ubon Ratchathani, Thailand.
2. **Preechakasedkit, P.;** Siangproh, W.; Chaicumpa, W.; Tongtawe, P.; and Chailapakul, O. (26-28 February, 2010) Dot Blot Assay coupled with Optical Detection for Low-cost Diagnosis. Proceeding of International Conference on Chemical Engineering and Applications (CCEA 2010), Singapore, Singapore.