การพัฒนาชุดตรวจแอนติเจนของเชื้อเลปโตสไปราสายพันธุ์ก่อโรคด้วย
เทคนิค Lateral Flow Immunoassay โดยอาศัยอนุภาคทองคำระดับนาโน

นายประยูร แลงี

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Development of Gold Nanoparticle-Based Lateral Flow Immunoassay for Detection of Pathogenic Leptospiral Antigen

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ประยูร แลงี : การพัฒนาชุดตรวจแอนติเจนของเชื้อเลปโตสไปราสายพันธุ์ก่อโรคด้วยเทคนิค Lateral Flow Immunoassay โดยอาศัยอนุภาคทองคำระดับนาโน (Development of Gold Nanoparticle-Based Lateral Flow Immunoassay for Detection of Pathogenic Leptospiral Antigen) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. พญ. กนิษฐา ภัทรกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. นพ.อมรพันธุ์ เสรีมาศพันธุ์, 156 หน้า.

โรคเลปโตสไปโรซิสซึ่งมีสาเหตุจากเชื้อเลปโตสไปราสายพันธุ์ก่อโรค เป็นโรคติดต่อจากสัตว์สู่คนที่เกิดอุบัติซ้ำ มีการแพร่กระจายทั่วโลกและมักเป็นโรคประจำถิ่นของประเทศ โดยเฉพาะอย่างยิ่งบริเวณเขตร้อนและกึ่งเขตร้อนรวมถึงประเทศไทย การตรวจทางห้องปฏิบัติการ ณ จุดดูแลผู้ป่วย (Point-of-Care Testing, POCT) อาศัยเครื่องมือในการตรวจวินิฉัยโรคอย่างง่าย เพื่อให้การวินิฉัยโรคอย่างรวดเร็วและให้การรักษาผู้ป่วยอย่างเหมาสม โดยเฉพาะอย่างยิ่งในพื้นที่ขาดแคลนเครื่องมือ ชุดแถบตรวจโรคแบบแลทเทอรัลโฟลว์ (Lateral flow immunoassay, LFA) เป็นเครื่องมือที่ให้ผลการวิเคราะห์ที่รวดเร็ว ใช้ง่าย ราคาถูก มีขั้นตอนเดียวในการวิเคราะห์ และไม่จำเป็นต้องใช้เครื่องมือที่ยุ่งยากซับซ้อน ลิโพโปรตีนของเชื้อเลปโตสไปราขนาด 32 กิโลดาลตัน หรือโปรตีน LipL32 เป็นโปรตีนเป้าหมายที่นิยมนำมาใช้เพื่อพัฒนาชุดตรวจโรคในรูปแบบต่างๆสำหรับวินิจฉัยโรคเลปโตสไปโรซิส เนื่องจาก LipL32 เป็นโปรตีนสำคัญหลักของผนังเซลล์ชั้นนอกที่จำเพาะต่อเชื้อเลปโตสไปราสายพันธุ์ก่อโรคเป็นส่วนใหญ่ การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาชุดตรวจหาแอนติเจนของเชื้อเลปโตสไปราสายพันธุ์ก่อโรคด้วยชุดแถบตรวจโรคแบบแลทเทอรัลโฟลว์โดยอาศัยอนุภาคทองคำระดับนาโน โดยใช้แอนติบอดีที่จำเพาะต่อโปรตีน LipL32 ได้แก่ โมโนโคลนอลแอนติบอดีผลิตในหนู โคลนที่ 3 และ 82 และโพลิโคลนอลแอนติบอดีผลิตในกระต่าย ซึ่งสามารถจับอย่างจำเพาะต่อเชื้อเลปโตสไปราสายพันธุ์ก่อโรคที่เป็นสายพันธุ์อ้างอิงในประเทศไทยทั้งหมด 22 ซีโรวาร์ แต่ไม่จับกับเชื้อเลปโตสไปราสายพันธุ์ไม่ก่อโรคทั้ง 2 ซีโรวาร์ การวิเคราะห์ด้วยเทคนิค competitive inhibition-based enzyme-linked immunosorbent assay พบว่าโมโนโคลนอลแอนติบอดีโคลนที่ 3 และ 82 จับเอพิโทปที่แตกต่างกันบนโปรตีน LipL32 จากการศึกษานี้พบว่าชุดแถบตรวจโรคแบบแลทเทอรัลโฟลว์ที่ประกอบด้วยโมโนโคลนอลแอนติบอดีต่อโปรตีน LipL32 โคลน 82 ติดบนอนุภาคทองคำระดับนาโน (anti-LipL32 mAb82 conjugated AuNPs) แล้วตรึงบน conjugate pad กับโพลิโคลนอลแอนติบอดีต่อโปรตีน LipL32 ที่ตรึงบนแถบทดสอบ (test line) ให้ผลการทดสอบดีที่สุด กล่าวคือให้ปริมาณต่ำสุดที่ตรวจพบได้ (limit of detection, LoD) มีค่าต่ำที่สุดเมื่อเทียบกับรูปแบบอื่น ในงานวิจัยนี้สามารถสังเคราะห์อนุภาคทองคำระดับนาโนเมตรด้วยวิธี seeded growth synthesis ได้ขนาดแตกต่างกันคือ 10, 20, 30 และ 40 นาโนเมตร จากการทดสอบอิทธิพลของขนาดอนุภาคทองคำระดับนาโนต่อความไว (sensitivity) ของชุดแถบตรวจโรคนี้ พบว่าอนุภาคทองคำระดับนาโนขนาด 40 นาโนเมตรซึ่งติดด้วยโมโนโคลนอลแอนติบอดีโคลนที่ 82 สามารถตรวจพบเชื้อเลปโตสไปราสายพันธุ์ก่อโรคที่เติมลงในตัวอย่างซีรัมได้ในปริมาณที่น้อยกว่า 10 และ 100 เท่า เมื่อเทียบกับใช้อนุภาคทองคำระดับนาโน ขนาด 30 และ 20 นาโนเมตร ตามลำดับ ในงานวิจัยนี้พบว่าชุดแถบตรวจโรคแบบแลทเทอรัลโฟลว์ที่ได้จากการปรับสภาวะที่เหมาะสมแล้ว สามารถตรวจพบเชื้อเลปโตสไปราสายพันธุ์ก่อโรคในตัวอย่างซีรัมได้น้อยที่สุดเท่ากับ 5×10^3 เซลล์ นอกจากนี้ เมื่อทดสอบเบื้องต้นด้วยตัวอย่างซีรัมจากผู้ป่วยโรคเลปโตสไปโรซีสระยะเริ่มต้นและซีรัมจากผู้ป่วยโรคต่างๆ ที่ไม่เกี่ยวข้อง เพื่อประเมินประสิทธิภาพของชุดแถบตรวจโรคแบบแลทเทอรัลโฟลว์นี้ พบว่าเมื่อมีการเตรียมตัวอย่างซีรัมของผู้ป่วยก่อนทดสอบด้วย Tween 20 ที่ความเข้มข้นสุดท้ายประมาณร้อยละ 4.5 สามารถเพิ่มความไวของชุดแถบตรวจโรคนี้ได้เมื่อเทียบกับตัวอย่างที่ไม่ได้เตรียมด้วย Tween20 โดยเพิ่มจากร้อยละ 6 (ผลบวก 3 ใน 50 ตัวอย่าง) เป็นร้อยละ 24 (ผลบวก 12 ใน 50 ตัวอย่าง) นอกจากนี้ พบว่าผลการทดสอบเป็นลบในทุกตัวอย่างซีรัมของผู้ป่วยโรคที่ไม่เกี่ยวข้องและคนสุขภาพดี ทั้งตัวอย่างที่มีการเตรียมและไม่ได้เตรียมด้วย Tween 20 ดังนั้น ชุดแถบทดสอบนี้มีความจำเพาะ (specificity) ร้อยละ 100 อย่างไรก็ตาม ชุดแถบตรวจโรคแบบแลทเทอรัลโฟลว์ที่พัฒนาขึ้นในการศึกษานี้ยังมีความไวต่ำ ดังนั้น จึงจำเป็นต้องนำกลยุทธ์ใหม่ๆ มาพัฒนาประสิทธิภาพของชุดแถบตรวจโรคต้นแบบนี้ให้มีความไวเพิ่มขึ้นในการตรวจจับแอนติเจนของเชื้อเลปโตสไปราในตัวอย่างผู้ป่วย เพื่อให้สามารถใช้ตรวจวินิจฉัยโรคเลปโตสไปโรซิสในระยะเริ่มต้นได้ดียิ่งขึ้นต่อไปในอนาคต

สาขาวิชา จุลชีววิทยาทางการแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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PRAYOON LAE-NGEE: Development of Gold Nanoparticle-Based Lateral Flow Immunoassay for Detection of Pathogenic Leptospiral Antigen. ADVISOR: ASSOC. PROF. KANITHA PATARAKUL, M.D., Ph.D., CO-ADVISOR: ASST. PROF. AMORNPUN SEREEMASPUN, M.D., Ph.D., 156 pp.

Leptospirosis, caused by pathogenic *Leptospira* spp., is a global re-emerging zoonosis and endemic disease in tropical and sub-tropical countries including Thailand. Point-of-care testing (POCT) for rapid diagnosis of leptospirosis is needed for prompt and appropriate treatment particularly in resource-poor settings. Lateral flow immunoassay (LFA)-based POCT devices are rapid, user friendly, cheap, have one-step analysis, and need no sophisticated equipment. LipL32 is a common target for diagnostic tests of leptospirosis because it is the major outer membrane protein (OMP) that is specific and highly conserved among pathogenic *Leptospira*. This study aimed to develop the gold nanoparticle (AuNP)-based LFA for detection of pathogenic leptospiral antigen. To develop the LFA, this study used two clones of anti-LipL32 mouse monoclonal antibodies (mAb3 and mAb82) and a rabbit anti-LipL32 polyclonal antibody (pAb) that showed specific binding to all 22 reference pathogenic *Leptospira* serovars found in Thailand and did not bind to 2 non-pathogenic serovars. These mAb3 and mAb82 bind different epitopes on LipL32 as demonstrated by the competitive inhibition-based enzyme-linked immunosorbent assay. The lowest limit of detection (LoD) was achieved by the LFA comprising anti-LipL32 mAb82-conjugated AuNPs on the conjugate pad and the anti-LipL32 pAb on the test line. Different sizes of AuNPs including 10, 20, 30 and 40 nm in diameter were successfully synthesized by seeded growth synthesis using citrate reduction. The LoD of 40-nm mAb82-conjugated AuNPs was 100- and 10-fold lower than that of 20-nm and 30-nm AuNPs, respectively, to detect sonicated *L. interrogans* serovar Pomona spiked in sera. In the present study, the lowest LoD obtained after various optimization of anti-LipL32 mAb82-conjugated AuNP-based LFA was 5×10^3 cells of whole cell lysates in sera. This AuNP-based LFA was evaluated with acute sera from patients with leptospirosis and unrelated diseases. Pretreatment of sera with 4.5% Tween 20 improved the sensitivity of the LFA from 6% (3 of 50) to 24% (12 of 50) to detect acute phase sera from known cases of leptospirosis. Negative detection was observed in all 30 (100%) pretreated and untreated sera of patients with unrelated diseases and healthy person resulting in 100% specificity of this LFA. The new strategies are required to improve the sensitivity of this potential anti-LipL32 mAb82 conjugated AuNP-based LFA for better diagnosis of acute phase leptospirosis in the future.

Field of Study: Medical Microbiology
Academic Year: 2017

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I

INTRODUCTION

Leptospirosis, caused by pathogenic *Leptospira* species, is a worldwide reemerging zoonosis. It is an endemic disease with increasing outbreaks in tropical and sub-tropical regions (1-3) including Southeast Asia such as Thailand, Indonesia, India, and Malaysia and also Central and South America (4, 5). The genus *Leptospira* is classified according to DNA-DNA hybridization analysis into at least 22 pathogenic and non-pathogenic species. Based on serological typing using agglutination of serovar-specific antibodies with lipopolysaccharide (LPS) antigens, *Leptospira* are classified into more than 300 serovars, including 200 pathogenic strains (6, 7). Rats and other rodents are the most important reservoir animals of pathogenic *Leptospira* spp. Humans are susceptible to leptospirosis and become accidental hosts. Pathogenic *Leptospira* are transmitted to human by direct contact with blood, urine or infected animal tissue or less commonly by ingestion. The carrier animals can shed bacteria into the environment via urine and humans may indirectly contact the contaminated water and soil (6, 8, 9). The major risk factors for human leptospirosis are occupation-related activities such as agriculture, livestock, and sewer workers (10, 11). Leptospires can penetrate abraded skin or mucous membrane and establish a systemic infection by disseminating through the bloodstream (12). Symptoms and severity of leptospirosis range from mild, flu-like illness to severe and potentially

lethal infection with multiple target organ involvement such as liver, lung, and kidney dysfunction (13-15). Leptospirosis is a biphasic disease with incubation period of approximately 7-12 days. In the acute or septicemic phase during 3-7 days of illness, patients presents with non-specific symptoms such as high fever, headache, myalgia, arthralgia, and vomiting (16). In the convalescent or immune phase, the bacteria disappear from the bloodstream and antibodies are produced against leptospiral antigens (6, 16, 17). Clinical manifestations in this phase are associated with target organ damage such as jaundice, meningitis, and renal failure. Current diagnostic tools for leptospirosis include direct examination of clinical samples, culture, nucleic acid detection, and antibody-based tests. For direct examination at least 10^4 cells/mL of leptospires in blood or urine is required for visualization under dark-filed microscopy (18). Bacterial load in blood was shown to range from 10^2 to 10^6 cells/mL during the acute bacteremic phase (19). Therefore, direct examination of the spirochete in clinical samples is poorly sensitive. In addition, it is not specific and requires a dark-filed microscope, which is rarely available. Culture of leptospires are difficult due to slow-growing and requiring complicated media. Microscopic agglutination test (MAT) used to detect anti-*Leptospira* agglutinating antibodies after the second week of infection is currently the standard method for diagnosis of leptospirosis (17). MAT has low sensitivity during early phase of the disease and possibly requires paired sera resulting in delayed diagnosis (20-22). Moreover, this technique is performed only in the reference laboratory because it requires expensive instruments, trained staff, and

maintaining several viable *Leptospira* serovars for antigen sources (8, 23). Other techniques for antibody-based detection, such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay, the slide agglutination test (24), indirect hemagglutination assay (25), and the lateral flow assay (26, 27), are also not sensitive for the early phase detection (28). Antigen or DNA detection assays such as ELISA and PCR are more useful for diagnosis of early acute leptospirosis. However, they requires special equipment, dedicated laboratory space, and highly skilled personnel (29, 30). Therefore, point-of-care testing (POCT) for rapid diagnosis of leptospirosis is needed so that patients are early diagnosed and appropriately treated to reduce morbidity and mortality especially in the resource-poor setting.

Lateral flow assay (LFA)-based POCT devices has been widely used in detecting several targets, such as toxins, pathogens, pesticides, heavy metals and cancer markers (31-33). The advantages of LFA are user friendly formats, rapid detection, low cost, no need of specialized personnel, and simple reading by naked eyes (34, 35). Gold nanoparticles (AuNPs) have been widely used for LFAs due to easy synthesis, biocompatibility, ability to attach to diverse molecules (36, 37), and enhancing visual detection. Therefore, AuNP-based LFAs provide rapid and reliable on-site analysis (38, 39).

LipL32, a 32 kDa lipoprotein, is the major outer membrane protein (OMP) which is highly conserved among pathogenic *Leptospira* and absent in non-pathogenic strains (40, 41). LipL32 is expressed at high level in leptospires during

infection and is highly immunogenic (42-44) as shown by the presence of antibodies against LipL32 in sera of patients in acute and convalescent phases of leptospirosis (42). LipL32 has been used as an antigen for enzyme-linked immunosorbent assay (ELISA) and its encoding gene has been used as a target gene for PCR (24, 45, 46).

In this study, we aim to develop a gold nanoparticle-based lateral flow immunoassay as a POCT that can detect pathogenic leptospiral antigen for diagnosis of leptospirosis at the acute stage.

CHAPTER II

OBJECTIVES

I. To develop a gold nanoparticle-based lateral flow immunoassay for detection of pathogenic leptospiral antigen.

II. To determine optimal conditions, such as type of buffers, size of gold nanoparticles, type of antigen and antibodies, for development of gold nanoparticle-based lateral flow immunoassay for detection of pathogenic leptospiral antigen.

CHAPTER III

LITERATURE REVIEWS

Leptospira and Leptospirosis

Leptospirosis is a zoonosis that is caused by pathogenic *Leptospira*. This genus is separated into two species, pathogenic and saphophytic *Leptospira.* Leptospires are thin spirochetes, the size of cell range from 10 -20 μm in length and about 0.15 μm in thickness. In addition, leptospires are flexible helical rods that are actively motile, obligate aerobic, hook-ended, and slow-growing bacteria (Figure 1) (47, 48). Serological method has been used to determine serovars of *Leptospira*, more than 250 of which are considered pathogenic species. DNA sequence similarity is currently used to determine genospecies, the major of which is *L. interrogans*. Leptospirosis is a major public health problem in tropical and sub-tropical countries and outbreaks mostly occur in the rainy season and after floods (49). Human infection commonly is transmitted through contact with urine of infected animals or exposure to contaminated fresh water or soil (Figure 2) (6). Based on global data collection, the International Leptospirosis Society estimates that 300,000 – 500,000 cases of leptospirosis occur annually (50). Occupational exposure risk to this disease such as agriculture, livestock, and sewer workers is a common risk factor of this disease (11). Leptospirosis was found to be a major cause of acute undifferentiated

fever in Thai agricultural workers (51). The bacteria can enter the body through broken skin or mucous membranes. Pathogenic *Leptospira* have a large proportion of the structural and functional outer membrane proteins (OMPs) such as LipL32, LipL21, and LipL41, that can involve in adhesion (40). In vitro, *L. interrogans* adhere to a variety of cell lines including fibroblasts, endothelial cells, and kidney epithelial cells. *Leptospira* can swim through blood stream and disseminate to many organs such as kidney, liver and lung (13-15). These organs can offer a large lipid source because fatty acids are an important requirement for leptospiral growth (52). Symptoms and severity of leptospirosis range from mild, flu-like illness to lethal with severe target organ involvement such as liver, lung and kidney failure (53). Clinical manifestations of leptospirosis vary such as jaundice, swollen ankles, headaches, vomiting, seizures, shortness of breath and hemoptysis, respiratory dysfunction, kidney failure, and internal bleeding. Severe leptospirosis may be called Weil's disease. The clinical presentation of leptospirosis is usually biphasic (Figure 3), the first phase called “acute or septicemic phase” during approximately one week of bacteremia followed by the second phase called “convalescent or immune phase” considered by antibody production and secretion of leptospires in the urine (6).

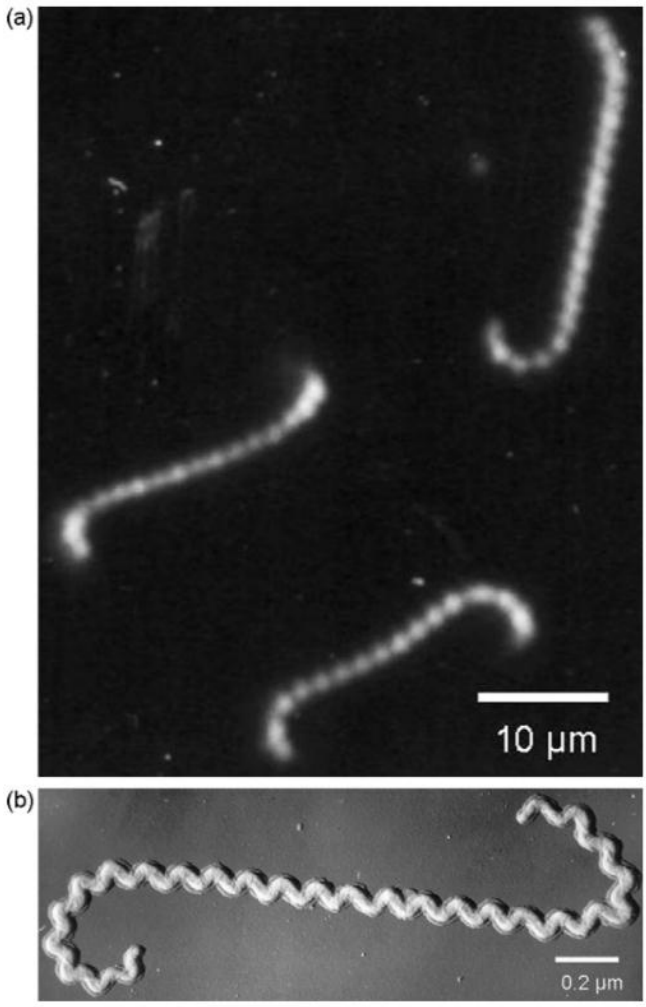


Figure 1. Morphology of *Leptospira* spp. by visualization under dark-field (a) and electron microscope (b) (54).

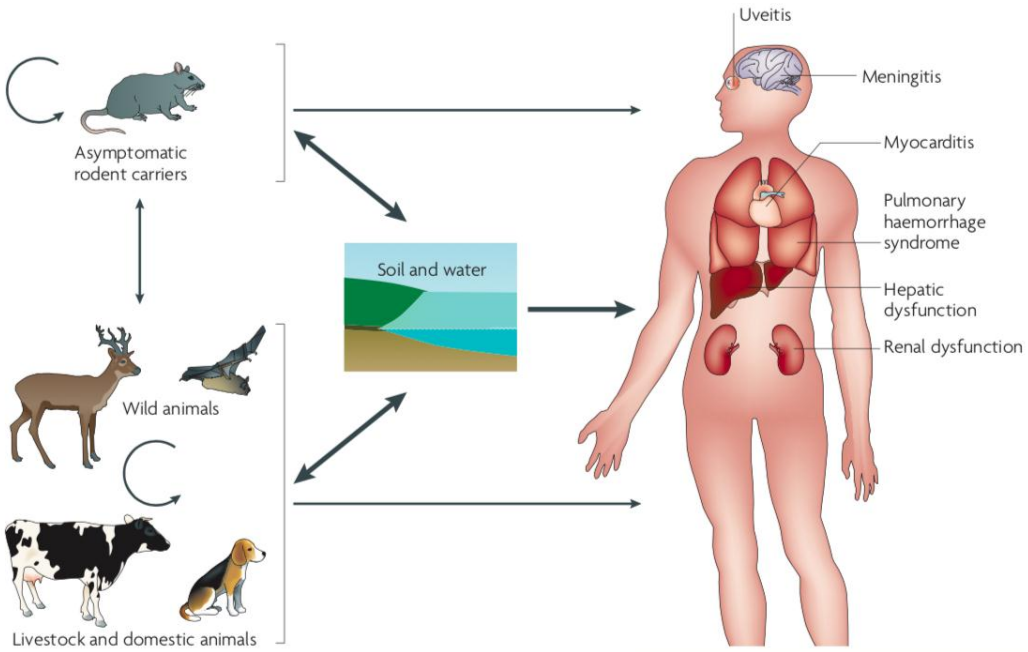


Figure 2. The cycle of leptospiral infection. Rodent species are carrier for pathogenic *Leptospira.* In these reservoirs showed that chronic and asymptomatic carriage. *Leptospira* can infect livestock and domestic and wild animals and cause a range of disease manifestations and carrier states. Human can infect with *Leptospira* by direct and indirect contact with infected animals and their urine contaminated environment. Leptospirosis causes an acute febrile illness during the early phase of infection and is develop to multisystem manifestations such as hepatic dysfunction and jaundice, acute renal failure, pulmonary haemorrhage syndrome, myocarditis and meningoencephalitis at immune phase (55).

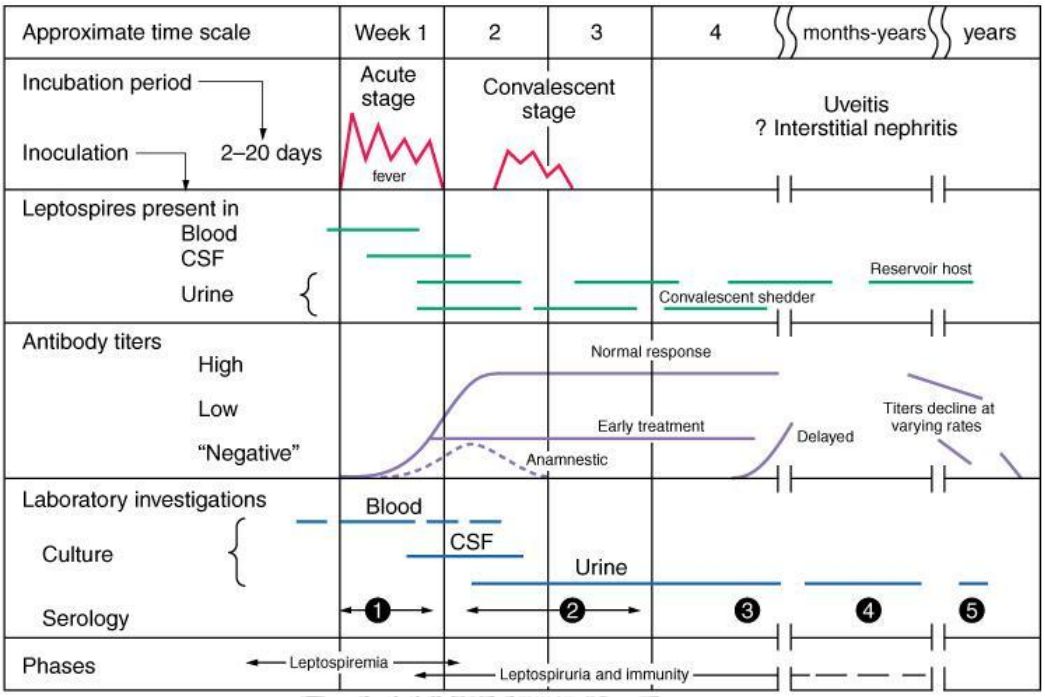


Figure 3. Biphasic nature of leptospirosis and relevant investigations at different stages of disease (6).

Laboratory diagnosis of leptospirosis

Culture

Leptospira can be isolated from blood and CSF samples during 7-10 days of illness and from urine after the second week of illness (56). Samples for cultivation should be collected before antimicrobial drug treatment. *Leptospira* spp. need complicated media such as Ellinghausen-McCullough-Johnson-Harris (EMJH) media and incubation in the dark at 28-30 ℃ for bacterial growth. The leptospires should be examined weekly by dark-field microscopy for up to 13 weeks. The sensitivity of culture for detection of leptospires is low because normally human leptospirosis have bacterial load in blood of 10^2 to 10^6 *Leptospira* per milliliter (19). To detect *Leptospira* by dark-field microscopy, the limit of detection was determined at least approximately 10^4 *Leptospira* per milliliter (16). Therefore, culture is not a gold standard for diagnosis of leptospirosis.

Molecular technique

Direct demonstration of leptospiral DNA in patient's specimens can be used for diagnosis. PCR can detect DNA of *Leptospira* from blood in the first 5-10 days after the onset of the disease even the patient has received an effective antimicrobial drug (57). Leptospiral DNA has been amplified from serum, urine and CSF depending on disease stage at the time of analysis (58). The advantage of PCR assay using specific primer sets is its ability to differentiate between pathogenic and

non-pathogenic species and its higher sensitivity and specificity than culture and direct examination under dark-filed microscopy. Recently, real-time PCR has been developed as a rapid and sensitive tool for detection of leptospiral DNA, and this technique can reduce the risk of carryover contamination (8). The DNA may not be detected in blood in some cases of leptospirosis because of a low level or short period of leptospiremia phase. However, PCR was recommended to be used in combination with serological tests to improve the sensitivity of the diagnosis of leptospirosis in the first phase of the disease (58).

Serological diagnosis

Antibodies are detected in the blood approximately 5–7 days after the onset of symptoms. IgM starts appearing early in the course of acute infection (6). However, level of IgM decreases in the third or fourth weeks of illness but IgG appears later and persist at low level of years. One research group demonstrated that ELISA, using recombinant LipL32 as a target to detect IgG antibodies in patient's sera and showed sensitivity and specificity more than 90% (45). Most commercially available serological tests, the definite serological investigation in leptospirosis is the microscopic agglutination test (MAT). The MAT procedure is laborious, requires the maintenance of viable *Leptospira*, and quality control must be employed. Furthermore, the sensitivity of MAT in the acute phase is low, serovar-specific, and

paired sera are needed to confirm the diagnosis. Current commercially available tests are whole *Leptospira* cell-based assays such as ELISA, dipstick, lateral flow, indirect hem-agglutination assay and latex agglutination test (26, 27, 59). The limitations of common diagnostic tests for leptospirosis by using antigen-based tests are shown in (Table 1) and using antibody-based tests in (Table 2).

Table 1. Advantages and disadvantages of common diagnostic test for leptospirosis by using antigen-based tests (57).

	Microscopic	Culture	Molecular testing
Specimen collection	Blood, urine, CSF	Blood, urine, CSF, tissues	Blood, urine, CSF, tissues
Window of positivity	1st week: blood, CSF 2nd week: urine	1st 10 d	From Day 5–10 in blood
Processing time	Available in 1 h	2 weeks to 4 months	Available in 1 d
Early diagnosis	No	No	Yes
Definitive diagnosis if positive	No	Yes	Yes
Identification	No	Yes (if MAT or molecular testing available)	Yes (by additional molecular tests)
Remark	Low sensitivity and specificity	Low sensitivity, slow, difficult	The only sensitive test at the acute phase
Equipment required	Dark field microscope	Specific culture media	Special equipment, dedicated laboratory space, highly skilled personnel

Table 2. Advantages and disadvantages of common diagnostic test for leptospirosis by using antibody-based tests (57).

	Serology MAT	Serology ELISA IgM	Serology rapid tests IgM
Specimen collection	Blood	Blood	Blood
Window of positivity	From Day 10–12	From Day 6–8	From Day 6–8
Processing time	Several weeks if not locally available	Available in 1 d	Available in 15–30 min
Early diagnosis	No	No	No
Definitive diagnosis if positive	Yes (seroconversion)	Yes (seroconversion)	Yes (seroconversion)
Identification	Yes	No	No
Remark	Gold standard but very difficult	Needs confirmation by MAT	Needs confirmation by MAT
Equipment required	Reference laboratory only	Standard laboratory	Laboratory equipment not required

Lateral flow assay

The Lateral flow assay (LFA)-based POCT devices emerged for the first time in 1960s, being used for detection of serum protein. LFA has been widely used for detecting various targets, such as cancer marker, microorganisms, heavy metal, mycotoxins and pesticides (34). The advantages of LFA are low cost, easy operation, user friendly, rapidly test and visual detection by naked eyes (34, 60, 61). The LFA is composed of a chromatography (separation of mixed components in environment), immuno-chromatography reaction (reaction between specific antigen-antibody, and nucleic acid-target analyses (62). The components of LFA consist sample pad, conjugate pad, reaction membrane (usually nitrocellulose membrane) and adsorbent pad (Figure 2a). The sample pad, area for sample loaded, usually made from cellulose or glass fiber. Cellulose membrane shows low affinity while glass fiber shows no affinity for protein binding (63). In addition, sample pads can be designed to pre-treated the sample for separation of sample components, removal of interference, adjustment of pH, etc. (35). Conjugate pad; this part containing of nanoparticles labeled bio-recognition molecules to specific targets. Nanomaterials are most commonly used for optical signal, including fluorescence or color changes by aggregations (64-66). Colloidal gold (31, 67, 68), silver and carbon nanoparticles are widely used for developing LFA for many analyses (69, 70). Colloidal gold or gold nanoparticles are the most commonly used as detection system in LFA for

visualization of signals because of many advantages, such as easy synthesis, high chemical stability, easy to attach many molecules, and biocompatibility. These properties support the analysis in a short period of time and provide reliable on-site analysis (38, 71, 72). Reaction membrane; nitrocellulose membrane is the one of most commonly used for development of LFA. Test line and control line were capture on this part. Nitrocellulose membrane are available in several pore sizes, control the flow rate of mobile phase and provide support binding of reaction (35). Final part of FLA strip is absorbent pad (or wick). It maintains flow rate of the liquid over the membrane and prevents back flow of the sample (Figure 4). The key process of LFA are antibody preparation, bio-recognition molecules labeling, and all LFA components assembling. LFA formats consist sandwich and competitive formats (Figure 5 and 6) (73).

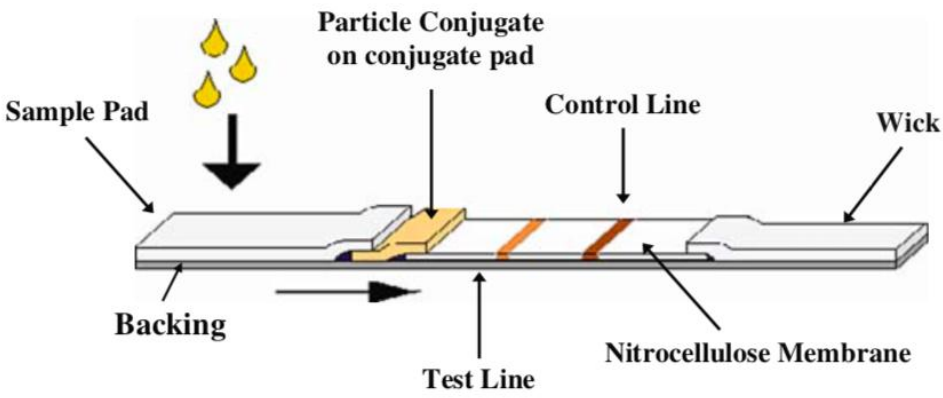


Figure 4. Typical configuration of a lateral flow immunoassay test strip (73).

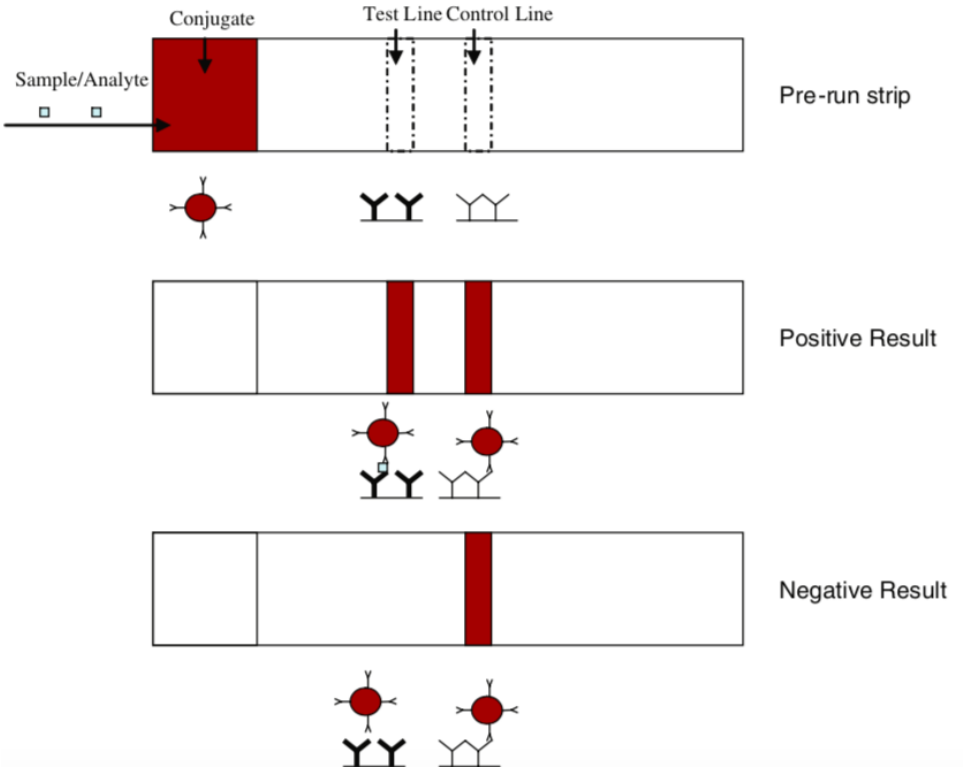


Figure 5. Direct solid-phase immunoassay (73).

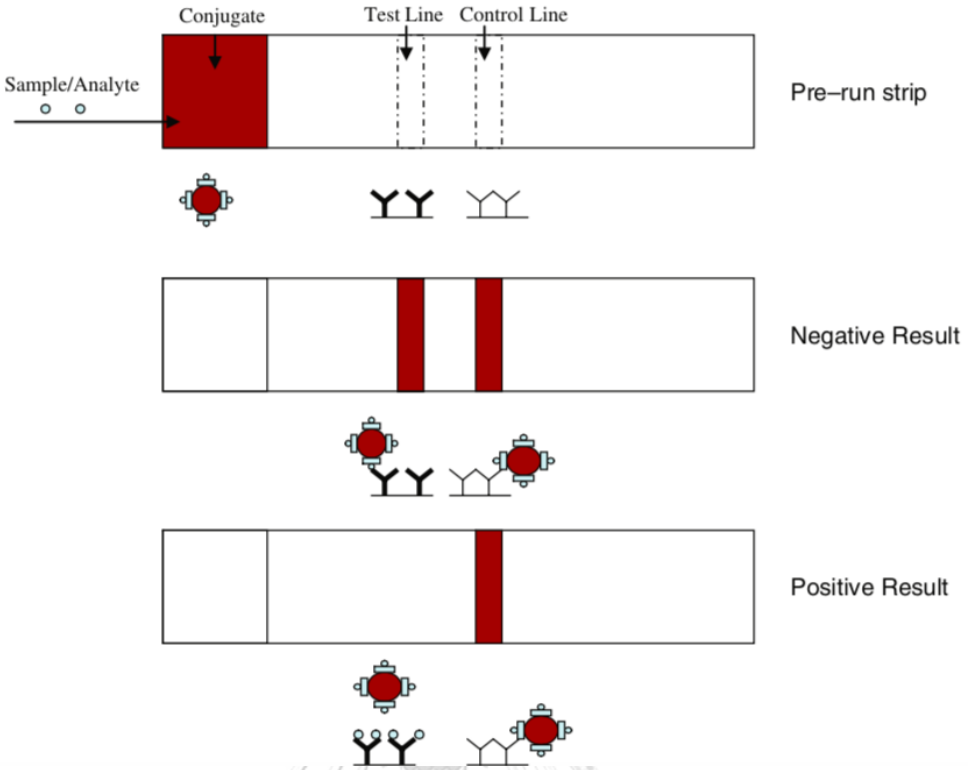


Figure 6. Competitive solid-phase immunoassay (73).

Gold nanoparticles (AuNPs) for LFA

Gold nanoparticles are most commonly used for development of LFA for visualization of signals. Advantages of gold nanoparticles are easy synthesis, chemical stability, low cost, biocompatibility, and easy preparation steps (34). In term of AuNP-based LFA, AuNPs easily attached to another molecule such as protein, nucleic acid and chemical agent etc. Moreover, AuNPs are red color enhances visual detection. AuNPs are most commonly synthesized by citrate reduction method to reduce Au3+ to Au0 (nanoparticles form). After synthesis of AuNPs, surface of AuNPs were covered with negatively charge (OH^-) to keep AuNP stability (37, 74) (Figure 7). The elaborate chemical reaction can be presented as

$$6Au^{3+} + C_6H_5O_7^{3-} + 15OH^- \text{----------} > Au + 6CO_2 + 10H_2O \qquad (37)$$

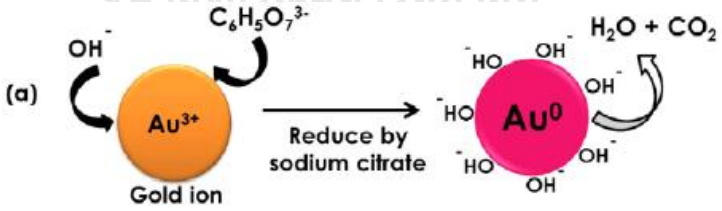


Figure 7. Schematic of AuNP formation during synthesis using citrate reduction method.

The way for confirm diameter and distribution of AuNPs have been measured via TEM and characteristic absorbance peak of AuNPs is observed at 520 nm by using spectrophotometer (75). However, different sizes of AuNPs were showed that diverse properties. The AuNPs with diameter smaller than 15 nm were strong stability but generate faintly color. However, 20 nm in diameter of AuNPs were used as a detector reagent. AuNPs with diameter larger than 60 nm were easily aggregated (70). In addition, seeded-growth method by citrate stabilization were used for synthesize of larger sizes of AuNPs. This technique can produce AuNPs with various sizes from 10 up to 180 nm in diameter (74) (Figure 8).

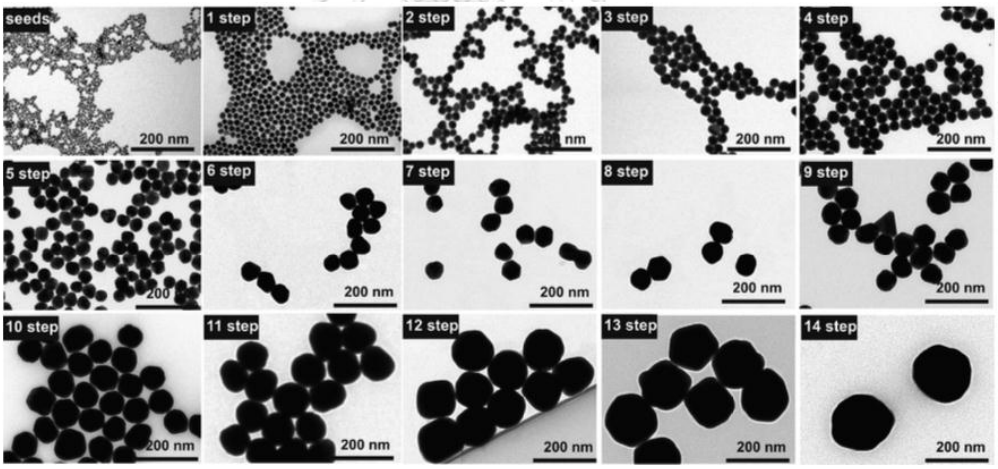


Figure 8. Monodisperse citrate-stabilized gold nanoparticles with a uniform quasi-spherical shape of up to 200 nm (74).

Seeded-growth synthesized AuNPs were shown that higher stability than traditional citrate reduction method (37). The shape, size and stability of AuNPs are key parameters that affect the success of the LFA. The most popular shape and size of AuNPs for LFA development are globular and 20 - 40 nm in diameter of AuNPs. Part of antibody binding to gold surface, antibody bind strongly to AuNP surface with non-covalent interaction such as London- Van der Waals force and hydrophobic interaction (76) (Figure 9).

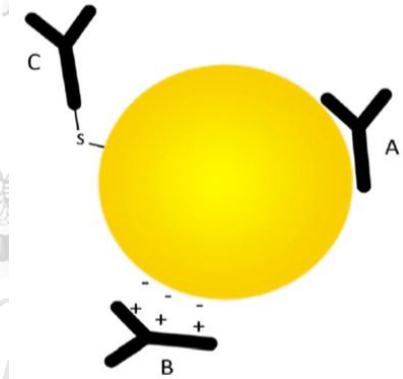


Figure 9. Hydrophobic and ionic interactions between antibody and gold nanoparticle surface. A) hydrophobic interaction B) ionic interaction C) a covalent bond is formed due to dative binding (77).

Table 3. AuNPs based LFA for detection of pathogens

Pathogen	Biorecognition element	LoD	Analysis time	Reference
S. aureus	antibody	500 cfu/mL	5 min	(78)
M. Tuberculosis	antibody	5ng/mL	-	(79)
Samonella	aptamer	10 cfu/mL	5 min	(80)
Vibrio cholerea O1	antibody	10^8 cfu/mL	10 min	(81)
Vibrio cholerea O139		10^7 cfu/mL		
Streptococcus suis	antibody	10^4 cfu/mL	15 min	(82)
E. coli O157:H7	aptamer	10 cfu/mL	5 min	(83)
Listeria monocytogenes	RNA	0.5 pg/μL	5 min	(84)
Hepatitis B virus	oligonucleotides	5 ng/mL	-	(85)
Samonella typhimurium	antibody	10^4 cfu/mL	5 - 15 min	(86)
Herpes simplex virus type 2	antibody	-	15 - 20 min	(87)

Previous studies of LFA development for diagnosis of leptospirosis

Development of antibody-based LFA for diagnosis of leptospirosis

Sehgal et al. evaluated sensitivity and specificity of LFA for rapid serodiagnosis of human leptospirosis (88). The criteria for diagnosis of human leptospirosis were isolation of leptospires from blood culture, seroconversion in microscopic agglutination test (MAT) with a 4-fold rising titer of paired sera or MAT titer of 400 or more of a single serum. In this study, LFA had sensitivity of 52.9% (37/70) in the first week of illness and 86% (49/57) during week 2–4. In addition, the sensitivity was 34.3% (12/35) on day 2–3 of the illness, 63.3% (14/22) on day 4–5 and 84.6 % (11/13) at the end of the first week. In addition, sensitivity of LFA was similar to IgM ELISA and LEPTO Dipstick. However, the test has lower sensitivity in the first week of illness.

Smits et al. developed LFA for rapid serodiagnosis of human leptospirosis (27). They constructed LFA by immobilizing heat-resistant antigens of *L. biflexa* serovar Patoc I on the test line and using anti-human IgM antibody-conjugated AuNPs as a detection system. The sensitivity and specificity of developed LFA were performed using 268 sera from laboratory-confirmed cases of leptospirosis by MAT or IgM ELISA, 212 sera of healthy controls, and 167 sera from unrelated diseases. The results showed that the overall sensitivity of developed LFA was 85.8% (95% confidence interval [CI], 79 to 91%), and the overall specificity was 93.6% (95% CI, 88

to 97%). In addition, the specificity of developed LFA for detection with sera of unrelated diseases was 88.4% (95% CI, 82 to 93%). However, the sensitivity of this LFA was 65.9% for sera collected during the first 10 days of the illness and 80.9% for sera collected 10 to 30 days after the onset of the disease. Therefore, sera in early phase of illness were demonstrated lower sensitivity than convalescent phase sera.

Vanithamani et al. developed lipopolysaccharide (LPS)-specific immunochromatography (ICG)-based LFA for serogroup specific diagnosis of leptospirosis in India (89). The extracted LPS from five locally predominant circulating serogroups including Australis, Autumnalis, Ballum, Grippotyphosa, and Pomona were used as antigens to detect IgM antibodies in patient sera. The 200 sera of clinically suspected and laboratory confirmed leptospirosis, 120 sera of clinically suspected and laboratory negative leptospirosis, 174 sera of unrelated disease, and 121 sera of healthy controls were tested for evaluation of sensitivity and specificity. The sensitivity and specificity of LFA for detecting antibodies against target LPS in homologous sera were shown to be in the range of 93 to 100% and more than 99%, respectively. However, the Wilcoxon analysis showed that the ICG based LFA developed with leptospiral LPS was not significantly different from the MAT ($P > 0.05$).

Development of antigen-based LFA for diagnosis of leptospirosis

Widiyanti at el. developed AuNP-based LFA for detection of leptospiral LPS antigen in urine (90). In this study, the developed LFA was evaluated the limit of detection (LoD) by using various concentrations of leptospiral whole cell lysates and showed the detection limit to be 10^6 cells. The LFA did not cross-react with non-pathogenic serovars used in this study. In addition, the 46 urine from *Leptospira*-infected hamster, 44 urine from patients with suspected leptospirosis, and 14 urine from healthy controls were used for evaluation of sensitivity and specificity of this test. The sensitivity and specificity of this AuNP-based LFA for detecting leptospiral LPS antigen in human urine was 80% and 74%, respectively, while that of infected animal urine was 76% and 65%, respectively.

Available POCTs for diagnosis of leptospirosis and their advantages and disadvantages

Wagenaar et al. validated the commercial rapid diagnostic tests including LeptoTek Lateral Flow® assay (Organon Teknika, Durham, NC) and Dri Dot® card-agglutination test (Organon Teknika) for detection of specific antibodies against *Leptospira* (91). In this study, blood samples from 44 patients with undifferentiated fever and 83 healthy controls were examined for anti-*Leptospira* antibodies. The MAT was used as a gold standard test for anti-*Leptospira* antibodies to determine

sensitivity and specificity of this commercial lateral flow to be 86% and 94%, respectively, whereas the card-agglutination test showed a sensitivity of 72%–88% and a specificity of 90%. High seroprevalence of anti-*Leptospira* IgM antibodies in southern Vietnam restricted the ability of a recently developed LFA to confirm active leptospirosis. In addition, the card-agglutination test was less sensitive, especially when seroprevalences are high and needs further evaluation.

Chang et al. validated sensitivity and specificity of two commercial rapid tests for acute leptospirosis detection in Malaysia (3). The Leptorapide® (Linnodee, Northern Ireland) as a latex agglutination-based test and VISITECT®-LEPTO (Omega Diagnostics, Scotland, UK) as a LFA-based test, which are commonly used in Malaysia to detect human specific antibody against *Leptospira* with 58 sera from known cases of leptospirosis (MAT+ and PCR+), 29 sera from healthy controls and 41 sera form unrelated diseases. Performance of two commercial tests in detecting of leptospirosis showed low diagnostic sensitivity for both tests, 34% and 24% for Leptorapide® and VISITECT®-LEPTO, respectively. However, the specificity of VISITECT®-LEPTO (94%) was remarkably higher than Leptorapide® (69%). In this study, both kits showed sensitivity of less than 35% in detection of acute phase sera from human leptospirosis indicating their limited diagnostic values for patients infected within the first 10 days.

Lizer et al. validated sensitivity and specificity of LFA (WITNESS Lepto, Zoetis) as a commercially available to detect *Leptospira*-specific IgM in canine sera (92). Positive MAT of sera and/or blood or urine PCR were used as standard criteria for clinical diagnosis of leptospirosis upon receipt of samples. This commercial LFA diagnosed leptospirosis in 37 acute phase sera and 9 corresponding convalescent phase sera showing sensitivity to be 89.7% and 100% in acute and convalescent phase sera, respectively. In addition, this LFA showed 98.3% specificity. However, only 5 of the corresponding acute-phase sera were positive (55.6 %). Therefore, the false-negative LFA results might be due to inadequate interval for primary immune response after the onset of symptoms.

Gloor et al. validated the diagnostic performance of two commercial LFAs including Test-it TM and Witness® Lepto in the early diagnosis of canine leptospirosis using 108 cases of canine leptospirosis (positive MAT, or RT-PCR, and or silver staining) and 53 canines without leptospirosis (93). In this study, Test-it TM and Witness® Lepto has sensitivity of 82 and 76% and specificity of 91 and 100%, respectively. However, the correct interpretation of this rapid diagnostic tests-based LFA was difficult if the reaction was weakly positive.

Dahanayaka et al. validated the diagnostic performance of a commercial LFA (Immunemed *Leptospira* rapid, Korea) for detection of human antibodies against *Leptospira* using 78 sera of patients with leptospirosis (positives MAT as single titer >1:400 or seroconversion of 4-fold rising in antibody titers of MAT) (94). The commercial LFA had 95.6% sensitivity and 63.6% specificity. Therefore, this commercial LFA had a high sensitivity as a screening test for leptospirosis. In addition, high negative predictive value (NPV) of 91% is also important in clinical practice, suggesting clinicians to look for other causes of leptospirosis-like illness in the region.

Improvement of sensitivity of AuNP-based LFA

AuNP-based LFA is an effective method based POCT device that is widely used in many fields. However, the traditional AuNP-based LFA is limited due to its low sensitivity. Novel techniques have been developed for improving sensitivity, such as increasing sizes of AuNPs, signal amplification by using reaction between enzyme and substrate (70, 95), sensitizer by using gold solution enhancement (96), a dual AuNPs enhancement (97, 98), silver deposition (99-101), and Thermal contrast (102).

Makhsin et al. studied the effect of AuNP sizes on test sensitivity by using mouse anti-human IgG_4 conjugated AuNP-based LFA for detection of *Brugian filariasis* as a model (37). Three different sizes of AuNPs including 20, 30 and 40 nm in

diameter were tested. The result showed that the 30- and 40-nm AuNPs had a detection time of less than 15 min while 20-nm AuNPs needed at least 23 min. However, 30-nm AuNPs showed the highest sensitivity with 4-fold higher than that of 40-nm AuNPs.

For signal amplification by a dual AuNPs conjugated-based LFA, Choi et al. developed a new and simple method utilizing two AuNPs-antibody conjugates to detect troponin I as a target model (97). To develop a dual AuNPs, the 1st AuNPs (10 nm) conjugated to anti-troponin I followed by BSA and the 2nd AuNPs (40 nm) conjugated to anti-BSA antibody were prepared. The strategy to increase the LFA sensitivity was to add the 2nd conjugation pad to the conventional AuNP-based LFA (Figure 10). The result showed that troponin I was detectable at 0.01 ng/mL compared to 1 ng/mL detectable by the conventional LFA. Therefore, the signal amplification of this dual AuNP-based LFA was 100-fold more sensitive than the conventional AuNP-based LFA. In this study, not only a dual BSA conjugated AuNPs and anti-BSA conjugated AuNPs was described but also a dual biotin-AuNPs conjugates and streptavidin-AuNP conjugates. In addition, Maneeprakorn et al. also demonstrated that dual biotin labeled BSA conjugated AuNPs and the second AuNP-conjugated streptavidin showed 8-fold improvement (103).

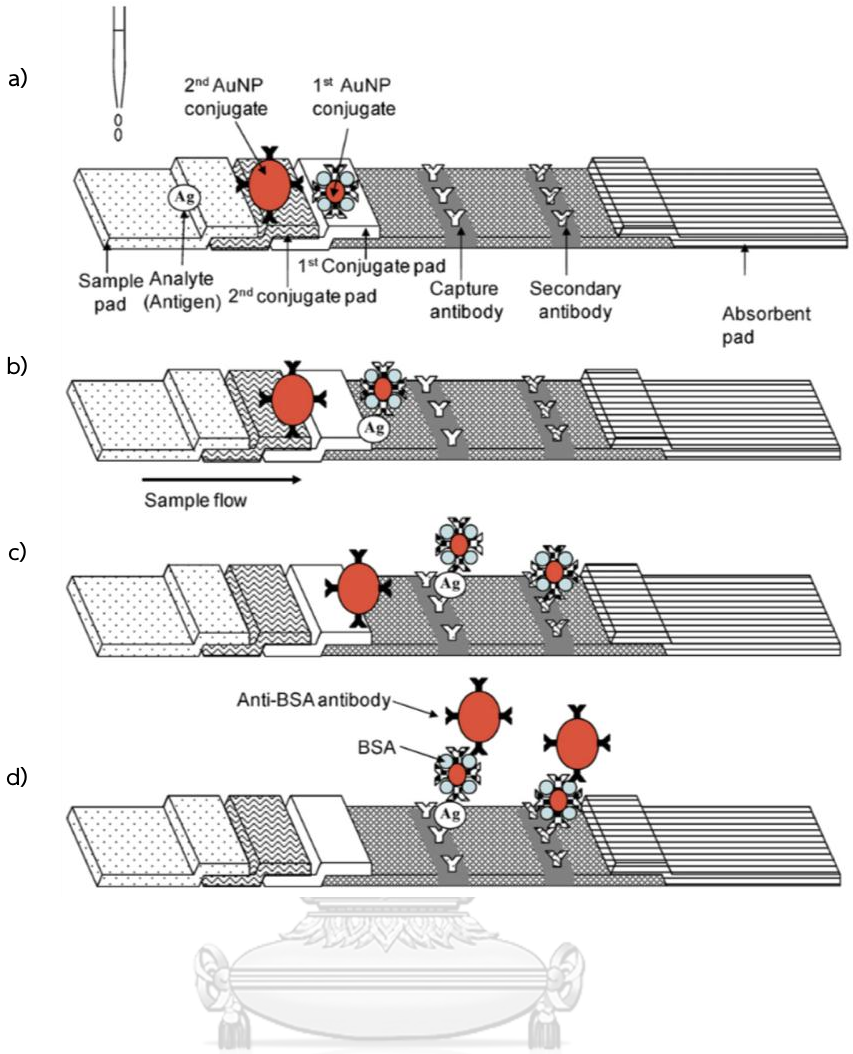


Figure 10. Schematic illustration of a dual AuNP-based LFA method developed in this study (97). The LFA strip is comprised of a sample pad, two conjugate pads, nitrocellulose (NC) membrane, and an adsorbent pad. When an antigen solution is first applied to the sample pad (a), the antigen will interact with the 1st AuNPs (b). The complex of the antigen and 1st AuNPs conjugate binds to antibody immobilized on the test line (c). The 2nd anti-BSA antibody conjugated AuNPs move to interact with BSA on 1st AuNPs conjugate (d).

The AuNP signal amplification using a novel ‘sensitizer’ have been reported by Nagatani et al. to detect the human chorionic gonadotropin hormone (hCG) as a model (96). Conventional LFA consists of monoclonal anti-human hCG (MabhcG)-conjugated AuNPs deposited on the conjugate pad and monoclonal anti-human α-subunit of follicle-stimulating hormone (MabHαS) immobilized on the test line. The sensitizer comprised AuNPs conjugated to Mab-HαS (primary antibody) and hCG (antigen). The sample was applied onto the sample pad for conventional LFA and then following by sensitizer (Figure 11). This study examined the signal of analytes by using surface plasmon resonance (SPR). The result showed that sensitizer can increase sensitivity more than conventional LFA for around 40-fold improvement while observing the test line intensity.

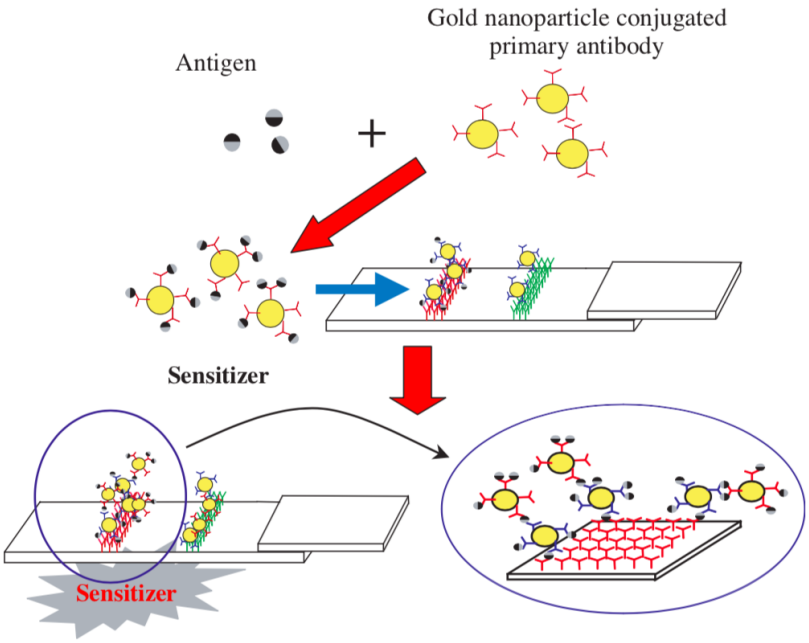


Figure 11. The schematic illustration for the sensitizer enhancing the signal on the test line (96).

Signal amplification of AuNPs using Thermal contrast have been reported by Qin et al. (102). The FDA-approved LFA for detection of cryptococcal antigen was used as a model. After dropping the clinical specimen, the antigen-AuNPs complex was accumulated on the test line and created visualization detection (Figure 12). Thermal contrast showed a 32-fold greater improvement in the analytical sensitivity than the colorimetric detection.

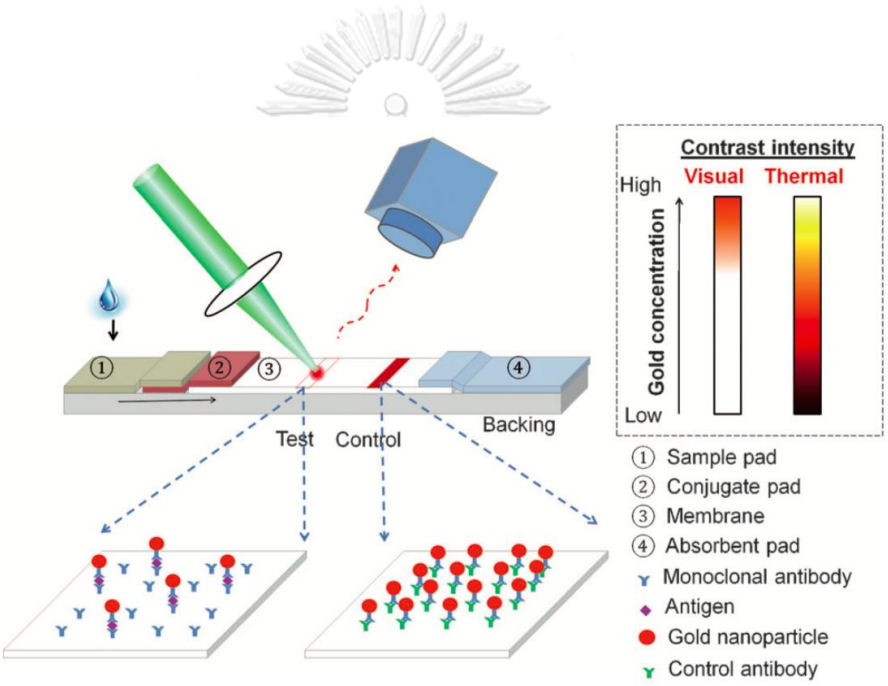


Figure 12. Concept of thermal contrast for immunochromatographic lateral flow assays. Firstly, the specific mAb-conjugated AuNPs bind the target antigen. Secondly, the antigen-Ab-AuNPs complex binds to mAb immobilized on the test line, thus accumulating of AuNPs on the test line leading to visible color change (visual contrast) at the test line. Finally, a low-cost laser or light-emitting diode (LED; shown

in green) and an infrared temperature gun (shown as a blue box) were applied. The control band ensures the validity of the assay (102).

For signal amplification of AuNPs by labelling carriers and enzymatic activity, Parolo et al. developed a AuNP-based LFA using HRP-labeled anti-human IgG (αHIgG) γ chain conjugated-AuNPs for detection of HIgG as a model (70). Three different substrates including 3,3',5,5'-Tetramethylbanzidine (TMB), 3-Amino-9-ethylcarbazole (AEC), and 3,3'-Diaminobanzidine tetrahydrochloride (DAB) were used in this study. The result showed that traditional AuNP-based LFA can detect HIgG at LoD of 50 ng/mL by visualization. However, each substrate including TMB, AEC and DAB was applied and showed that LoD was similar to the traditional AuNP-based LFA when detected by naked-eyes. In addition, after TMB, AEC and DAB was individually applied, the LoD was at 200 pg/mL, 310 pg/mL, and 1.6 ng/mL, respectively by using a strip reader. The color signal was not dispersed along the strip when TMB was applied, so the strip reader was required. Moreover, in case of high colored background due to enzymatic reactions it was difficult to distinguish between a blank and the signals by naked eyes.

Wada et al. amplified signal of AuNPs using silver deposition (104). The AuNP-based LFA developed to detect H5 influenza virus hemagglutinin was demonstrated for silver amplification. After the antigen-antibody-AuNP complex accumulated at the

test line region, silver nitrate solution (Ag ion) and ammonium iron (II) sulfate (a reducing agent) were applied onto this LFA (Figure 13). The result showed that silver amplification allowed detection of recombinant HA protein within 15 minutes and 500-fold increased sensitivity compared with the conventional AuNP-based LFA.

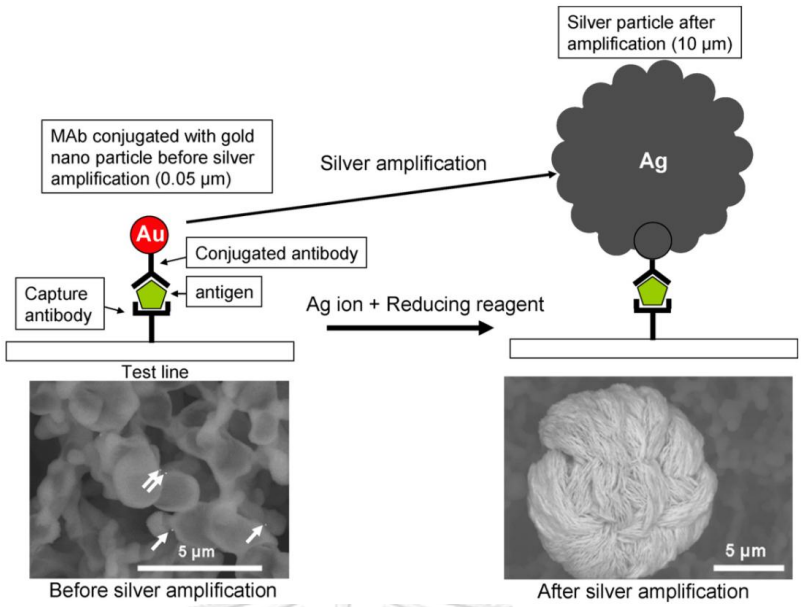
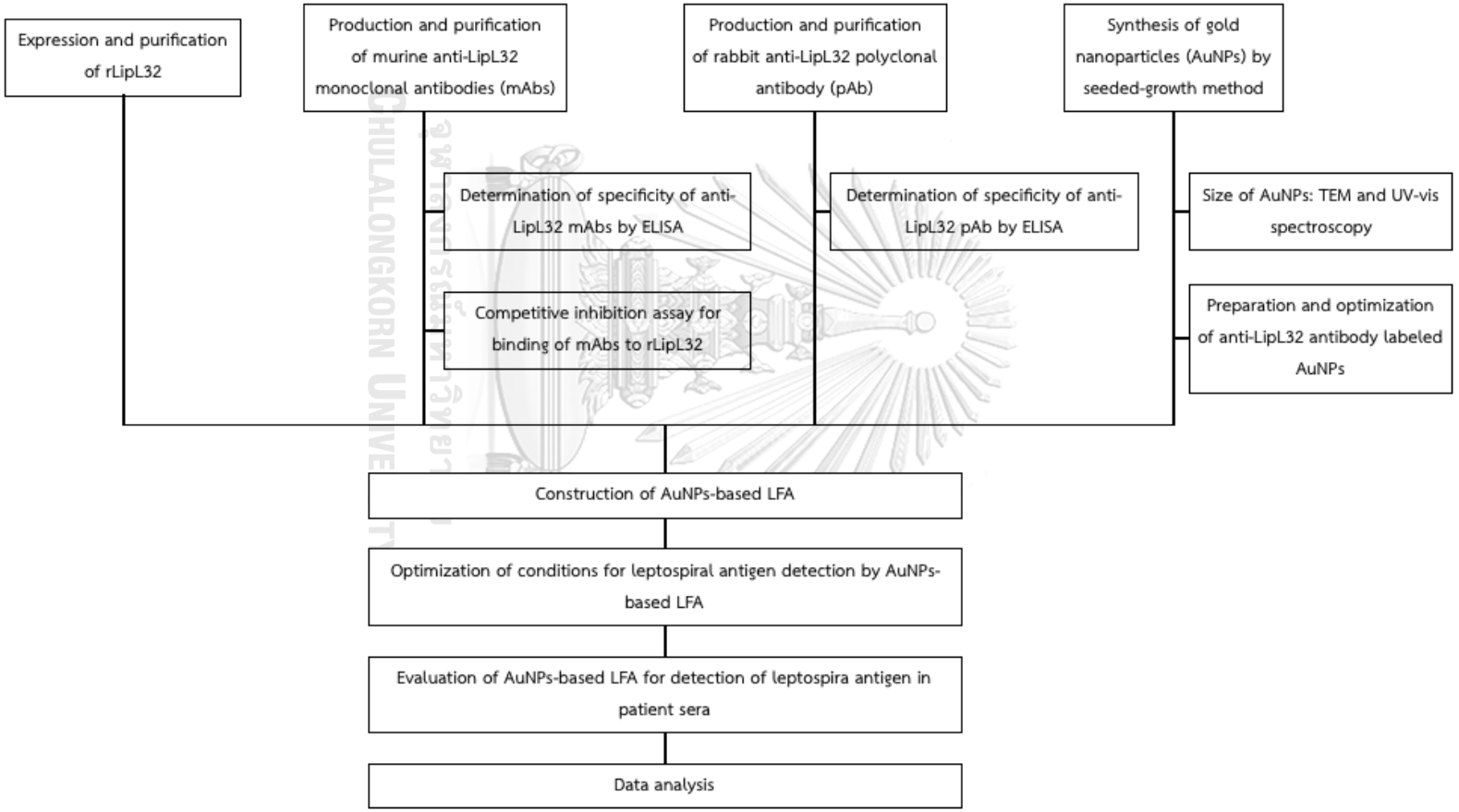


Figure 13. The silver amplification of AuNP-based LFA for detection of H5 influenza virus hemagglutinin. After a sample is applied, target antigen is captured with specific mAb-conjugated AuNPs and are then captured with specific antibody immobilized on the test line (left). Reagent A (reducing agent) and Reagent B (silver ions) run through the membrane forming silver clusters around the gold particles. The SEM images were taken before (left) and after (right) silver amplification. The arrow indicates AuNPs captured on the membrane. The larger particles around the gold particles are nitrocellulose (104).

CHAPTER IV

MATERIALS AND METHODS

Experimental designs



Leptospira culture

Twenty-two serovars of pathogenic *Leptospira* spp. including fourteen serovars of *L. interrogans*; Autumnalis, Bratislava, Bataviae, Canicola, Djasiman, Hebdomadis, Icterohaemorrhagiae, Javanica, Louisiana, Manhao, Mini, Panama, Pomona, and Pyrogenes,. Others pathogenic *Leptospira* including *L. borgpetersenii* serovar Ballum, *L. borgpetersenii* serovar Tarassovi, *L. borgpetersenii* serovar Sejroe, *L. weilii* serovar Celledoni, *L. weilii* serovar Sarmin, *L. kirshneri* serovar Grippotyphosa, *L. kirshneri* Cynopteri. *L. santarosai* serovar Shermani, and two serovars of non-pathogenic *Leptospira* including *L. meyeri* serovar Ranarum and *L. biflexa* serovar Patoc. All serovars of *Leptospira* were kindly provided by Asst. Prof. Thareerat Kalambaheti, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Thailand. All *Leptospira* spp. were cultured in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with 10% bovine serum albumin (BSA) fatty acid and were incubated at 28-30^{o}C until the density reached approximately 1x10^{8} to 5x10^{8} cell/mL (log phase). *Leptospira* spp. were harvested the cells by centrifugation at 10,000 x g for 5 minutes and sonicated at 40% amplitude for 10 seconds pulse on and pulse off for 20 cycles on ice.

Patient sera

Fifty acute phase sera from confirmed cases of leptospirosis (four-fold rising MAT titers of paired sera or MAT titer $\geq$ 1:400 of single serum or positive real-time PCR of *lipL32*), 20 acute phase sera from patients with unrelated diseases (negative MAT and PCR for *lipL32*), and 10 sera from healthy persons were obtained from Asst. Prof. Nattachai Srisawat, Excellence Center for Critical Care Nephrology, Faculty of Medicine, Chulalongkorn University, Thailand (COA-CREC 005/2017)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein sample was mixed with 6xSDS sample buffer to make a final concentration of 1xbuffer. Then, the sample was boiled at 100°C for 5 minutes followed by centrifugation at 10,000 x g for 2 minutes. Next, the supernatant containing soluble proteins was loaded into the well of 15% polyacrylamide gel assembled in electrophoresis running systems (Mini-PROTEIN Tetra Cell, Bio-Rad, U.S.A.) under 1x running buffer. Proteins were separated at 120 voltages (V) for 90 - 120 minutes. The gel was stained with Coomassie Brilliant Blue R-250 for 30 minutes and de-stained with de-staining buffer until the background was clear.

Western blotting

Protein samples in the SDS-PAGE gel were electrophoretically transferred to nitrocellulose membranes with semi-dry transfer cell (Semi-Dry Transblot, Bio-Rad, U.S.A.) at 15V for 30 minutes using blotting buffer. The membranes were blocked with 1% BSA for 1 hour and washed three times with 1x phosphate buffer saline pH 7.4 containing Tween 20 at a final concentration of 0.05% (PBST) for 5 minutes. After blocking, the membranes were incubated with primary antibodies; mouse anti-LipL32 mAb82 to detect rLipL32 protein or AP-labeled goat anti-mouse IgG antibody to detect mouse anti-LipL32 mAbs or AP-labeled goat anti-rabbit antibody to detect rabbit anti-LipL32 pAb. Each antibody was diluted with a blocking buffer (1% BSA dissolve in 1xPBST). The membranes were incubated with the primary antibody for 1 hour at room temperature and washed three times with PBST for 5 minutes. Then, membranes were incubated with the corresponding secondary antibody, AP-labeled goat anti-mouse IgG antibody for detection of rLipL32, for 1 hour at room temperature and washed three times with PBST for 5 minutes. Lastly, the blots were stained with phosphatase substrate, nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (NBT/BCIP, KPL, U.S.A.), for 10 - 15 minutes and stop reaction using distilled water. The positive (purple) bands of interest were observed by visualization.

Antibody biotinylation

The mouse anti-LipL32 mAb82 was biotinylated using succinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (NHS-SS-Biotin) (EZ-Link NHS-SS-Biotin, Thermo scientific, U.S.A.). Firstly, the vial of NHS-SS-Biotin was equilibrated at room temperature for 30 minutes and then dissolved NHS-SS-Biotin with 1 mL of dimethylsulfoxide (DMSO) to prepare 10mM solution of NHS-SS-Biotin. Secondly, 26.8 μL of 10 mM solution of NHS-SS-Biotin was mixed with 2 mg of anti-LipL32 mAb82, making sure that the volume of organic solvent does not exceed 10% of the final reaction volume. Then, the mixed solution of anti-LipL32 mAb82 and HNS-SS-Biotin was incubated on ice for 2 hours. Finally, non-reacted NHS-SS-Biotin was removed by dialysis using 10K dialysis bag (SnakeSkin Dialysis Tubing, Thermo scientific, U.S.A.) at 4 ℃ for 24 hours. The biotinylated anti-LipL32 mAb82 was stored at -20 ℃ until use.

Preparation of rLipL32 coupled on NHS-activated Sepharose column

The rLipL32 was coupled to NHS-activated Sepharose column using HiTrap NHS-activated HP (GE Healthcare Life Sciences, Sweden). Firstly, the column was washed with 5 mL of 1 mM HCl, ice-cold for twice. Be sure not to exceed flow rates of 1 mL/minute. Then, the column was immediately injected with 1 mL of the ligand solution containing 2 mg of rLipL32 and sealed the column at 4 ℃ for 4 hours and repeated with 1 mL of the ligand solution containing 2 mg of rLipL32 again for

coupled protein amount of rLipL32 for 4 mg on NHS-activated Sepharose column. Washing and deactivation steps, the column containing ligand solution was deactivated and washed with 5 mL of buffer A for twice and followed with 5 mL of buffer B for twice. Then, the column was deactivated again with 5 mL of buffer A for twice and incubated at 4°C for 4 hours. After incubation, the column was washed with 5 mL of buffer B and 5 mL of buffer A for twice, respectively. Finally, the column was washed again with 5 mL of buffer B and 5 mL of 0.05 M disodium hydrogen phosphate (Na_2HPO_4) containing 0.1% (sodium azide) NaN_3, pH 7.4, respectively. The rLipL32 coupled on NHS-activated Sepharose column was stored at 4 °C until use.

Expression of recombinant LipL32 (rLipL32)

E. coli strain BL21(DE3) pLysS containing *lipl32* gene in pRSET C vector obtained from our previous study was grown at 37 °C in LB broth containing 100 μg/ml ampicillin and 35 μg/ml chloramphenicol. After continuous shaking at 200 rpm for 16-18 hours, the overnight culture was added to fresh LB medium for optical density (OD600 = 0.1) and cultured until OD600 reached 0.4-0.6. Then, protein expression was induced with 0.5 mM of isopropyl-β-D thiogalactopyranoside (IPTG, Fermentas, U.S.A.). The induced culture was incubated with shaking at 200 rpm for 3 hours. *E. coli* culture was harvested by centrifugation at 8,000 x g for 30 minute and

followed by high pressure homogenizer for break the cells. Protease inhibitor cocktail was added for prevent protein denaturation (complete tablets, mini EDTA-free, EASYpack, Roche, U.S.A.). Finally, culture supernatant was harvested by ultracentrifugation at 45,000 x g for 30 minutes.

Purification of rLipL32

The supernatant containing 6xHis-tagged LipL32 protein was collected for purification by affinity chromatography, a nickel-charged Sepharose column, using the AKTAPrime chromatography system (Amersham Bioscience, USA). The rLipL32 protein was eluted with 25, 50, 100, 200 and 400 mM imidazole. The eluted fraction containing His-tagged LipL32 protein was dialyzed through 10K dialysis bag (SnakeSkin Dialysis Tubing, Thermo scientific, U.S.A.) with 1xPBS, pH 7.4. The amount of protein was determined by Bradford method (Quick Start Bradford protein assay, Bio-Rad, U.S.A.). The purified rLipL32 protein was separated with 15% polyacrylamide gel SDS-PAGE. Then, purified rLipL32 was detected with Western blotting using the anti-LipL32 mAb82.

Production and purification of murine anti-LipL32 monoclonal antibodies (mAbs)

Murine mAbs against LipL32 secreted from hybridoma cell lines clone number 2, 3, 81, and 82 (mAb2, mAb3, mAb81 and mAb82) obtained from our laboratory were produced by using conventional hybridoma procedures (105). Four clones of hybridoma cells were cultured individually in 750 ml cell culture flask (NEST Scientific, USA) containing 100 ml of RPMI medium supplemented with 10% fetal bovine serum (FBS) for 5 days. The culture supernatant containing mAbs secreted from those clones of hybridoma cell lines were used for purification by HiTrap Protein G HP antibody purification columns (GE health care, USA) as small scale purification. In addition, scale up for purification of anti-LipL32 mAbs, Protein G Sepharose 4 Fast Flow antibody purification resin was packed into column, using the AKTAPrime chromatography system.

For small scale purification of anti-LipL32 mAbs, the 1 ml HiTrap Protein G columns were equilibrated with 5 column volume of phosphate buffer saline (PBS) at pH 7.4. Then, each of culture supernatant containing anti-LipL32 mAb was applied into the column and then collect flow through for analysis of unbound proteins. Next, the anti-LipL32 mAb captured protein G were washed with 5 column volume of PBS at pH 7.4. Finally, each of anti-LipL32 mAb was eluted with 1 column volume of 0.1 M of glycine, pH 2.7 for 5 fractions. The eluted anti-LipL32 mAb of each

fraction was neutralized with 1M Tris-HCl, pH 9.0 for a ratio of Tris-HCl to glycine as (1:10). For large scale purification of anti-LipL32 mAbs, Protein G Sepharose 4 Fast Flow antibody purification resin was packing into XK 16/20 column (GE health care, USA), using the AKTAPrime chromatography system. The purification steps of larger scale followed those of the small scale purification as described above.

Production and purification of rabbit anti-LipL32 polyclonal antibody (pAb)

Anti-LipL32 pAb production, two New Zealand white rabbits were immunized three times at 2-week interval with 200 micrograms of rLipL32 with complete Freund's adjuvant and incomplete Freund's adjuvant (Sigma-aldrich, USA). Rabbit sera were collected after the first, second, and third immunization for detection with rLipL32 and whole cell lysates of *L. interrogans* serovar Pomona by indirect ELISA. When the anti-LipL23 pAb was reached to high titer, two rabbits were sacrificed and collected of whole blood. Rabbit sera containing anti-LipL32 pAb were collected by centrifugation at 1000 x g of whole blood from rLipL32 immunized rabbits. The rabbit anti-LipL32 pAb were purified by affinity chromatography (rLipL32 coupled on NHS-activated Sepharose column). The purification procedures for rabbit anti-LipL32 pAb by using 1 ml HiTrap Protein G column was followed from purification of anti-LipL32 mAbs procedure as described above.

Binding of anti-LipL32 mAbs and anti-LipL32 pAb to *Leptospira* spp. by enzyme-linked immunosorbent assay (ELISA)

Ninety-six-well plate was coated individually with 100 μL of sonicated whole cell lysates (at concentration 10^7 cell/mL) of 22 pathogenic *Leptospira*, 2 non-pathogenic *Leptospira*, *E. coli* BL-21 (DE3)pLysS and 100 ng of rLipL32 as a positive control. Then, samples were incubated at 4 °C for overnight. After coating, wells were blocked with 1% BSA (1g BSA dissolved in 1X PBS containing 0.05% tween20) at 37 °C for 1 hour. Then, each of mouse anti-LipL32 mAbs (mAb2, mAb3, mAb81 and mAb82) and rabbit anti-LipL32 pAb were used as primary antibody source and incubated at 37 °C for 1 hour. Finally, HRP labeled goat anti-mouse IgG (1: 5,000) was added. Every step were washed for 6 times with 1X PBS containing 0.05% Tween20 (PBST) to remove non-specific binding. The absorbance values were measured at the wavelength 450 nm using spectrophotometer.

Binding of anti-LipL32 mAbs to *Leptospira* spp. by dot blot assay

To determine binding of anti-LipL32 mAb3 and mAb82 for development of AuNP-based LFA, the binding of each mouse anti-LipL32 mAb to 24 representative serovars of *Leptospira* found in Thailand was determined by dot blot. Nitrocellulose membranes were spotted individually with 10^6 cells of 24 serovars of *Leptospira* whole cell lysates including 22 serovars of pathogenic and 2 serovars of non-

pathogenic *Leptospira*. The rLipL32 at a concentration 100 ng was used as positive control. After spotting, the nitrocellulose membranes containing spotted *Leptospira* spp. were dried at 37 °C for 1 hour. Then, membrane was blocked with 1%BSA in PBST for 30 minute at room temperature. Each mouse anti-LipL32 mAb3 and mAb82 was used as a primary antibody and followed with AP labeled goat anti-mouse IgG (1: 5,000). Every step must be washed for 6 times with 1X PBST to remove non-specific binding. The spots were detected by visualization.

Competitive inhibition assay for binding of mAb3 and mAb82 to LipL32

To determine whether mouse anti-LipL32 mAb3 and mAb82 bind to different or common epitopes on rLipL32 protein, the competitive inhibition assay-based ELISA was performed. Firstly, 96-well plate was coated with 100 μL of rLipL32 (at concentration 1 μg/mL). Then, each well was blocked with 1% BSA in PBST. Next, anti-LipL32 mAb3 at serial dilution from 1:250, 1:500, 1:1,000, 1:2,000 and 1:4,000 were added into each well and then incubated at 37°C for 1 hour. The anti-LipL32 mAb82 was used as inhibition control. After that, biotin labeled mAb82 (dilution 1:5,000) was added and incubated at 37°C for 1 hour. Then, HRP labeled streptavidin (dilution 1:5,000) was added and incubated at 37°C for 1 hour. Every step was washed for 6 times with 1X PBST to remove non-specific binding. Lastly, TMB

substrate was added and measured absorbance at the wavelength 450 nm using spectrophotometer.

Synthesis of colloidal gold nanoparticles (AuNPs)

The colloidal AuNPs were prepared by seeded growth synthesis of citrate-stabilized AuNPs as described previously (74). Briefly, a solution containing 50 mL of (2.2 mM) trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in a 250 mL flask was heated to 100 °C with stirring condition and then 300 μL of 25 mM $HAuCl_4 \cdot 3H_2O$ was added. When the solution color changed to red, then temperature was reduced to 90 °C and the solution was stirred continuously for 30 minutes. Next, 2 mL aliquot was harvested (G0). After that, 300 μL of trisodium citrate dihydrate (60 mM) and 300 μL of a $HAuCl_4 \cdot 3H_2O$ solution (25 mM) were added and maintained temperature of the solution at 90 °C for 30 minutes. Finally, 2 mL aliquot was harvested (G1) after each step to acquire further samples with larger sizes. All steps of seeded growth preparation involved in addition of 300 μL trisodium citrate dihydrate (60 mM) and 300 μL of $HAuCl_4.3H_2O$ (25 mM) at 90°C with 30 minutes of stirring as described above.

Characterization of AuNPs

The size and morphology of AuNPs was observed by transmission electron microscopy (TEM) using an H-7650 Hitachi TEM. Samples were prepared by depositing drops of AuNPs on formva/carbon grid. The UV-vis spectroscopy was used for measuring the size and size distribution of AuNPs.

Optimization of pH for anti-LipL32 mAb82 conjugated AuNPs

The optimal pH of colloidal AuNPs was investigated by various pH of 100 μL of 20-nm AuNPs (at OD_{520} = 1) ranging from 5.7 to 10.5 with 0.2 M sodium bicarbonate (Na_2CO_3). Then, colloidal AuNPs at each pH condition was measured absorbance at the wavelength 520 nm using spectrophotometer (before conjugation values). Next, 10 μg of anti-LipL32 mAb82 was added into each pH condition of colloidal AuNPs and incubated for 10 minute at room temperature. After incubation, the mixer between anti-LipL32 mAb82 and various pH of colloidal AuNPs were measured their absorbance at the wavelength 520 nm using spectrophotometer (after conjugation values). The optimal pH of colloidal AuNPs for conjugation to anti-LipL32 mAb82 was observed by visualization and different values between (before conjugation values) and (after conjugation values).

Optimization of antibody concentration for anti-LipL32 mAb82 conjugated AuNPs

An optimal concentration of anti-LipL32 mAb82 for conjugation to 20-nm AuNPs (at OD_{520} = 1) was determined by UV-vis spectroscopy. The anti-LipL32 mAb82 at various concentrations ranging from 0, 0.125, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, and 3 μg were added individually into 100 μL of colloidal AuNPs (OD_{520} = 1) at optimal pH (pH 9.0) and incubated for 10 minute at room temperature. Then, the anti-LIpL32 conjugated AuNPs mixer were measured absorbance at the wavelength 520 nm using spectrophotometer (value before adding NaCl). After that, 100 μL of 10% sodium chloride (NaCl) was added into mixed anti-LipL32 mAb82 conjugated AuNPs solution and incubated for 2 minute at room temperature. The mixture of anti-LipL32 conjugated AuNPs were measured at absorbance at the wavelength 520 nm using spectrophotometer again (value after adding NaCl). The optimal concentration of anti-LipL32 mAb82 conjugated AuNPs was investigated by visualization and different value between (value before adding NaCl) and (value after adding NaCl).

Conjugation of 20-nm AuNPs to anti-LipL32 mAb82 at the optimal condition

The 20-nm AuNPs were conjugated with anti-LipL32 mAb82 as described previously (106). Briefly, 1 mL of colloidal AuNPs (at OD_{520} = 1) was adjusted the pH 9.0 (at the optimal pH) with 0.2 M Na_2CO_3. Then, 15 μg of anti-LipL32 mAb82 (at the optimal amount) was mixed with AuNP solution and incubated at room temperature

with gentle mixing for 1 hour. Next, the mixed anti-LipL32 mAb82 with AuNP solution was blocked with 100 μL of 10% BSA and mixed continuously for 30 minutes. Finally, the anti-LipL32 mAb82-conjugated 20-nm AuNPs was centrifuged at 10,000 x g for 20 minutes and then pellet was re-suspended in gold storage buffer. The anti-LipL32 mAb82 conjugated AuNPs was stored at 4 °C until use.

Optimization of gold storage buffer

The sediment of anti-LipL32 mAb82 conjugated AuNPs from previous experiment was re-dispersed with various formulae of gold storage buffer (Table 4). The aggregation and color change of AuNPs were observed by visualization within 5 minutes. For longer storage time, anti-LIpL32 conjugated AuNPs in each formula of gold storage buffer were stored at 4 °C for 2 weeks. The aggregation and color change of AuNPs were observed by visualization.

Table 4. The formulae of gold storage buffer

Formula	Ingredients
1	1 M phosphate buffer, pH 6.0 + 1% BSA + 2 mM boric acid
2	1 M phosphate buffer, pH 6.8 + 1% BSA + 2 mM boric acid
3	1 M phosphate buffer, pH 8.0 + 1% BSA + 2 mM boric acid
4	100 mM phosphate buffer, pH 6.0 + 1% BSA + 2 mM boric acid
5	100 mM phosphate buffer, pH 6.8 + 1% BSA + 2 mM boric acid
6	100 mM phosphate buffer, pH 8.0 + 1% BSA + 2 mM boric acid

Construction of lateral flow assay (LFA) test strip

A lateral flow strip was assembled on a plastic backing pad composing of a sample pad, a conjugate pad, a nitrocellulose membrane, and an absorbent pad as shown (Figure 14a). The specific anti-LipL32 antibodies and goat anti-mouse IgG were immobilized on the test and control lines, respectively (Figure 14b) by using BioJet Elite dispensing (BioDot, Irvine, CA, USA). Then, the membranes were blocked with blocking agent. After that, the strips were incubated at room temperature for 30 minutes and washed membranes twice with double distilled water pH 8.0. The conjugate pad was deposited with anti-LipL32 antibodies conjugated AuNPs and dried at 45 ^{o}C for 1 hour. The sample pad, conjugate pad, nitrocellulose membrane and absorbent pad were assembled with 2 mm overlapping between each component (Figure 14b). The strips were stored in a desiccator dry cabinet (Auto dry cabinet, Korea Ace Scientific Crop.) until use. A sample was applied onto sample pad. The positive result is an appearance of two red lines at the test and control lines

(Figure 14c) while the negative result showed only a single line at the control line (Figure 14d).

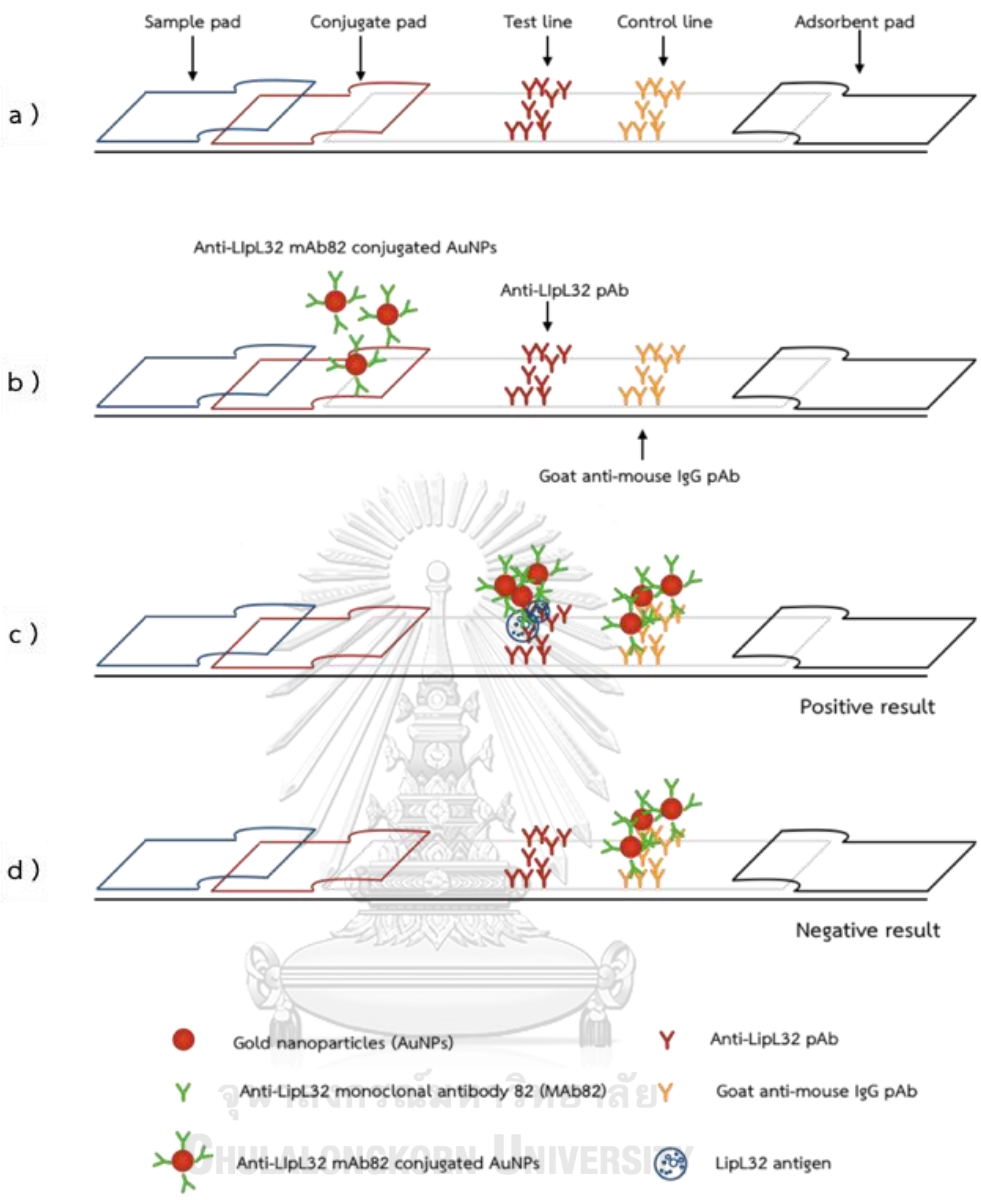


Figure 14. Typical configuration of a LFA test strips for detection of pathogenic leptospiral antigen. (a) Typical configuration of a LFA test strips. (b) AuNP-based LFA for detection of pathogenic leptospiral antigen. (c) Interpretation of LFA for detection of pathogenic leptospiral antigen, (c) positive result, and (d) negative result.

The effect of storage buffer for binding of anti-LipL32 mAb82-conjugated 20-nm AuNPs to rLipL32 on LFA test trips

The 15 μg of anti-LipL32 mAb82 were used for conjugation with 1 mL of 20-nm AuNPs (at OD_{520} =1) as described in previous experiment. The anti-LipL32 mAb82 conjugated AuNPs were incubated for 1 hour and blocked with 1% BSA for 30 minutes. After that, the mixed were centrifuged at 10,000 x g for 20 minutes. Next, the sediments were re-dispersed with 100 μL of gold storage buffer as 10 mM sodium phosphate buffer at pH 6.8 or pH 8.0. Lastly, 10 μL of each anti-LipL32 mAb82 conjugated AuNPs in different gold storage buffers was mixed with 90 μL of double distilled water (DDW) containing 100 ng of rLipL32 and load on sample pad of LFA test strips. The 90 μL of DDW without antigen was used as negative control. The LFA test strips were prepared following in previous experiment (construction of LFA test strip). Double band appearing at the test line (T) and the control line (C) was interpreted as positive result, and single band only at the control line was interpreted as negative result. The bands were observed by visualization for within 15 minutes.

Optimization of membrane blocking solution of LFA test strip

To find optimal blocking agents, the BSA, glycine, sucrose, trehalose and skim milk, at final concentration 1% (w/v) in sodium phosphate buffer pH 8.0 were tested to reduce non-specific binding. Construction of LFA strip was described previous experiment, after anti-LipL32 antibodies and anti-mouse IgG antibody were immobilized on test line and control line, respectively, membranes were blocked individually with BSA, glycine, sucrose, trehalose or skim milk for 30 minutes. Finally, the LFA strips were stored in a desiccator dry cabinet until use. The anti-LipL32 mAb82 conjugated AuNPs were immobilized on conjugate pad. The optimal membrane blocking solution of the LFA test strip was determined by visual detection using various amounts of rLipL32 ranging from 1 to 100 ng. The 10 mM phosphate buffer pH 8.0, buffer with no antigen (buffer) was used as negative control. The 100 μL of each sample was drop on sample pad. Double band appearing at the test line (T) and the control line (C) was interpreted as positive result, and single band only at the control line was interpreted as negative result. The bands were observed by visualization for within 15 minutes.

Selection of the best pair of antibodies and their positions on LFA strip

The best pair of antibodies for the LFA strip were selected from three antibodies including mouse anti-LipL32 mAb3 and mAb82, and rabbit anti-LipL32

pAb. Firstly, each antibody was immobilized on two different positions, the conjugated pad (in the form of Ab-conjugated 20-nm AuNPs) or the test line and incubated at 45 ^{o}C for 1 hour. Then, membranes were blocked with 1%BSA (an optimal membrane blocking buffer) for 30 minutes. Lastly, the LFA strips were stored in a desiccator dry cabinet until use. The LFA test strips were tested with various amounts of sonicated *L. interrogans* serovar Pomona at 10^4 to 10^6 cells. The sodium phosphate buffer pH 8.0 without antigen was used as negative control. The 100 μL of each sample was drop on sample pad. Double band appearing at the test line (T) and the control line (C) was interpreted as positive result, and single band only at the control line was interpreted as negative result. The bands were observed by visualization for within 15 minutes.

Evaluation of LFA strip using leptospiral antigen-spiked serum samples

The LFA strip was prepared as described previous experiment. Pre-treated sample pad with borate buffer pH 8.0 at various concentrations of 10 and 100 were tested to reduce non-specific binding of serum. The 100 μL of borate buffer pH 8.0 at concentration 10 or 100 was drop on sample pad and dried at 45 ^{o}C for 1 hour. Then, pre-treated sample pad was put forward of LFA strip. The anti-LipL32 mAb82 conjugated 20-nm AuNPs was immobilized on conjugate pad. The 1 μg of anti-LipL32 pAb and anti-mouse IgG antibody were immobilized on test line and control line,

respectively. The 10 μL of serum spiked with 100 ng rLipL32 and serum with no antigen were individually mixed with 90 μL of running buffer (1% BSA in sodium phosphate buffer pH 8.0) and applied on sample pad.

Modified running buffer with NaCl at various concentrations from 1 to 1,000 mM were tested to reduce non-specific biding in serum. The LFA strips were prepared as described above using pre-treated sample pad. The 10 μL of serum spiked with 100 ng rLipL32 and serum with no antigen were individually mixed with 90 modified μL of running buffer and applied on sample pad.

To determine limit of detection (LoD) of the LFA strip, sera spiked with sonicated whole cell lysates of 10^3, 10^4, 10^5, and 10^6 of pathogenic *L. interrogans* serovar Pomona were applied to the previously optimized AuNPs-based LFA. Double band appearing at the test line (T) and the control line (C) was interpreted as positive result, and single band only at the control line was interpreted as negative result. The bands were observed by visualization for within 15 minutes.

Enhancement of AuNP-based LFA for detection of pathogenic leptospiral antigen in serum

Different sizes of AuNPs were used for enhancement of sensitivity of the LFA strip. The anti-LipL32 mAb82 were conjugated individually with 20, 30 and 40-nm AuNPs following conjugation process as described previous experiment. The LFA strip using various combinations of pre-treated sample pads and modified running buffers were used in this experiment. To determine limit of detection (LoD) of the LFA strip, sera spiked with sonicated whole cell lysates of 10^3, 10^4, 10^5, and 10^6 of pathogenic *L. interrogans* serovar Pomona were applied onto the sample pad.

Modified running buffers with Tween 20 at various concentrations from 1.25, 2.5, 5.0 and 10 % (v/v) were used for enhancement of sensitivity. Optimal LFA test strips using 40-nm AuNPs conjugated with anti-LipL32 as described above were used for this experiment. To determine limit of detection (LoD) of the LFA strip, the 10 μL of serum spiked with intact cells of various amount from 10^3, 10^4, 10^5, and 10^6 of pathogenic *L. interrogans* serovar Pomona were mixed with 90 μL of the modified running buffer and applied on sample pad. Double band appearing at the test line (T) and the control line (C) was interpreted as positive result, and single band only at the control line was interpreted as negative result. The bands were observed by visualization within 15 minutes.

Evaluation of the AuNP-based LFA strip for detection of leptospiral antigen in clinical specimens

To determine sensitivity and specificity, the optimal LFA strips from previous experiment with pre-treated sample pad and modified running buffer were used for detection of leptospiral antigen in patient sera. Fifty acute phase sera from confirmed cases of leptospirosis, 20 acute phase sera from patients with unrelated diseases, and 10 sera from healthy persons were tested by using the optimal LFA strips. The 10 μL of sera from human leptospirosis, unrelated diseases or health persons were pretreated with 90 μL of Tween 20 at a final concentration about 4.5% and untreated samples before loading onto the sample pad. The bands were observed by visualization within 15 minutes.

Data analysis

The diagnostic sensitivity and specificity of the LFA test strip was determined using the following formulae:

Sensitivity (%) = true positive / (true positive + false negative) x 100%

Specificity (%) = true negative / (false positive + true negative) x 100%

CHAPTER V

RESULTS

Expression and purification of recombinant LipL32 (rLipL32) protein

E. coli strain BL21(DE3)pLysS containing *lipl32* gene in pRSET C vector obtained from our laboratory was successfully induced with 0.5 mM IPTG, which was subsequently purified by nickel column affinity chromatography. The purified rLipL32 was analyzed by 15% SDS-PAGE with Coomassie blue staining showing more that 95% purity (Figure 15a). The purified protein was confirmed to be rLipL32 by immunoblotting with anti-LipL32 monoclonal antibody (mAb82) (Figure 15b).

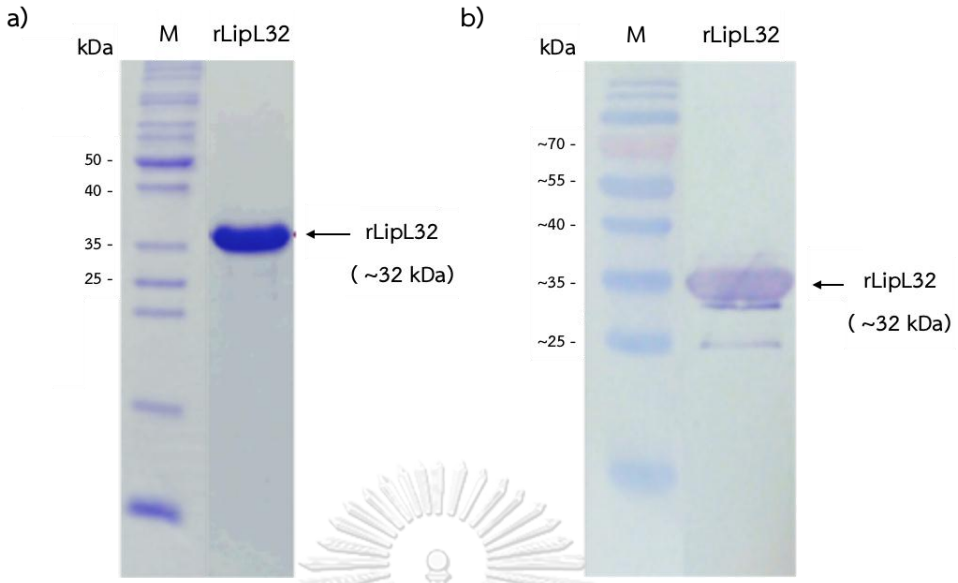


Figure 15. Determination of rLipL32 by SDS-PAGE and Western blot. Purity of rLipL32 was analyzed by 15% SDS-PAGE and Coomassie blue staining (a). Lane M: molecular weight (MW) marker, and lane rLipL32: purified rLipL32 was eluted with 200 mM imidazole. The rLipL32 was confirmed by Western blotting using anti-LipL32 mAb82 (b). Lane M: pre-strained MW marker, and lane rLipL32: purified rLipL32 was eluted with 200 mM imidazole. The arrows indicate the position of suspected rLipL32 protein. Molecular weight marker is shown in kilodaltons (kDa).

Production and purification of anti-LipL32 mAbs

Four clones of hybridoma cell lines were obtained from our previous study. Anti-LipL32 mAbs secreted from hybridoma clones 3 (mAb3) and 82 (mAb82) were higher than those from clones 2 (mAb2) and 81 (mAb81) (Figure 16).

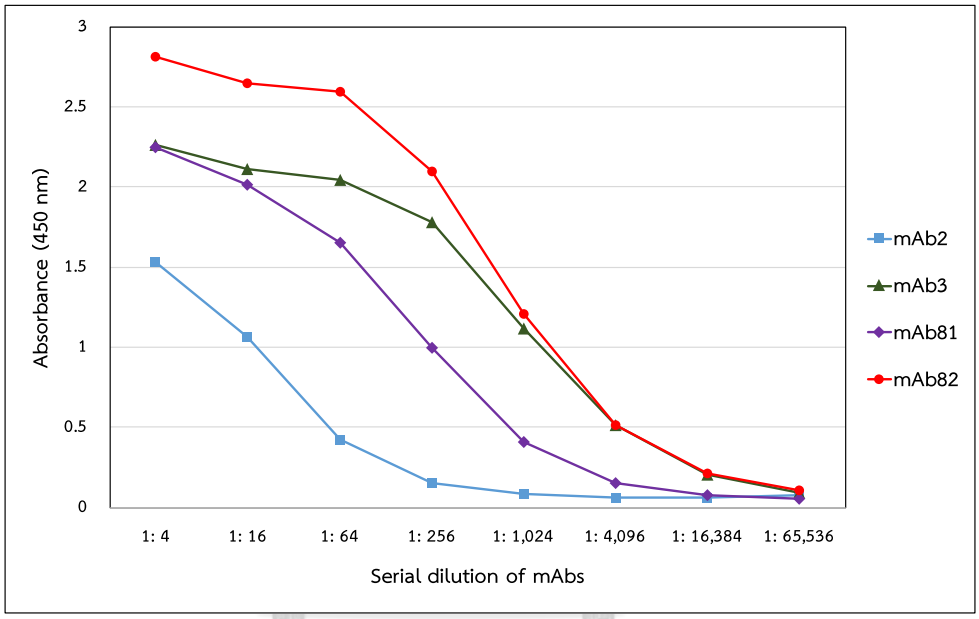


Figure 16. Production of anti-LipL32 mAbs. Culture supernatant containing anti-LipL32 mAbs from clones 2, 3, 81 and 82 were individually incubated with rLipL32 at a concentration 1 μg/mL. Then, HRP labeled goat anti-mouse IgG pAb was added and incubated for 1 hour. Finally, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added. The absorbance was measured at the wavelength 450 nm (OD_{450}) using a spectrophotometer.

Production and purification of anti-LipL32 pAb

The pAb against LipL32 was produced in a rabbit model. Rabbits were immunized three times with rLipL32 and Freund's adjuvant. Sera obtained after the first, second, and third immunization was shown to bind to both rLipL32 and native LipL32 protein of *L. interrogans* serovar Pomona (Figure 17). The anti-LipL32 pAb was purified with rLipL32-coupled NHS-activated Sepharose column (Figure 18a and 18b).

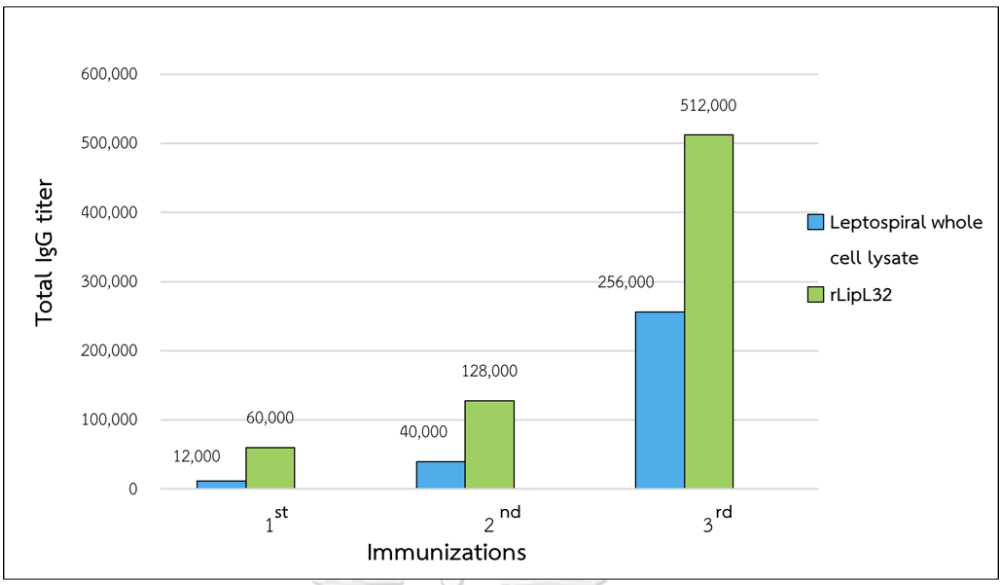


Figure 17. Production of rabbit anti-LipL32 pAb. A New Zealand white rabbit was immunized three times at 2-week interval with 200 μg of rLipL32 and Freund's adjuvant. Rabbit sera were collected after the first, second, and third immunization for detection of antibody titer against rLipL32 at a concentration 1 μg/mL and sonicated *L. interrogans* serovar Pomona at a concentration 10^7 cells/mL by ELISA. The absorbance was measured at the wavelength 450 nm (OD_{450}) using a spectrophotometer.

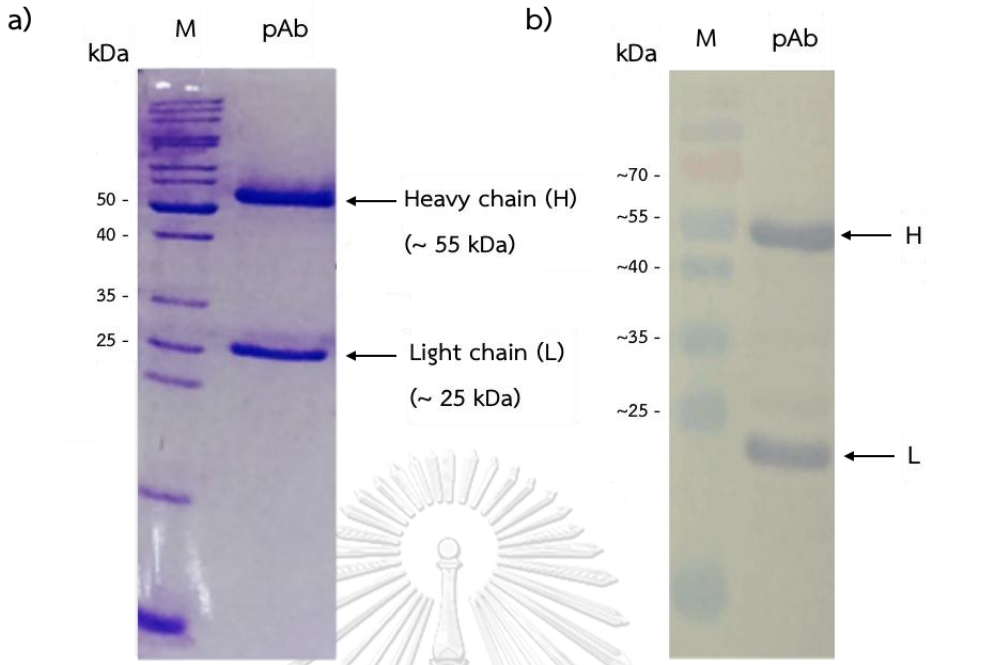


Figure 18. SDS-PAGE and Western blot analysis of purified anti-LipL32 pAb. The purified anti-LipL32 pAb was analyzed by SDS-PAGE using 15% acrylamide gel (a). Lane M: MW marker, and lane pAb: purified anti-LipL32 pAb was eluted with 0.1 M glycine pH 2.7. The purified anti-LipL32 pAb was confirmed by Western blotting using alkaline phosphatase (AP) labeled anti-rabbit IgG antibody (b). Lane M: pre-stained MW marker, and lane pAb: purified anti-LipL32 pAb was eluted with 0.1 M glycine pH 2.7. The arrows indicate the position of suspected heavy chain (H) and light chain (L) of rabbit anti-LipL32 pAb.

Binding of anti-LipL32 mAbs to *Leptospira* spp. by enzyme-linked immunosorbent assay (ELISA)

Binding of the anti-LipL32 mAbs to 24 reference serovars of *Leptospira* was performed by indirect ELISA. Anti-LipL32 mAbs secreted from hybridoma clones 3 and 82 were able to bind all tested pathogenic *Leptospira* spp. but did not bind to 2 serovars of non-pathogenic *Leptospira* (Ranarum and Patoc). The anti-LipL32 mAbs secreted from hybridoma clones 2 and 81 bound to 20 serovars of pathogenic *Leptospira* spp. except serovar Manhoa and Tarassovi (Figure 19 and Table 5). No clones showed binding to *E. coli* BL21(DE3)pLysS host strain used for recombinant protein production. Therefore, mAbs from clones 3 and 82 were used further for LFA development.

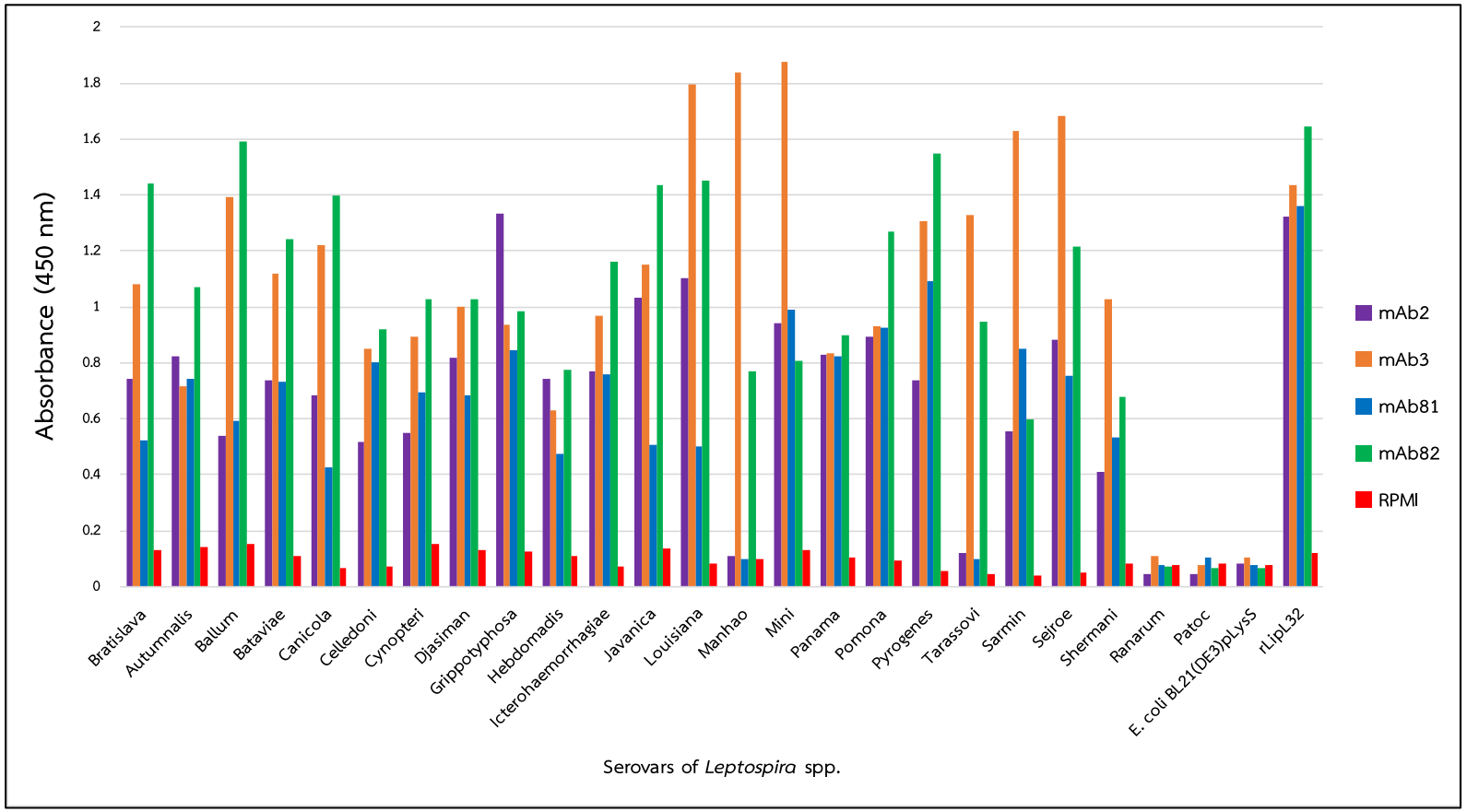


Figure 19. Binding of anti-LipL32 mAbs to *Leptospira* spp. by ELISA. The 24 representative serovars of *Leptospira* found in Thailand were used as a concentration of 10^7 cell/mL, rLipL32 was used at a concentration 1 μg/mL. Culture medium (RPMI 1640) was used as a negative

No.	Serovar	ELISA			
		mAb2	mAb3	mAb81	mAb82
1	Bratislava	+	+	+	+
2	Autumnalis	+	+	+	+
3	Ballum	+	+	+	+
4	Bataviae	+	+	+	+
5	Canicola	+	+	+	+
6	Celledoni	+	+	+	+
7	Cynopteri	+	+	+	+
8	Djasiman	+	+	+	+
9	Grippotyphosa	+	+	+	+
10	Hebdomadis	+	+	+	+
11	Icterohaemorrhagiae	+	+	+	+
12	Javanica	+	+	+	+
13	Louisiana	+	+	+	+
14	Manhao	-	+	-	+
15	Mini	+	+	+	+
16	Panama	+	+	+	+
17	Pomona	+	+	+	+
18	Pyrogenes	+	+	+	+
19	Tarassovi	-	+	-	+
20	Sarmin	+	+	+	+
21	Sejroe	+	+	+	+
22	Shermani	+	+	+	+
23	Ranarum	-	-	-	-
24	Patoc	-	-	-	-

Table 5. Binding of anti-LipL32 mAbs to *Leptospira* spp. by ELISA. The 24 representative serovars of *Leptospira* found in Thailand were used as a concentration of 10^7 cell/mL, rLipL32 was used at a concentration 1 μg/mL. Culture medium (RPMI 1640) was used as a negative control. + = the absorbance value at the wavelength

450nm (OD_{450}) was higher than that of the background (RPMI 1640 control); - = OD_{450} was not different from that of the background (RPMI 1640 control)

Binding of anti-LipL32 pAbs to *Leptospira* spp. by ELISA

Binding of the anti-LipL32 mAbs to 24 reference serovars of *Leptospira* was performed by indirect ELISA. The purified rabbit anti-LipL32 pAb was able to bind all 22 tested pathogenic *Leptospira* spp. but did not bind to 2 serovars of non-pathogenic *Leptospira* spp. (Ranarum and Patoc) (Figure 20). No binding to *E. coli* BL21(DE3)pLysS host strain was observed.

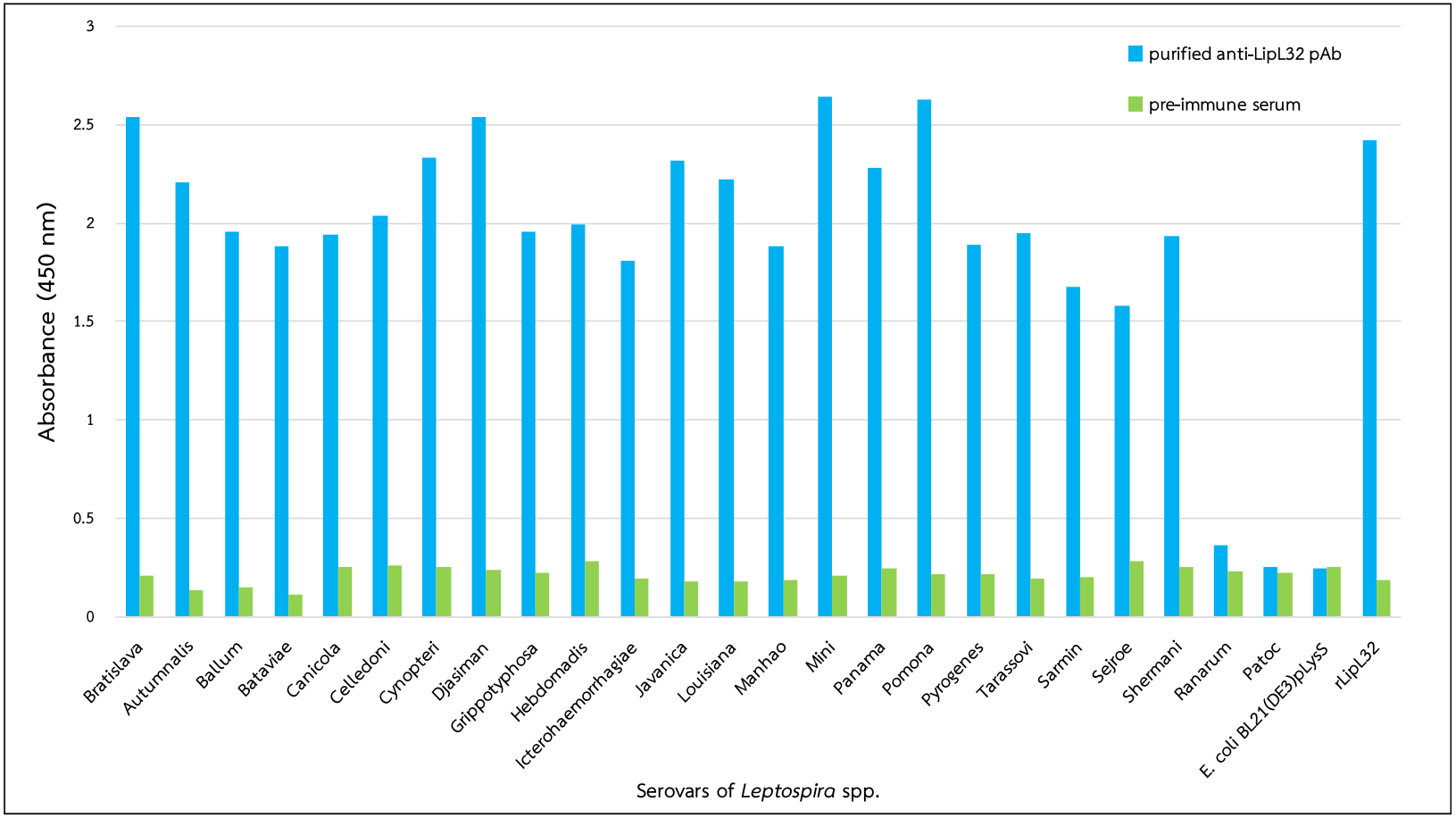


Figure 20. Binding of anti-LipL32 pAb to *Leptospira* spp. by ELISA. The 24 representative serovars of *Leptospira* found in thailand were used as a concentration of 10^7 cell/mL, rLipL32 was used at a concentration 1 g/mL. Culture medium (RPMI 1640) was used as a negative control.

Binding of anti-LipL32 mAbs to *Leptospira* spp. by dot blot assay

The binding of anti-LipL32 mAbs against sonicated *Leptospira* spp. were determined by dot blot assay using 22 serovars of pathogenic *Leptospira*, 2 non-pathogenic serovars of *Leptospira*, and rLipL32 as a positive control. The mAbs clone 3 and 82 were able to bind to all tested pathogenic serovars. In addition, both mAbs did not bind to non-pathogenic *L. meyeri* serovar Ranarum and *L. biflexa* serovar Patoc (Figure 21).

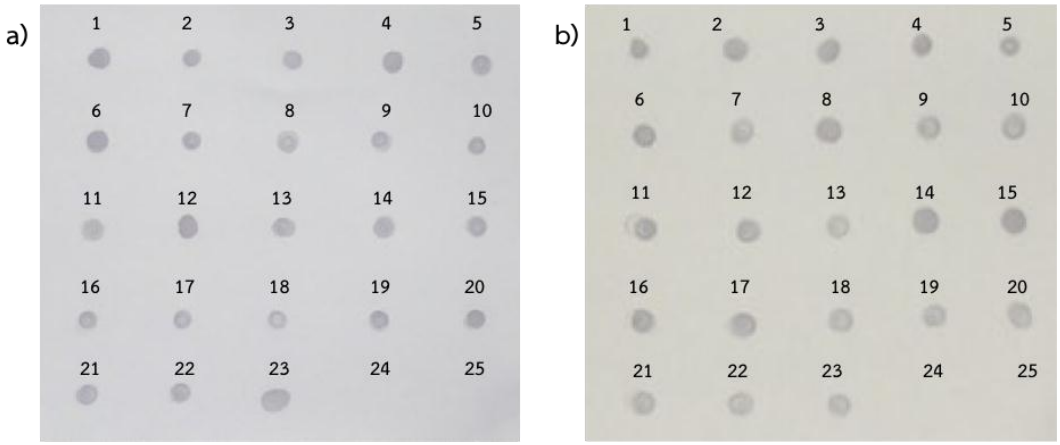


Figure 21. Determination of binding of anti-LipL32 mAbs by dot blot assay using anti-LIpL32 mAb3 (a) and anti-LipL32 mAb82 (B). The 10^6 cells of 24 serovars of sonicated *Leptospira* was individually spotted on a nitrocellulose membrane. The 100 ng of rLipL32 was used as a positive control (1). The 24 tested serovars includes Bratislava (2), Autumnalis (3), Ballum (4), Bataviae (5), Canicola (6), Celledoni (7), Cynopteri (8), Djasiman (9), Grippothyphosa (10), Hebdomadis (11), Icterohaemorragiae (12), Javanica (13), Louisiana (14), Manhao (15), Mini (16), Panama (17), Pomona (18), Pyrogenes (19), Sarmin (20), Sejroe (21), Shermani (22), Tarassovi (23), Ranarum (24), and Patoc (25). The spots were detected by visualization.

Competitive inhibition assay for binding of mAb3 and mAb82 to LipL32

To determine whether mAb3 and mAb82 bind to a common epitope on LipL32, a competitive inhibition assay was performed. The result showed that unlabeled mAb3 at various concentrations was unable to compete the binding of biotin labeled mAb82 to rLipL32 indicating that the mAb3 and mAb82 bound to different epitopes of rLipL32 (Figure 22). Therefore, both mAbs were used for development of AuNP-based LFA for detection of pathogenic leptospires.

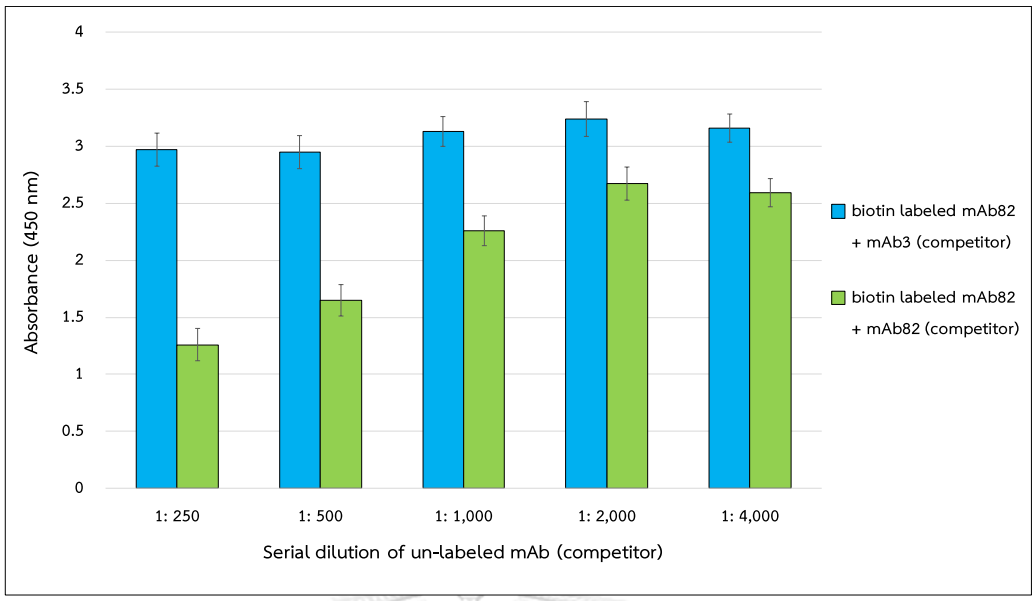


Figure 22. Competitive inhibition assay for common epitope binding of mAb3 and mAb82 to rLipL32 by ELISA. Each well was coated with 100 ng of purified rLipL32. Competitive binding between various dilutions of unlabeled mAb3 and biotin labeled mAb82 to rLipL32 was determined. The unlabeled mAb82 was used as a control. The absorbance was measured at the wavelength 450 nm (OD45) using a spectrophotometer.

Synthesis and characterization of the AuNPs

Colloidal AuNPs were successfully synthesized by seeded growth synthesis of citrate-stabilized AuNPs. Colors of the gold solution changed from light yellow to light red when it was completely reduced as becoming nanoparticles (Figure 23). This study, synthesized AuNPs at each of four generation growth steps resulted in four

different sizes of AuNPs. The colors of gold solution were correlated with the sizes of AuNPs (Figure 24). Morphology and particle size of anhydrous gold particles obtained after different growth steps were measured by TEM. The sizes of AuNPs gradually increased, 11.00±2.89, 20.74±3.54, 28.91±3.74, and 41.13±3.99 nm in diameter in the first, the second, the third, and the fourth growth steps, respectively (Figure 25). The globular shape of AuNPs were observed in all growth steps. In addition, UV-vis spectroscopy used to measure optical properties and extrapolate size of AuNPs showed that the maximum absorption peak shifted to longer wavelengths when the AuNP size increased. In this study, the synthesized AuNPs showed maximum absorption wavelengths at 518, 520, 524 and 532 nm in the 1^{st}, 2^{nd}, 3^{rd} and 4^{th} growth step, respectively (Figure 26).

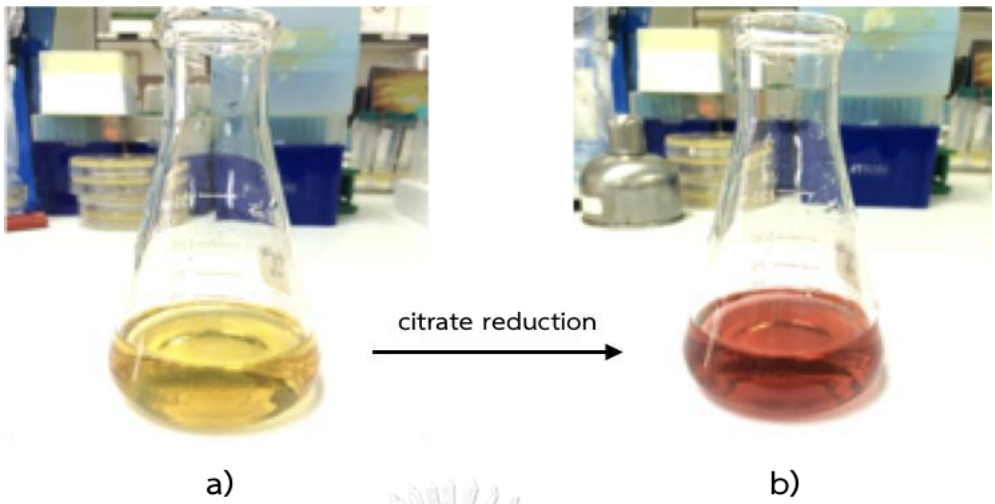


Figure 23. Synthesis of AuNPs by seeded growth method. Gold (III) chloride trihydrate ($HAuCl_4 \cdot 3H_2O$) were reduced with trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) in 90 ^{o}C with stirring condition. The color of gold solution changed from yellow to red as becoming nanoparticles.

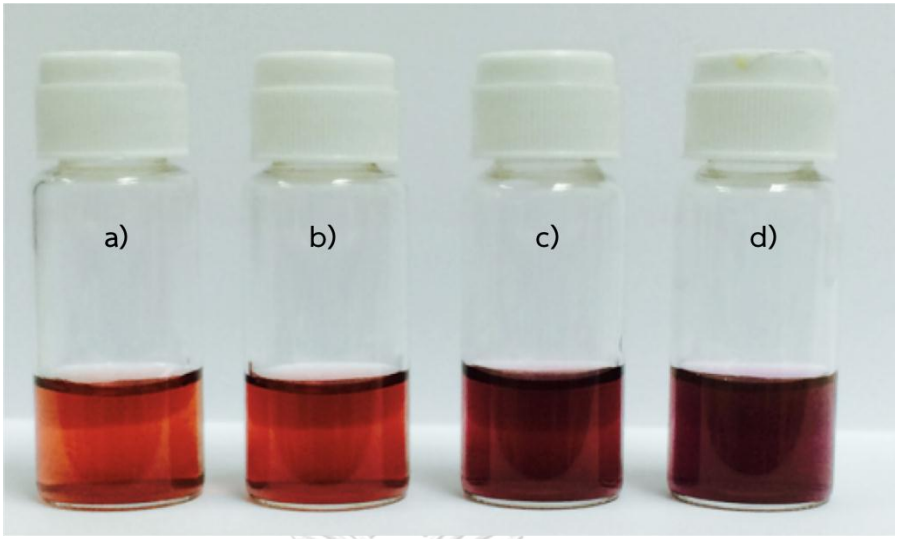


Figure 24. The color of different sizes of colloidal AuNPs. The AuNPs were synthesized by seeded growth method for four growth steps with citrate reduction. The smaller first generation AuNPs (a) were shown to be light red and changed to darker red color in the second (b), third (c), and fourth (d) generation AuNPs with larger sizes.

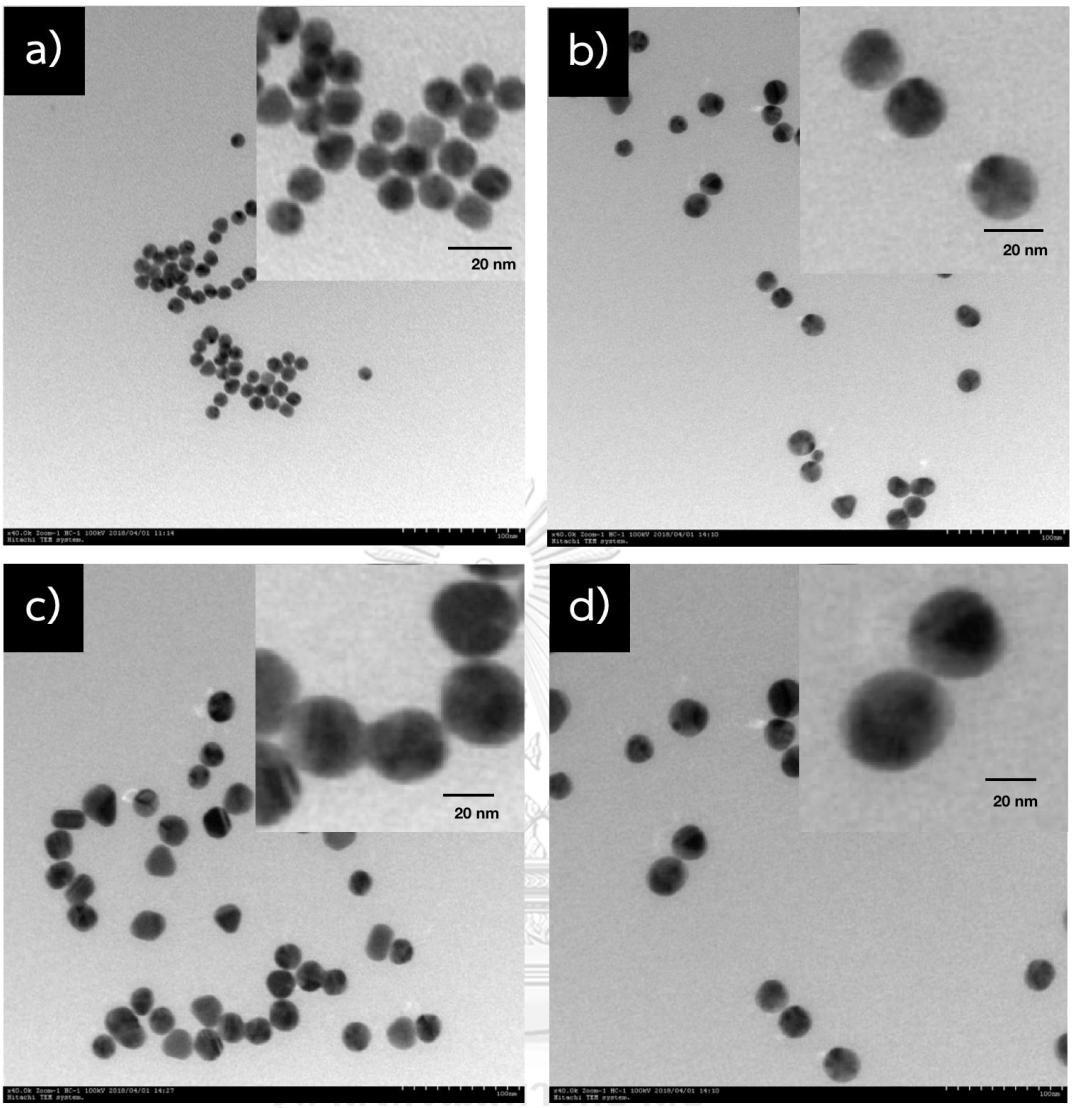


Figure 25. Morphology and sizes of AuNPs at different growth steps were analyzed by TEM. The different sizes of AuNPs were observed for the first (a), second (b), third (c), and fourth (d) growth step, respectively.

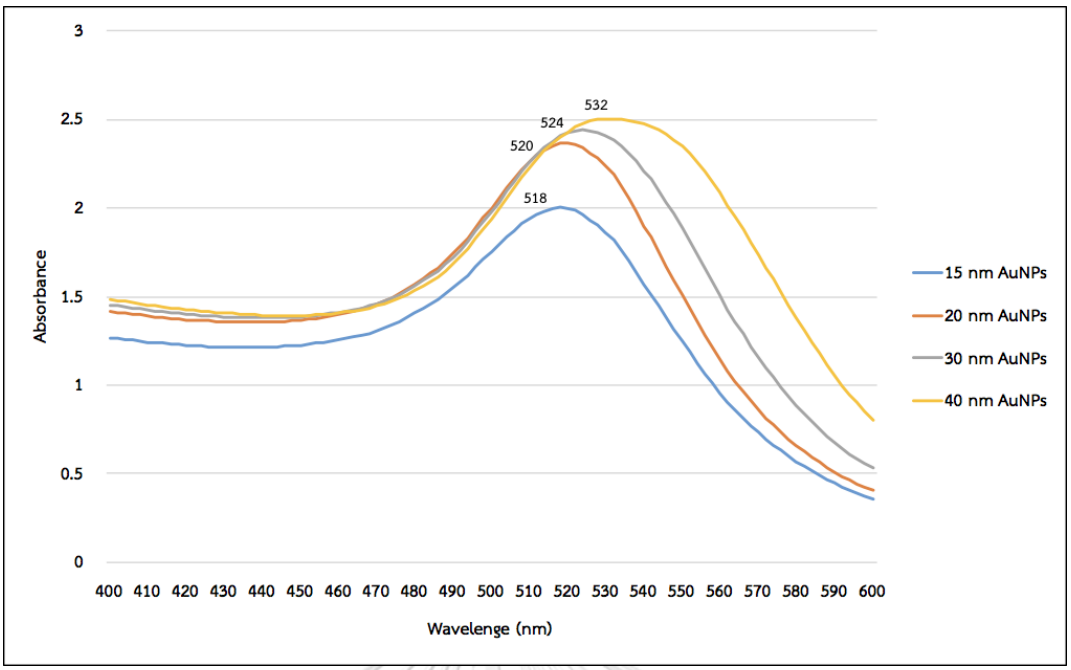
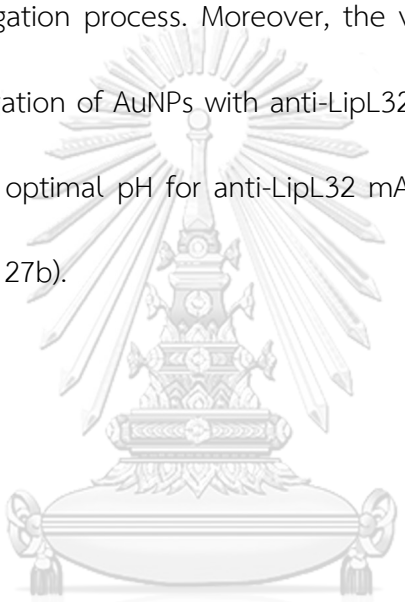


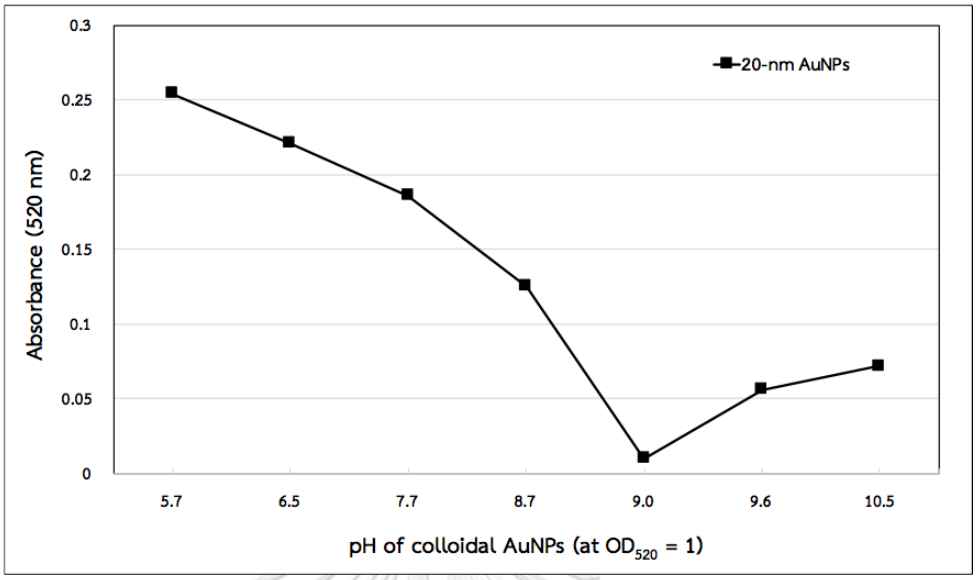
Figure 26. Absorbance spectra of AuNPs at different growth steps were analyzed by spectroscopy using scanning mode of spectrophotometer. The different absorbance spectra of AuNPs at 15, 20, 30, and 40 nm in diameter were observed in the first, second, third, and fourth growth step, respectively.

Optimization of pH for anti-LipL32 mAb82 conjugated AuNPs

The 20-nm AuNPs (at OD_{520} = 1) were used for optimization of the LFA in the following experiments. Various pH ranging from 5.7 to 10.5 were tested for conjugation of AuNPs to the antibodies. At the optimal pH, aggregation of AuNPs was at the minimum as determined by spectrophotometer. No color change indicated no aggregation after conjugation process. Moreover, the values of absorbance at OD_{520} before and after conjugation of AuNPs with anti-LipL32 mAb82 at various pH did not change. Therefore, the optimal pH for anti-LipL32 mAb82 conjugated 20-nm AuNPs was 9.0 (Figure 27a and 27b).



a)



b)

Figure 27. Optimization of pH for 20-nm colloidal AuNPs (at OD_{520} = 1) conjugated with anti-LipL32 mAb82 (10 μg). The effect of various pH ranging from 5.7 to 10.5 for 20-nm AuNPs conjugated to anti-LipL32 mAb82 were analyzed at the wavelength 520 nm (OD_{520}) using a spectrophotometer (a). Visual changes of color at different pH for 20-nm colloidal AuNPs conjugated with anti-LipL32 mAb (b).

Optimization of antibody concentration for anti-LipL32 mAb82-conjugated AuNPs

At the optimal pH (pH 9.0) of 20-nm colloidal AuNPs (at OD_{520} = 1), various concentrations of anti-LipL32 mAb82 from 0 to 30 μg per 1 mL of colloidal AuNPs were tested for conjugation process. The absorbance values of colloidal AuNPs after conjugation to anti-LipL32 mAb82 were reduced to nearly zero when saturation of anti-LipL32 conjugated mAb on AuNPs was reached. The saturated amount of anti-LipL32 mAb for conjugation was at 15 μg per 1 mL of 20-nm AuNPs (at OD_{520} = 1) (Figure 28).

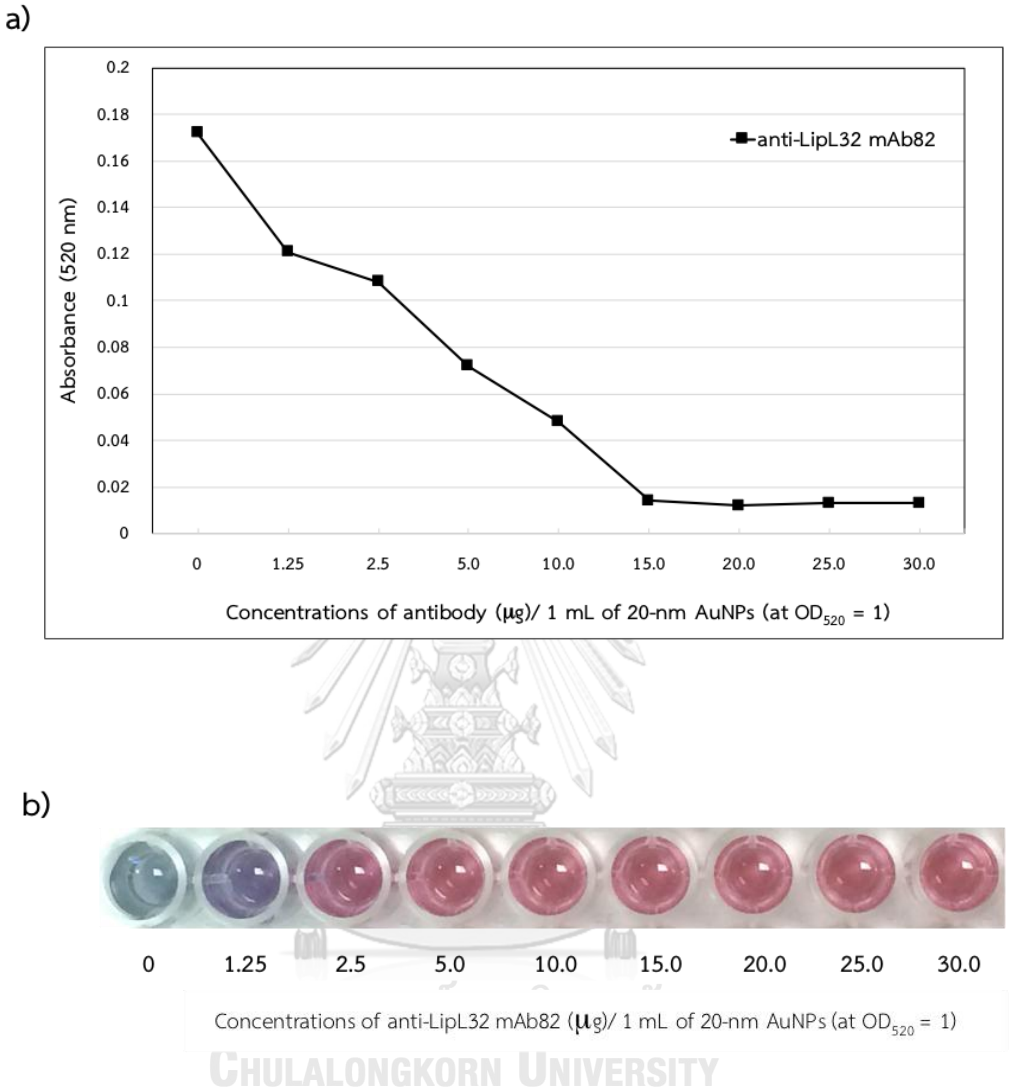


Figure 28. Optimization of anti-LipL32 mAb82 concentrations for conjugation to 20-nm AuNPs (at OD_{520} =1). The effect of anti-LipL32 mAb82 concentration from 1.25 to 30.0 µg for conjugation per 1 ml of 20-nm AuNPs was analyzed at the wavelength 520 nm (OD_{520}) using a spectrophotometer (a) with corresponding visual changes of colors at different amount of anti-LipL32 mAb82 (b).

Conjugation of 20-nm AuNPs to anti-LipL32 mAb82 at the optimal condition

The optimal pH (pH 9.0) of 20-nm AuNPs (at OD_{520} = 1) and concentration of anti-LipL32 mAb82 (15 μg/mL) was used for the conjugation process. After conjugation of the mAb82 to AuNPs, the solution of AuNPs was visually observed for aggregation. The anti-LipL32 mAb82-conjugated AuNPs showed no aggregation, well re-dispersed, and no change of color (Figure 29a) at the optimal pH (pH 9.0). In contrast, aggregation of AuNPs and color changes were observed at lower pH (6.5) (Figure 29b) and higher pH (10.5) (Figure 29c).

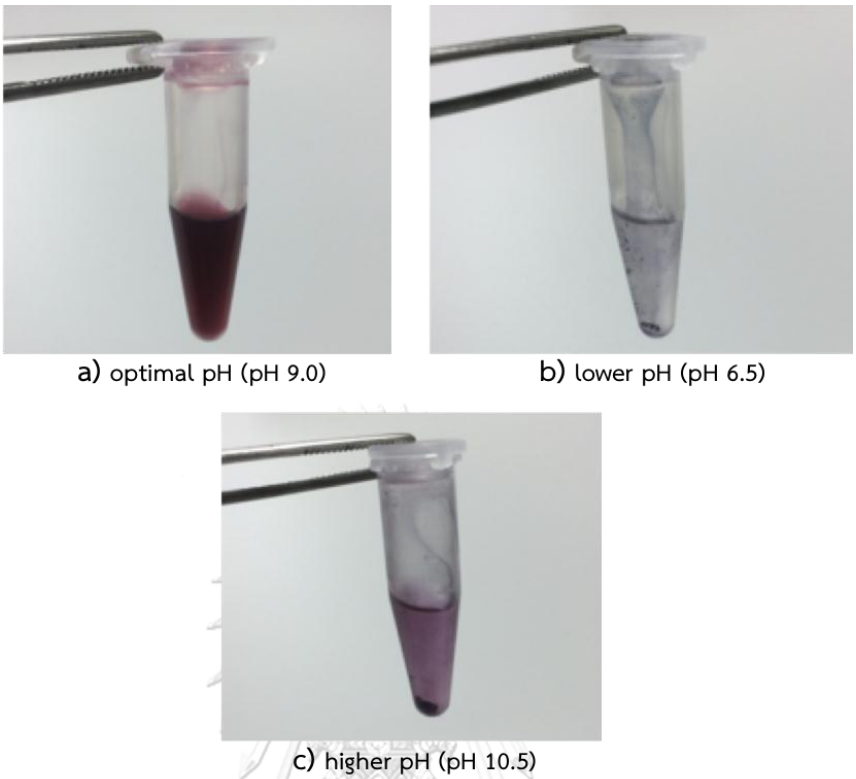


Figure 29. Visual analysis of anti-LipL32 mAb82-conjugated 20-nm AuNPs. At the optimal pH (pH 9.0) and optimal amount of antibody, the gold solution showed no aggregation and no color change (a). At the low pH (pH 6.5) (b) and high pH (pH 10.5) (c), the gold solution revealed aggregation and color changes. The aggregation and color change were observed by visualization within 5 minutes.

Optimization of gold storage buffer

In this study, 10 and 100 mM sodium phosphate buffer at pH 6.0, 6.8, and 8.0 were evaluated for 20-nm AuNP conjugation to anti-LipL32 mAb82 (Figure 30). In 10 mM sodium phosphate buffer, all tested pH did not cause aggregation of AuNPs (Figure 30a). In contrast, color changes and aggregation of AuNPs were observed in 100 mM sodium phosphate buffer at pH 6.0 and 8.0 (Figure 30b) within 5 minutes. For longer storage time, color changes and flocculation of mAb82-conjugated AuNPs were observed up to two weeks. Flocculation and color changes were observed in 10 mM phosphate buffer pH 6.0 and in 100 mM phosphate buffer pH 6.8 after storage at 4 ^{o}C for over six days. Therefore, 10 mM sodium phosphate buffer pH 6.8 and 8.0 were tested as a storage buffer for mAb82-conjugated AuNPs on LFA test strip platform in next experiment.

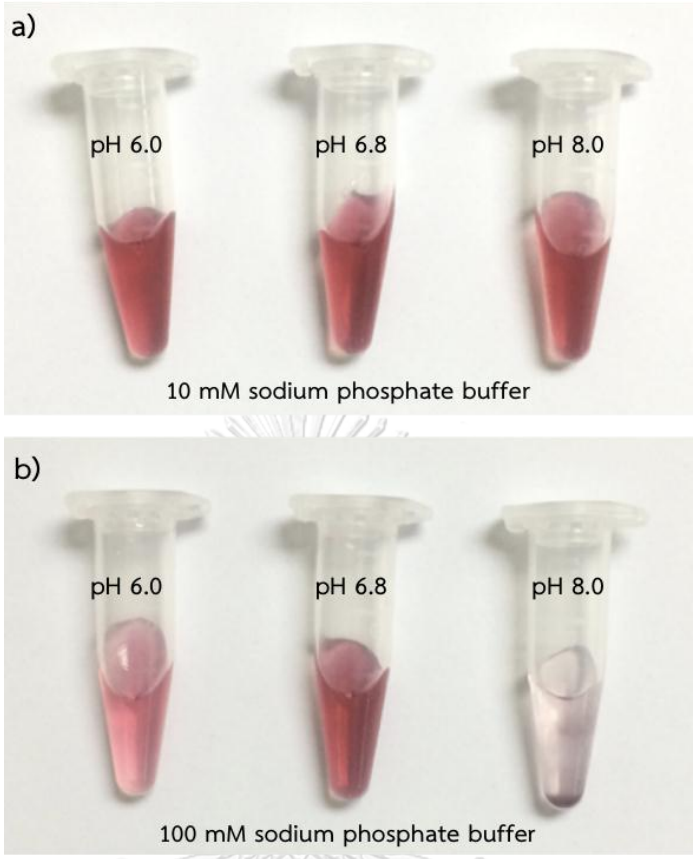


Figure 30. Stability of anti-LipL32 mAb82-conjugated 20 nm-AuNPs in gold storage buffers. Visualization of aggregation and color change of anti-LipL32 mAb82-conjugated AuNPs was determined at pH 6.0, 6.8, and 8.0 in 10 mM sodium phosphate buffer (a) and 100 mM sodium phosphate buffer (b). The aggregation and color change were observed by visualization within 5 minutes.

The effect of storage buffer for binding of anti-LipL32 mAb82-conjugated 20-nm AuNPs to rLipL32 on LFA test trips

Binding of mAb82 in different storage buffers to rLipL32 was observed on the LFA test strip using anti-LipL32 pAb immobilized on the test line. Using 10 mM sodium phosphate buffer pH 8.0, anti-LipL32 mAb82-conjugated AuNPs showed specific binding to 100 ng of rLipL32 (Figure 31a). In contrast, false positive result was observed in 10 mM sodium phosphate buffer pH 6.8 (Figure 31b). Therefore, 10 mM sodium phosphate buffer pH 8.0 is optimal for specific binding of anti-LipL32 mAb82 conjugated 20-nm AuNPs to LipL32 antigen on the LFA test strip.

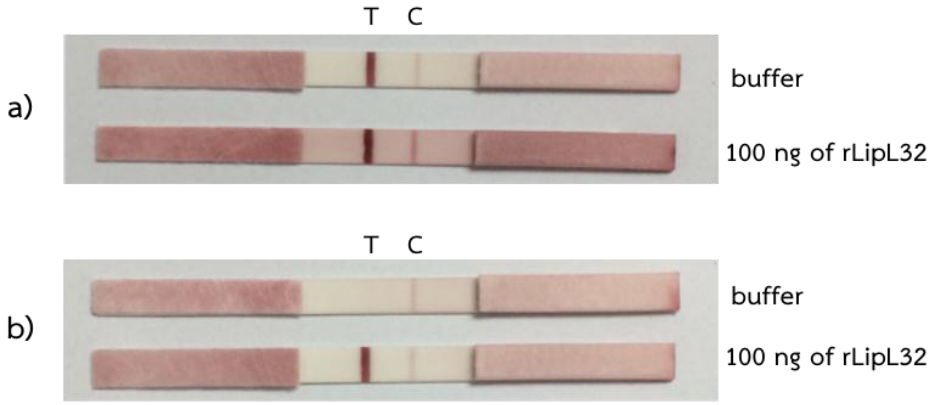


Figure 31. Effect of storage buffer for binding of anti-LipL32 mAb82-conjugated 20-nm AuNPs to rLipL32 on LFA test trip. The sediments of anti-LipL32 mAb82-conjugated AuNPs were re-dispersed in 10 mM sodium phosphate buffer pH 6.8 (a) and in 10 mM sodium phosphate buffer pH 8.0 (b). Then, 100 ng of rLipL32 was added in mixed antibody-AuNPs solution and applied on the sample pad (100 ng of rLipL32). Double distilled water was used as negative control (buffer). The 1 μg of each anti-LipL32 pAb and goat anti-mouse IgG pAb was immobilized onto test line and control line, respectively. Double band at the test line (T) and the control line (C) is interpreted as positive result, and single band at the control line (C) is interpreted as negative result. The bands were observed by visualization within 15 minutes.

Optimization of membrane blocking solution for LFA strip

To prevent non-specific binding on LFA test strips five reagents including bovine serum albumin (BSA), casein, glycine, trehalose, and sucrose were used in blocking buffers. All blocking reagents were individually dissolved in 10 mM sodium phosphate buffer pH 8.0, which was shown previously to be the optimal storage buffer for anti-LipL32 mAb82-conjugated AuNPs. The results showed that 1% BSA and 1% casein were able to block non-specific binding. Moreover, the limit of detection (LoD) of the membrane blocked with 1% BSA was 10 times lower than that blocked with 1% casein. Blocking buffers containing glycine, trehalose, and sucrose showed inhibition of mobile phase onto the nitrocellulose membrane. Therefore, the optimal membrane blocking solution of LFA test strip in this study was 1% BSA in 10 mM sodium phosphate buffer pH8.0 (Figure 32).

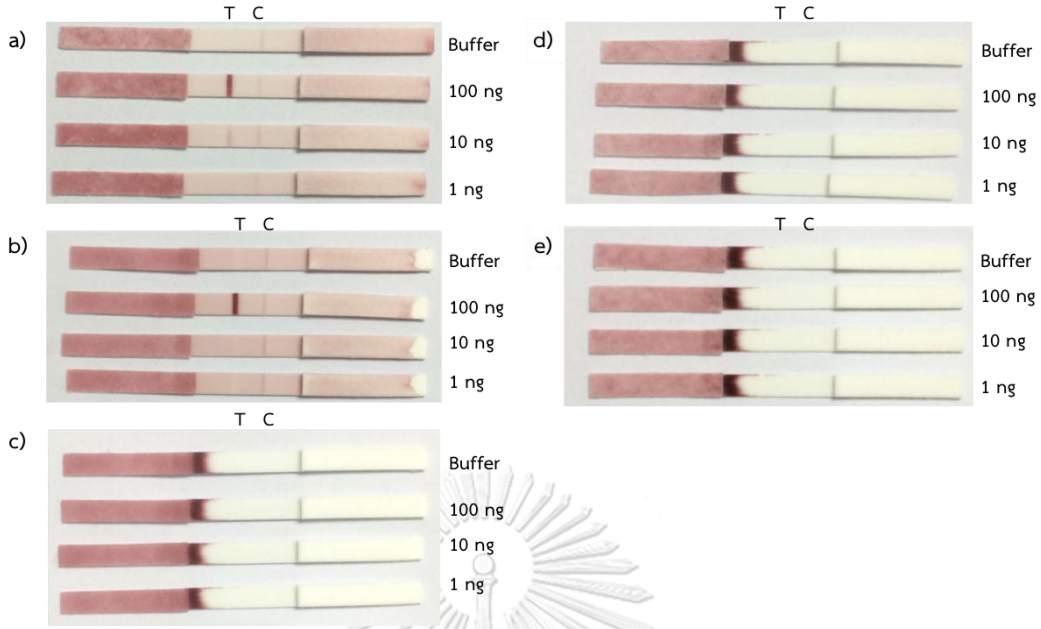


Figure 32. Determination of membrane blocking solution for detection of rLipL32 by LFA test strip. Nitrocellulose membranes were individually blocked with 5 different blocking solutions containing 1% BSA (a), 1% casein (b), 1% glycine (c), 1% trehalose (d), and 1%sucrose (e). The optimal membrane blocking solution of the LFA test strip was determined by visual detection using various amounts of rLipL32 ranging from 1 to 100 ng. The 10 mM phosphate buffer, pH 8.0 buffer with no antigen (buffer) was used as negative control. The 1 μg of each anti-LipL32 pAb and goat anti-mouse IgG pAb was immobilized onto test line and control line, respectively. Double band at the test line (T) and the control line (C) is interpreted as positive result, and single band at the control line (C) is interpreted as negative result. The bands were observed by visualization within 15 minutes.

Selection of the best pair of antibodies and their positions on LFA strip

The best pair of antibodies for the LFA strip were selected from three antibodies including mouse anti-LipL32 mAb3 and mAb82, and rabbit anti-LipL32 pAb. Each antibody was immobilized on two different positions, the conjugated pad (in the form of Ab-conjugated AuNPs) or the test line, and then tested with various amounts of sonicated *L. interrogans* serovar Pomona. Of all patterns, the LFA strip with either mAb-conjugated AuNPs on the conjugate pad and anti-LipL32 pAb on the test line (pattern 3 and 5) showed the lowest LoD at 10^5 leptospiral cell lysates. The highest LoD, more than 10^6 leptospiral cell lysates, was obtained when anti-LipL32 pAb was on the conjugate pad and either mAb3 or mAb82 was on the test line (Figure 33 and Table 6).

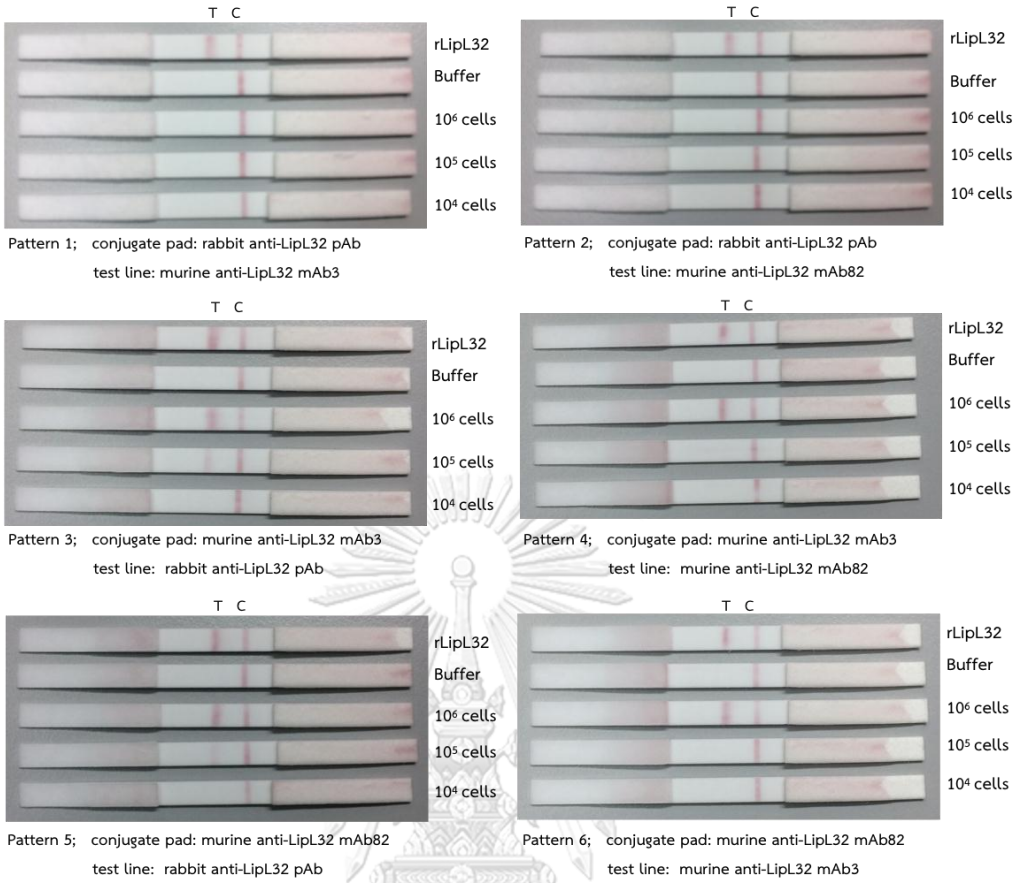


Figure 33. The best pair selection of anti-LipL32 antibodies immobilized on the conjugate pad and the test line of the LFA strip. The limit of detection of each pattern of LFA was determined using various amounts of sonicated pathogenic leptospires. 10 mM sodium phosphate buffer, pH 8.0 (buffer) was used as a negative control and 100 ng of rLipL32 was used as a positive control. Double band at the test line (T) and the control line (C) is interpreted as positive result, and single band at the control line (C) is interpreted as negative result. Double band at the test line (T) and the control line (C) is interpreted as positive result, and single band at the

control line (C) is interpreted as negative result. The bands were observed by visualization within 15 minutes.

Pattern	Conjugate	Test line	Limit of detection
1	pAb	mAb3	> 10^6 cells of sonicated pathogenic *Leptospira*
2	pAb	mAb82	> 10^6 cells of sonicated pathogenic *Leptospira*
3	mAb3	pAb	10^5 cells of sonicated pathogenic *Leptospira*
4	mAb3	mAb82	10^6 cells of sonicated pathogenic *Leptospira*
5	mAb82	pAb	10^5 cells of sonicated pathogenic *Leptospira*
6	mAb82	mAb3	10^6 cells of sonicated pathogenic *Leptospira*

Table 6. The limit of detection of each pattern of anti-LipL32 antibody immobilized on the conjugate pad or the test line of the LFA strip as determined using various amounts of sonicated pathogenic leptospires.

Evaluation of LFA strip using leptospiral antigen-spiked serum samples

The AuNP-based LFA strip was evaluated using 100 ng rLipL32 protein-spiked serum. The antigen-spiked were applied onto the LFA strip comprising sample pads treated with 10 and 100 mM borate buffer pH 8.0 compared to the strip with untreated sample pads. Healthy serum from normal persons without spiked antigen were used as a negative control. False positive results (positive test line with healthy

serum) were observed in the LFA strips comprising untreated (Figure 34a) and 10 mM borate buffer pH 8.0 pre-treated sample pads (Figure 34b). The LFA strip comprising a sample pad treated with 100 mM borate buffer pH 8.0 showed weakly positive when serum from healthy person was tested (Figure 34c).

To further reduce non-specific detection, in addition to the borate buffer-treated sample pad, the running buffer (1% BSA, 0.05% Tween20 in phosphate buffer pH 8.0) used to mix with samples before loading was modified by adding various concentrations of 1, 10 100 and 1000 mM NaCl. Only 100 mM NaCl in modified running buffer showed no band at the test line when healthy serum was added for 15 minutes. 1 M NaCl in the running buffer made gold aggregation on the conjugate pad. The combination of 100 mM NaCl in running buffer and 100 mM borate buffer pH 8.0 pre-treated sample pad did not showed false positive test line after applying the healthy serum sample for at least 15 minutes (Figure 35).

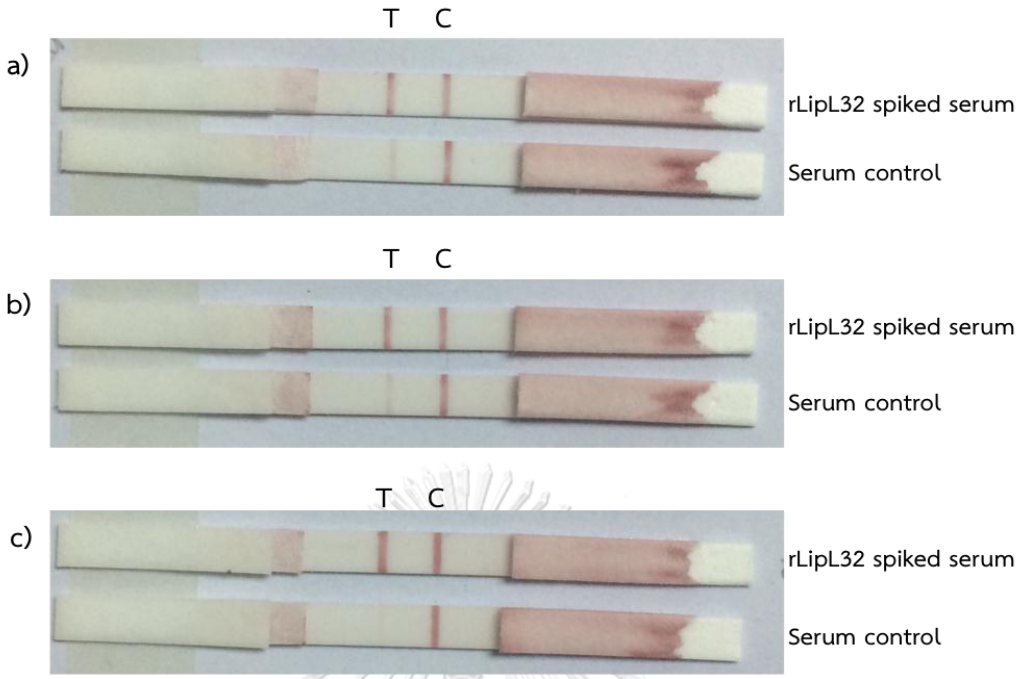


Figure 34. The 20-nm AuNP-based LFA strip with a sample pad pretreated with borate buffer. Untreated sample pad (a), or treated sample pad with 10 mM (b), and 100 mM borate buffer pH 8.0 (c) were applied with sera spiked with 100 ng rLipL32. Unspiked healthy serum was used as a negative control (serum control). The anti-LipL32 mAb82-conjugated 20-nm AuNPs was immobilized on the conjugate pad. The 1 μg of each anti-LipL32 pAb and goat anti-mouse IgG pAb was immobilized onto the test line and the control line, respectively. Double band at the test line (T) and the control line (C) is interpreted as positive result, and single band at the control line (C) is interpreted as negative result. The bands were observed by visualization within 15 minutes.

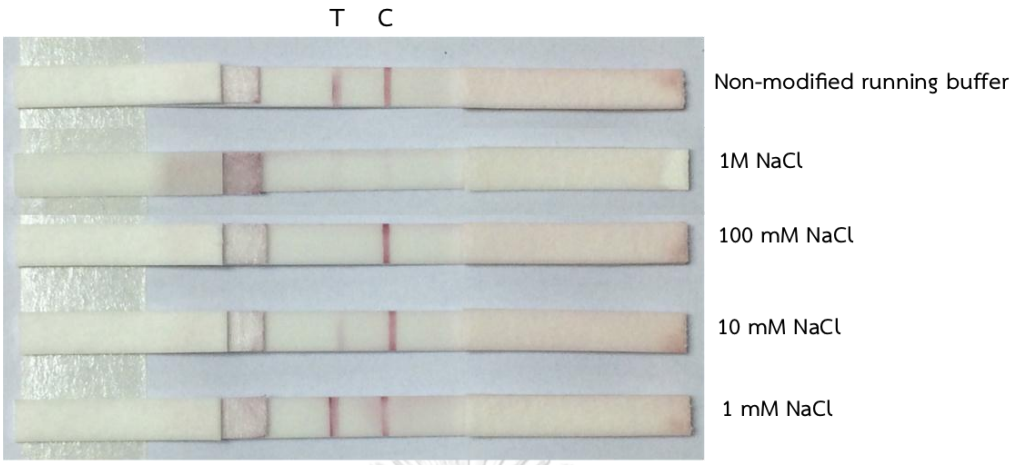


Figure 35. The AuNP-based LFA with sample pad pretreated with 100 mM borate buffer pH 8.0 and modified running buffer using 1, 10, 100 mM, and 1 M NaCl. Results were observed after healthy serum was applied onto leptospirosis test strip for 15 minutes. All LFA test strips were tested with the samples containing 10 μL of healthy serum and 90 μL of running buffer. The anti-LipL32 mAb82-conjugated 20-nm AuNPs was immobilized on the conjugate pad. The 1 μg of each anti-LipL32 pAb and goat anti-mouse IgG pAb was immobilized onto the test line and the control line, respectively. Double band at the test line (T) and the control line (C) is interpreted as positive result, and single band at the control line (C) is interpreted as negative result. The bands were observed by visualization within 15 minutes.

Next, to determine limit of detection (LoD) of the LFA strip, sera spiked with whole cell lysates of 10^3, 10^4, 10^5, and 10^6 of pathogenic *L. interrogans* serovar Pomona were applied to the previously optimized AuNP-based LFA. The LoD for this 20-nm AuNP-based LFA was shown to be 10^6 cells of sonicated whole cell lysates (Figure 36).

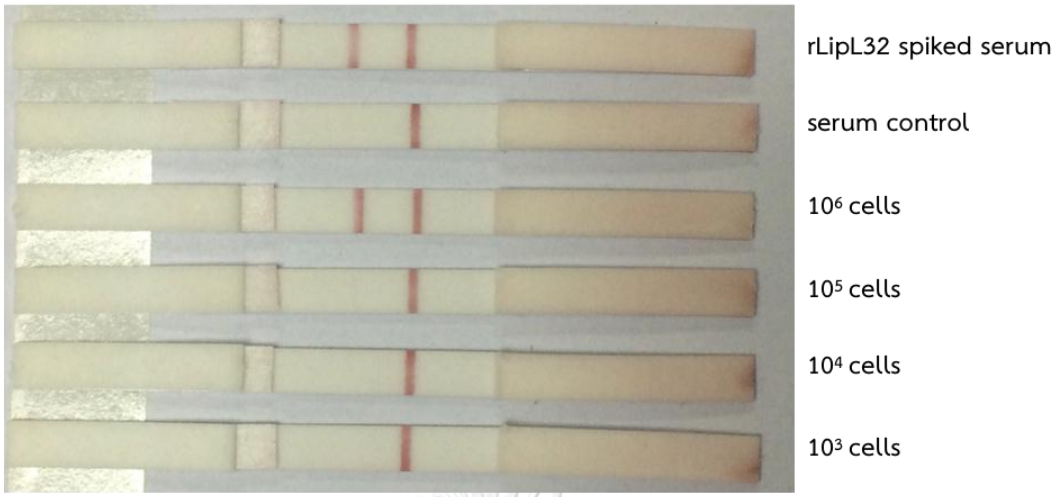


Figure 36. Limit of detection of the LFA test trips for detection with sonicated pathogenic leptospires. Sera spiked with 10^3, 10^4, 10^5, and 10^6 cells of pathogenic *Leptospira* whole cell lysates were applied to the LFA. Serum spiked with 100 ng of rLipL32 was used as a positive control and unspiked serum was used as a negative control (serum control). The results were observed by visualization within 15 minutes. The anti-LipL32 mAb82-conjugated 20-nm AuNPs was immobilized on the conjugate pad. The 1 μg of each anti-LipL32 pAb and goat anti-mouse IgG pAb was immobilized onto the test line and the control line, respectively. The bands were observed by visualization.

Enhancement of AuNP-based LFA for detection of pathogenic leptospiral antigen in serum

To improve LoD of the AuNP-based LFA strip, different sizes of gold nanoparticles; 20, 30, and 40 nm, were conjugated to anti-LipL32 antibodies. The pH of colloidal AuNPs and the concentration of anti-LipL32 antibodies conjugated to 30-nm and 40-nm AuNPs were optimized similar to those of 20-nm AuNPs as described earlier. The signal intensity of the test line of LFA strips was observed after applying sera spiked with whole cell lysates of 10^3 to 10^6 pathogenic *Leptospira*. The LoD of 40-nm AuNP-based strip was 10 and 100 times higher than that of 30-nm and 20-nm AuNPs, respectively (Figure 37). The LoD of 40-nm AuNP-based LFA strip was 10^4 cells of sonicated whole cell lysates spiked in serum (Figure 37c). In addition, to improve sensitivity of detection of *Leptospira* by the 40-nm AuNP-based LFA strip, serum samples were pretreated with mild detergent, Tween 20, before use. Optimal concentration of Tween 20 at a final concentration of 4.5 % (v/v) showed true positive and true negative results for detection of pathogenic and non-pathogenic *Leptospira*, respectively (Figure 38a and 38b). However, serum samples pretreated with 9.0% Tween 20 resulted in false positive detection (Figure 38b). In addition, pretreated samples with 4.5% Tween 20 rendered 2-fold reduction of LoD of the LFA strip. Therefore, the LoD of 40-nm AuNP-based LFA strips using sera pretreated with 4.5% Tween 20 was 5 x 10^3 cells of leptospires (Figure 39).

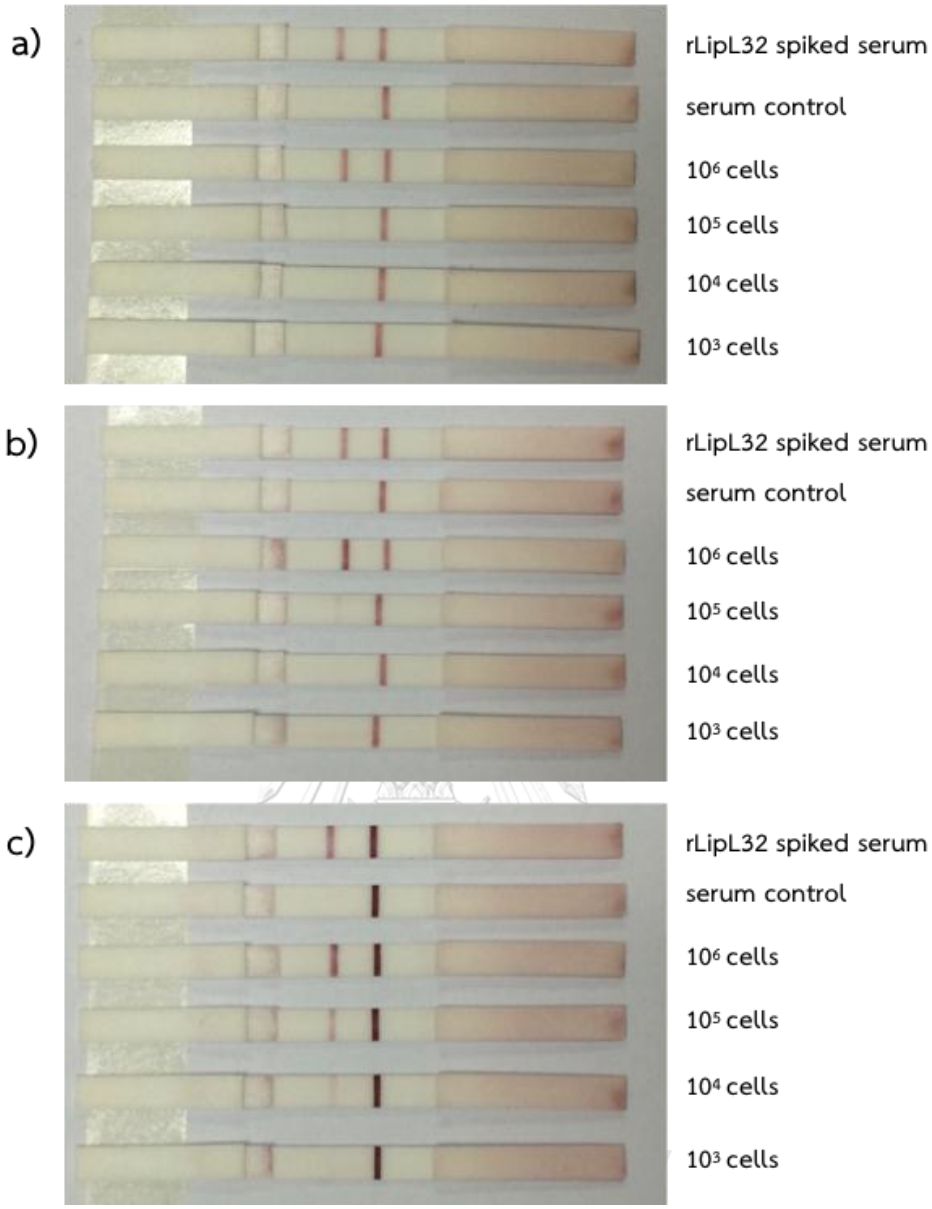


Figure 37. Limit of detection of 20-nm (a), 30-nm (b), and 40-nm (c) AuNP-based LFA strips. The gold particles at a particular size conjugated to anti-LipL32 mAb82 were used to develop LFA strips for detection of sera spiked with 10^3 to 10^6 cells of pathogenic *Leptospira* whole cell lysates. The bands were observed by visualization within 15 minutes.

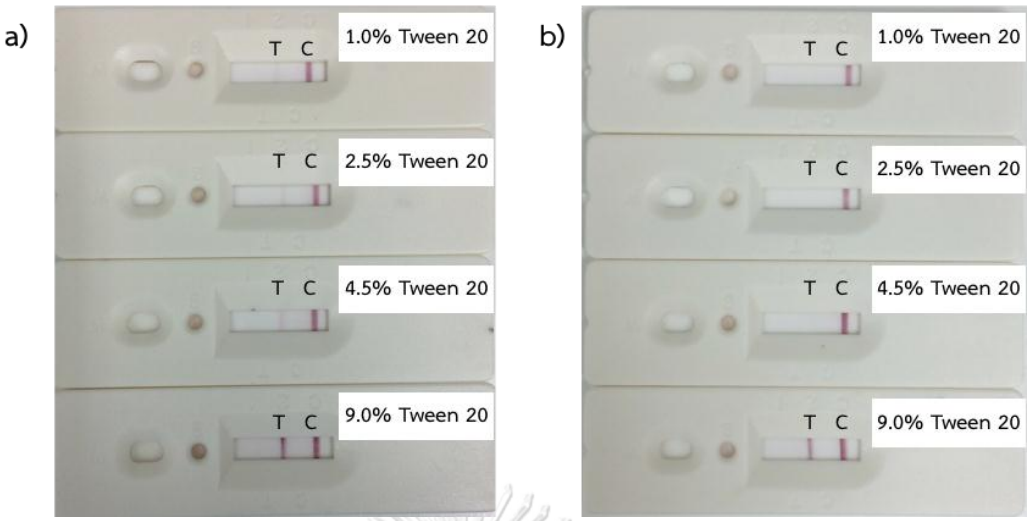


Figure 38. Optimization of Tween 20 concentration used for pretreatment of serum for the AuNP-based LFA. Tween 20 at various final concentrations of 1, 2.5, 4.5, and 9.0% (v/v) were used to pretreat serum spiked with 10^4 cells of pathogenic *L. interrogans* serovar Pomona (a) or non-pathogenic *L. biflexa* serovar Patoc (b) before loading onto the sample pad. Ten microliters of serum was individually pretreated with 90 μL of Tween 20 at different concentrations for 1 minute and applied onto the sample pad of the 40-nm AuNP-based LFA strips. The bands were observed by visualization within 15 minutes.

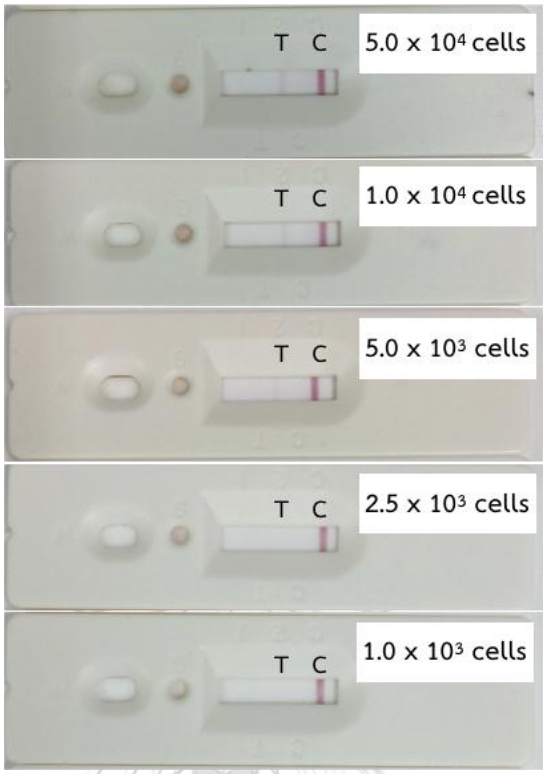


Figure 39. Limit of detection of the 40-nm AuNP-based LFA strip using pretreated samples with 4.5% Tween 20. Sera spiked with various numbers of *L. interrogans* serovar Pomona from 10^3 to 5 x 10^4 cells were pretreated with 4.5% Tween 20 for 1 minute and applied onto the sample pads. The bands were observed by visualization within 15 minutes.

Evaluation of the AuNP-based LFA strip for detection of leptospiral antigen in clinical specimens

To evaluate the sensitivity and the specificity, 50 acute phase sera from known cases of leptospirosis, 20 acute phase sera from unrelated diseases, and 10 sera from healthy persons were used to test the AuNP-based LFA strips developed in this study. If the patient sera were treated with Tween 20 (at a final concentration about 4.5 %), the positive results were increased from 3 (6%) to 12 (24%) samples (Figure 40 and Table 7). No cross detection was detected with sera from unrelated diseases and healthy persons (Table 7). The preliminary data showed that the sensitivity and specificity of the AuNP-based LFA for detection of pathogenic leptospiral antigen in acute phase sera of patients with leptospirosis is 24% and 100 %, respectively.

Table 7. Detection of leptospiral antigen by the AuNP-based LFA strips developed in this study using pretreated and untreated serum samples

LFA detection	Serum samples					
	Leptospirosis (n=50)		Unrelated diseases (n=20)		healthy persons (n=10)	
	Pretreated*	Untreated**	Pretreated*	Untreated**	Pretreated*	Untreated**
Positive	12 (24%)	3 (6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Negative	38 (76%)	47 (94%)	20 (100%)	20 (100%)	20 (100%)	20 (100%)

*Pretreated = sera treated with Tween 20 (at a final concentration about 4.5%) before use

**Untreated = no treatment of sera before use

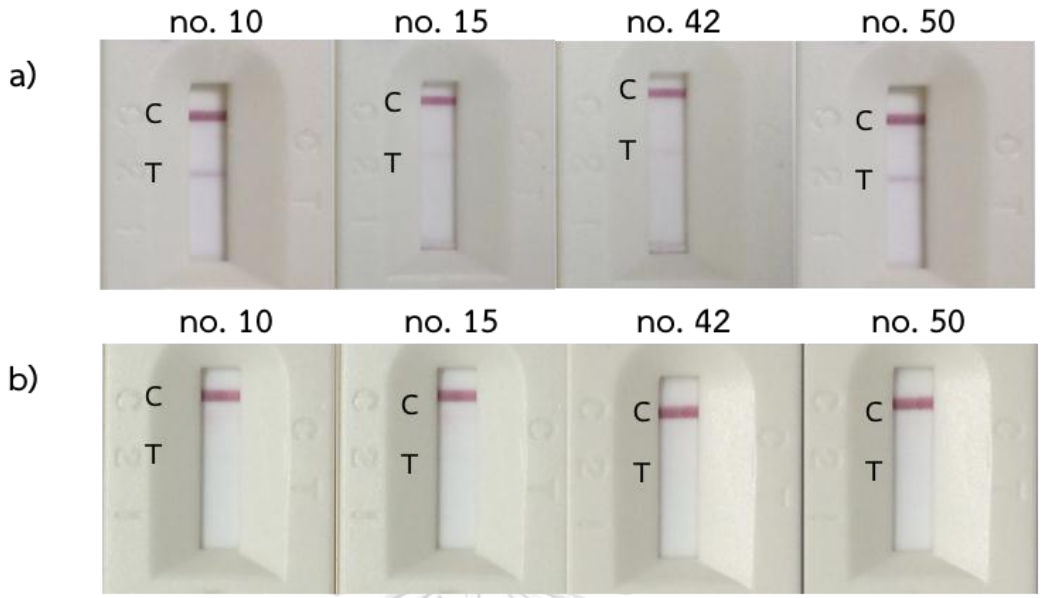


Figure 40. Representative of the AuNP-based LFA strips for detection of leptospiral antigen becoming positive after pretreating serum specimens with Tween20. Ten microliters of acute phase sera from known cases of leptospirosis pretreated with 90 μL of Tween 20 at a final concentration of 4.5% (a) and untreated samples (b), were applied onto the sample pad. The bands were observed by visualization within 15 minutes.

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DISCUSSIONS

Leptospirosis is the most widespread zoonotic disease in the world. This disease is one of acute febrile illnesses commonly found in Thailand because its clinical manifestations are typically non-specific. Currently, microscopic agglutination test (MAT) is the standard method for diagnosis of leptospirosis. This method has low sensitivity for acute phase of leptospirosis, requires technical expertise and instrument, needs to maintain several serovars of viable *Leptospira*, and is performed only in certain reference laboratories (20, 22). Recently, LFA-based POC devices are among rapidly growing strategies because of rapidity and one step analysis, user friendly format, low operational cost, and practicability in primary care settings where cases are commonly presented (34). Therefore, LFA for detection of leptospiral antigens should be useful particularly for diagnosis of acute phase of leptospirosis. LFAs for detection of leptospiral antigens have not been widely used or commercially available due to low sensitivity (3, 92).

Nanotechnology has been applied for design and creating new medical devices. AuNPs are most commonly used to prepare nano-platforms in smart sensor devices. Moreover, optical property of AuNPs enhances sensitivity and rapid result interpretation by visualization of LFA (107). Therefore, AuNP is a potential candidate of nanomaterial to be used for development of LFA for diagnosis of leptospirosis.

This study aimed to optimize and enhance sensitivity of AuNP-based LFA for detection of pathogenic leptospiral antigen in clinical samples.

In this study, leptospiral lipoprotein 32 kDa (LipL32) was used as a target to develop AuNP-based LFA because it is the most abundant and highly conserved outer membrane protein among pathogenic *Leptospira* but is not found in saprophytic leptospires or other bacteria (41), and express in both the acute and convalescent phases of illness (108). In our laboratory, murine monoclonal antibodies against LipL32 (anti-LipL32 mAbs) have been produced previously by hybridoma technology. This study selected 2 clones of mAbs, i.e. mAb3 and mAb82, to develop LFA because they were able to detect all 22 tested pathogenic serovars, which are reference strains found in Thailand, and did not bind 2 non-pathogenic serovars (Figure 19 and Table 5). In addition, the competitive inhibition assay showed that these two mAbs do not bind the same epitopes of LipL32 (Figure 22). Initially, only anti-LipL32 mAbs were intended to be used for LFA development because of their highly specificity, unlimited production, and no lot-to-lot variation compared to polyclonal antibody (pAb). However, to increase sensitivity of LFA based POC testing as a screening test, a rabbit anti-LipL32 pAb was also used in this study. To minimize lot-to-lot variation of pAb in the future, the pAb were purified by rLipL32-conjugated affinity chromatography instead of using protein G or protein A columns. These antibodies were tested at different sites of LFA, either coating on AuNPs that immobilized on the conjugate pad or immobilizing on the test line. The best pair and

their location to obtain the lowest LoD of the LFA was to use either mAb3 or mAb82-conjugated AuNPs on the conjugate pad and anti-LipL32 pAb immobilized on the test line (Figure 33 and Table 6). In case of using anti-LipL32 pAb-conjugated AuNPs on the conjugate pad, the ability of the pAb to bind multiple epitopes of LipL32 might hinder binding of anti-LipL32 mAb to the same antigen on the test line leading to higher LoD of the LFA.

Different sizes of AuNPs were successfully synthesized into approximately 10, 20, 30, 40 and 60 nm in diameter in this study. The synthesized AuNPs of all sizes showed high solubility in water and homogeneity in morphology (Figure 24 and 25) as described for high quality criteria of AuNPs (109, 110) However, 60-nm AuNPs were self-aggregated after storage for over six days similar to a previous report (70). In the beginning of this study, 20-nm AuNPs were first used for optimization of the AuNP-based LFA for detection of leptospiral antigen in the form of rLipL32 or sonicated whole cell lysates.

Antibody can conjugate to gold surface by electrostatic interaction depend on charge and specific isoelectric point (IEP) of protein (111, 112). The pH, salt concentration, and antibody concentration are key factors for binding between antibody and AuNPs and were optimized for conjugation of antibodies to the 20-nm AuNPs. The most suitable buffer for stabilizing 20-nm AuNPs was 10 mM borate buffer, pH 9.0 as indicated by no change of their absorbance (Figure 27a) or no visible color change (Figure 27b). The color change of colloidal AuNPs was used to indicate

their size change because the size and color of AuNPs depends on their surface plasmon absorption as a result of collective oscillation of the free conduction electrons (37), which is related to the incident photon frequency that causes specific absorbance in a visible range (113, 114).

In addition, the surface of AuNPs has to be fully covered with saturated antibody not only to obtain the highest sensitivity of detection but also to avoid aggregation. Anti-LipL32 mAb at a concentration of 15 ug/mL could fully cover 20-nm AuNPs (at OD520 = 1) as shown by no aggregation after adding NaCl (figure 28a and 28b). After synthesis by seeded growth method, the AuNPs have negatively charges surrounding the Au core. High concentration of NaCl can destroy the weak electrostatic force of AuNPs with free surface resulting in their aggregation (115) and therefore can be used to determine the full coverage of Ab on AuNPs.

To enhance sensitivity and specificity of LFA, buffers used for each component of the test strip needs to be optimized (116). In the present study, pre-treated sample pad with borate buffer pH 8.0 decreased non-specific binding because it can minimize test variation by controlling the pH of serum sample applied on the sample pad. Anti-LipL32 mAb82 conjugated-AuNPs on the conjugate pad required 10 mM phosphate buffer pH 8.0 containing 1% BSA as a gold storage buffer to prevent aggregation of AuNPs and to maintain functional antibodies for specific binding to the antigen. BSA in storage buffer can also bind the free surface of the mAb-conjugated AuNPs to increase their specificity and stability. To reduce non-

specific binding of contaminated proteins, 1% BSA was shown in this study to be the best blocking reagent of nitrocellulose membrane in comparison to casein, trehalose, glycine, and sucrose.

The anti-LipL32 conjugated AuNP-based LFA was evaluated for detection of leptospiral antigen in serum. The false positive result tested with negative serum control may be the result of contaminated proteins, pH, or salts concentration in sera causing non-specific antigen-antibody interactions. Pretreatment of serum with modified running buffer containing 100 mM NaCl could reduce non-specific binding because NaCl minimized sample variation by controlling ionic strength of the serum. The LoD of this optimized 20nm-AuNP based LFA was shown to be 10^6 cell of leptospiral sonicated whole cell lysates spiked in serum (figure 36). Previous studies reported that during acute phase of human leptospirosis the number of *Leptospira* present is 10^5-10^7 cells/mL in serum (19). Therefore, this LFA might not be sensitive enough for detection of leptospiral antigen in patient sera.

In this study, optimization strategies, i.e. sizes of AuNPs (30 and 40 nm in diameter) and modified running buffer to treat serum samples with mild detergent before use, were tested to reduce LoD of the LFA. The LFA using 40-nm AuNPs was shown to lowest LoD (figure 37) at 10^4 cell of leptospiral whole cell lysates in serum, which is 100-fold improvement compared with 20-nm AuNPs. In addition, pretreatment of serum sample with modified running buffer containing a mild detergent, Tween 20 at a final concentration about 4.5%, can further reduced of LoD

by 2-fold to 5 x 10^3 leptospiral cells. Previous study showed that LipL32 is a subsurface outer membrane protein (117) and the anti-LipL32 mAb82 may bind to the subsurface epitope of LipL32. Therefore, lysed cell of *Leptospira* with mild detergent can increased the accessibility of the antibody to bind to its target epitope on LipL32 protein. This optimized AuNP-based LFA shows the lowest LoD of 5 x 10^3 cells, which is 500-fold lower than that of a previously report (90) that allowed detection of leptspiral lippropolysaccharide (LPS) antigen in spiked urine.

Then, the optimized AuNP-based LFA was preliminary evaluated for its sensitivity and specificity using 50 sera of known patients with leptospirosis and 20 sera of patients with unrelated diseases. The sera were treated with 4.5% Tween 20 compared to untreated sera. Pre-treatment of sera with Tween 20 at a final concentration about 4.5% enhanced positive results from 3 of untreated sera to 12 (24%) out of 50 sera from known cases of acute leptospirosis (figure 40 and table 7). This LFA showed no cross reaction with untreated or pre-treated sera from patients with unrelated diseases (Table 7). Therefore, this LFA has sensitivity of 24% and specificity of 100%. There are several factors that might result in its low sensitivity. The average of leptospiral load in all 50 tested patient sera have been determined previously by real-time PCR using *lipl32* specific primers to be approximately 10^3 cell/mL or less, which is lower than the LoD of this LFA. Although the acute phase sera were collected at the first day of admission, the period of infection of each patient is unknown. It is possible that at the time of serum collected from the

patients *Leptospira* might be cleared from the blood after the first week of the onset or after patients had received antibiotics (57, 118, 119). In addition, the patient sera have been stored in a freezer for at least 2 years and the target protein in sera may be degraded.

The AuNP-based LFA has yet low sensitivity for diagnosis of human leptospirosis. It is potentially useful for detection of leptospiral antigen in urine of animals as reservoir or infected hosts. The concentration of *Leptospira* found in urine from infected dogs was reported to be up to 10^6 cells/mL (120). Rats excrete *Leptospira* at a high concentration (median = 5.7×10^6 cells/mL) (121). Large mammals such as pig, cattle, and sheep shed a larger number of leptospires in urine per day (5.1×10^8 to 1.3×10^9 cells) (121).

Recently, the AuNP-based LFA have been reported as an effective POCT-based device in many fields. However, the conventional AuNP-based LFA was limited due to its low sensitivity (3, 88, 92). Currently, the sensitivity of the AuNP-based LFA have been improved by several techniques such as silver incorporation with AuNPs enhancement (122, 123), sensitizer using secondary antibody-AuNPs conjugates (124), enzymes labeled AuNP-based LFA (70, 125), and modified surface of AuNPs for conjugated antibody (37). Therefore, new technologies can improve sensitivity of the anti-LipL32 conjugated AuNP-based LFA prototype developed in this study for detection of leptospiral antigen in patient sera and will be a promising tool as a POCT-based device for early and rapid diagnosis of acute phase leptospirosis.

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APPENDICES

APPENDIX A

BUFFER AND REAGENTS

Reagents for EMJH media

1. Albumin fatty acid supplement stock solution

$CaCl_2 + MgCl_2 \cdot 6H_2O$	0.076	g
$ZnSO_4 \cdot 7H_2O$	0.04	g
$CuSO_4 \cdot H_2O$	0.03	g
Vitamin B12	0.002	g
Tween 80	1	g
Glycerol	1	g

All reagents are stored at -20°C until use. Dissolve each reagent separately in 10 mL of distilled water.

2. Albumin fatty acid supplement solution, ready to use (50 ml)

BSA	5	g
$CaCl_2 + MgCl_2 \cdot 6H_2O$	750	µL
$ZnSO_4 \cdot 7H_2O$	500	µL
$CuSO_4 \cdot 5H_2O$	50	µL
$FeSO_4$	0.025	g
Sodium pyruvate	0.02	g

Vitamin B12	500	µL
Tween 80	6.25	mL
Glycerol stock	500	µL

Dissolve in distilled water and adjust pH 7.4-7.6 with concentrated HCl. Adjust volume with distilled water to make 50 mL. Sterilize the solution by filtration. Store at -20°C

3. Basal media (90 mL)

Bacto Leptospira Media Base EMJH dehydrated	0.23	g

Dissolve in distilled water and adjust volume to 90 mL. Sterilize the solution by autoclaving at 121°C for 15 minutes.

4. EMJH media

Base media	90	mL
Albumin fatty acid supplement solution	10	mL

Mix the solution and store at 4°C

Reagents for hybridoma cell culture

1. Complete RPMI 1640 (100 mL)

RPMI 1640	87	mL
Fetal bovine serum (FBS)	10	mL
Penicillin G/ Streptomycin 100 U/mL	1	mL
Sodium pyruvate	1	mL
Glucose	1	mL

Mix the solution and store at 4 ℃ before use.

2. Freezing media (100 mL)

RPMI 1640	67	mL
FBS	20	mL
Penicillin G/ Streptomycin 100 U/mL	1	mL
Sodium pyruvate	1	mL
Glucose	1	mL
DMSO	1	mL

Mix the solution and store at 4 ℃ before use.

3. Fetal bovine serum (FBS) inactivation

Before using FBS, FBS must be inactivated at 56 ℃ for 30 min using water bath.

Reagents for bacterial cell culture

1. LB medium (1 Liter)

Bacto-Tryptone	10	g
Yeast Extracted	5	g
NaCl	5	g

Dissolve in distilled water and adjust volume to 1 liter. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. Ampicillin (100 mg/mL)

Ampicillin	1	g
Double distilled water	10	mL

Mix the solution and filter through 0.2 µm syringe filter. Store at – 20 °C until use.

Reagents for SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. 1 M Tris-HCl pH 8.8

Tris base 12.11 g

Dissolve in distilled water and adjust pH to 8.8 with concentrated HCl. Adjust volume with distilled water to make 100 mL. Sterilize the solution by autoclaving at 121°C for 15 minutes.

2. 0.5 M Tris-HCl pH 6.8

Tris base 6.055 g

Dissolve in distilled water and adjust pH to 6.8 with concentrated HCl. Adjust volume with distilled water to make 100 mL. Sterilize the solution by autoclaving at 121°C for 15 minutes.

3. 4X Tris HCl/SDS pH 8.8 (100 mL)

Tris base 18.21 g

SDS 0.4 g

Dissolve in distilled water and adjust pH to 8.8 with concentrated HCl. Store at 4°C

4. Running Buffer (1 liter)

Tris base	15.1	g
Glycine	72	g
SDS	5.0	g

Dissolve in distilled water and adjust volume to 1,000 ml. Store at room temperature.

5. 6x SDS sample buffer with DTT (10 mL)

4X tris HCl/SDS pH 8.8	7	mL
Glycerol	3	mL
SDS	1	g
DTT	0.93	g
Bromophenol Blue	1.2	mg

Dissolve the solution and adjust volume to 10 mL. Store at room temperature.

6. 10% Ammonium persulfate (APS)

APS	1	g
Distilled water	10	mL

Mix the solution and store at -20°C

7. 10% Sodium lauryl sarcosine (SDS)

Sodium lauryl sarcosine	1	g
Distilled water	10	mL

Mix the solution and store at room temperature.

8. 30% Acrylamide/ 0.8% Bisacrylamide (100 mL)

Acrylamide	30	g
Bisacrylamide	0.8	g

Dissolve the solution in distilled water and adjust volume to 100 ml. Sterilize the solution by filtration. Store in the dark at room temperature.

9. 15% SDS-PAGE

Separating gel (15 mL)

Acrylamide/ bis	6.0	mL
1 M Tris-HCl pH 8.8	3.75	mL
10% SDS	0.15	mL
10% APS	75	µL
TEMED	7.5	µL
Distilled water	2.7	mL

Stacking gel (5 mL)

Acrylamide/ Bis	0.67	mL
0.5 M Tris-HCl pH 6.8	0.5	mL
10% SDS	40	µL
10% APS	40	µL
TEMED	4.0	µL
Distilled water	2.7	mL

10. Coomassie Blue R-250 Staining Solution Stock

Coomassie brilliant blue R-250	1	g
Methanol	25	mL
Acetic acid	5	mL

Mix the solution and stirrer for 2 hours at room temperature. Store at room temperature before use.

11. Coomassie Blue R-250 Staining Solution (Working Solution)

Coomassie blue stock	3	mL
Methanol	50	mL
Distilled water	40	mL
Acetic acid	10	mL

Mix the solution and store at room temperature before use.

12. De-stain Solution (2 liters)

Methanol	1000	mL
Acetic acid	200	mL
Distilled water	800	mL

Mix the solution and store at room temperature before use.

Reagents for Western blot

1. TBS (1 liter)

1 M Tris-base pH 7.5	20	mL
NaCl	29.22	g

Dissolve in distilled water and adjust volume to 1 liter. Sterile the solution by autoclaving at 121 °C for 15 minutes.

2. TBS-0.1% (v/v) Tween 20 (500 mL)

TBS	500	mL
Tween-20	500	μL

Mix the solution and store at room temperature.

3. Blotting buffer (1 liter)

Tris base	2.42	g
Glycine	11.24	g
Distilled water	800	mL

Dissolve in distilled water and add 200 mL methanol. Store at room temperature.

4. Alkaline phosphate buffer (1 liter)

1 M Tris base pH 9.5	50	mL
NaCl	2.922	g
2 M $MgCl_2$	625	μL

Dissolve in distilled water and adjust volume to 1 liter. Sterile the solution by autoclaving at 121 °C for 15 minutes.

Reagents for protein purification

1. 1x Phosphate buffer saline (PBS) pH 7.4

Na_2HPO_4	4.88	g
$NaH_2PO_4 \cdot H_2O$	1.54	g
NaCl	3.04	g

Dissolve in Milli Q water and adjust pH to 7.4 with HCl (conc.). Adjust volume with Milli Q water to make 10 liters volume.

2. 10 mM Immidazole binding buffer (50 mL)

10x Phosphate buffer stock solution pH 7.4	5	mL
1 M Immidazole stock solution pH 7.4	0.5	mL

Dissolve in distilled water and adjust pH to 7.4 with HCl (conc.). Adjust volume with distilled water to make 500 mL volume.

3. 400 mM Immidazole elution buffer (100 mL)

10x Phosphate buffer stock solution pH 7.4	10	mL
1 M Immidazole stock solution pH 7.4	25	mL

Dissolve in distilled water and adjust pH to 7.4 with HCl (conc.). Adjust volume with distilled water to make 100 mL volume.

4. 20% Ethanol (Metal-Affinity Chromatography)

Absolute Ethanol	100	mL

Adjust volume to 500 mL with distilled water.

Reagents for antibody purification

1. Washing Buffer, 1X Phosphate buffer saline (PBS) pH 7.4

Na_2HPO_4	4.88	g
$NaH_2PO_4 \cdot H_2O$	1.54	g
NaCl	3.04	g

Dissolve in Milli Q water and adjust pH to 7.4 with HCl (conc.). Adjust volume with Milli Q water to make 10 liters volume.

2. Elution Buffer, 0.1 M glycine pH 2.7 (500 mL)

glycine	3.75	g

Dissolve in distilled water and adjust pH to 2.7 with HCl (conc.). Adjust volume with distilled water to make 500 mL volume.

3. Neutralizing Buffer, 1 M Tris-HCl pH 9.0 (100 mL)

Tris base	12.11	mL

Dissolve in distilled water and adjust pH to 9.0 with HCl (conc.). Adjust volume with distilled water to make 100 mL volume.

4. 1X PBS pH 7.4 (containing 0.05% sodium azide)

Sodium azide	0.05	g

Dissolve in 1X PBS pH 7.4 and adjust volume with 1X PBS to 100 mL.

Reagents for ELISA

1. Coating Buffer

$NaHCO_3$	7.13	g
Na_2CO_3	1.59	g

Dissolve in distilled water to 1000 mL and adjust pH to 9.5 with 10N NaOH. Store at room temperature.

2. Blocking Buffer

1X PBS	100	mL
Tween 20	50	μL

Mix the solution and store at 4 °C before use.

3. Washing Buffer

1X PBS	100	mL
Tween 20	100	μL

Mix the solution and store at room temperature.

4. Stop Reaction Solution

0.5 M H_2SO_4	2.67	mL
Distilled water	97.33	mL

Mix the solution and store at room temperature.

5. 3,3',5,5'-tetramethylbenzidine (TMB) Substrate

Reagent A	5	mL
Reagent B	5	mL

Mix the solution and store at room temperature.

Antibody biotinylation

1. 10 mM NHS-SS-Biotin

NHS-SS-Biotin	5	mg
DMSO	1	mL

Mix the solution and store at room temperature.

2. 1x PBS, pH 7.4 (100 mL)

Na_2HPO_4	1.42	g
NaCl	0.876	g

Dissolve in distilled water to 100 mL and adjust pH to 7.4 with HCl. Store at room temperature.

Preparation of rLipL32 coupled on NHS-activated Sepharose column

1. Buffer A, pH 8.3 (100 mL)

Ethanolamine	3.054	g
NaCl	2.92	g

Dissolve in distilled water to 100 mL and adjust pH to 8.3 with NaOH.

Store at room temperature until use.

2. Buffer B, pH 4.0 (100 mL)

Sodium acetate	0.82	g
NaCl	2.92	g

Dissolve in distilled water to 100 mL and adjust pH to 4.0 with NaOH.

Store at room temperature until use.

3. Column storage buffer, pH 7.4 (100 mL)

Na_2HPO_4	0.71	g
NaN_3	1	g

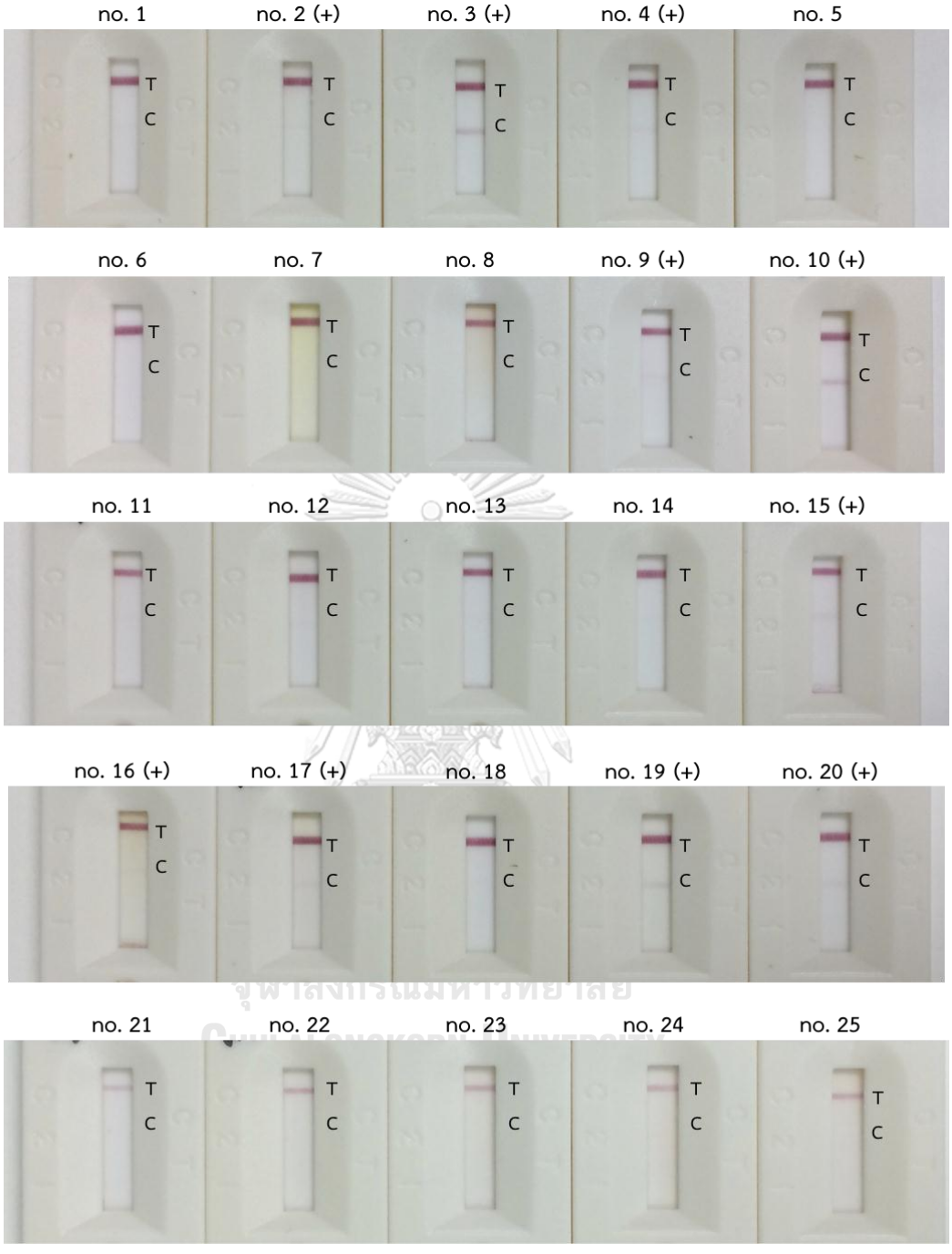
Dissolve in distilled water to 100 mL and adjust pH to 7.4 with NaOH.

Store at room temperature until use.

APPENDIX B

SUPPLEMENTARY DATA

Supplementary data 1. The 40-nm AuNP-based LFA strips for detection of leptospiral antigen becoming positive after pretreating serum specimens with Tween20. Ten microliters of acute phase sera from known cases of leptospirosis (n=50) pretreated with 90 μL of Tween 20 at a final concentration of 4.5% was applied onto the sample pad. The bands were observed by visualization within 15 minutes.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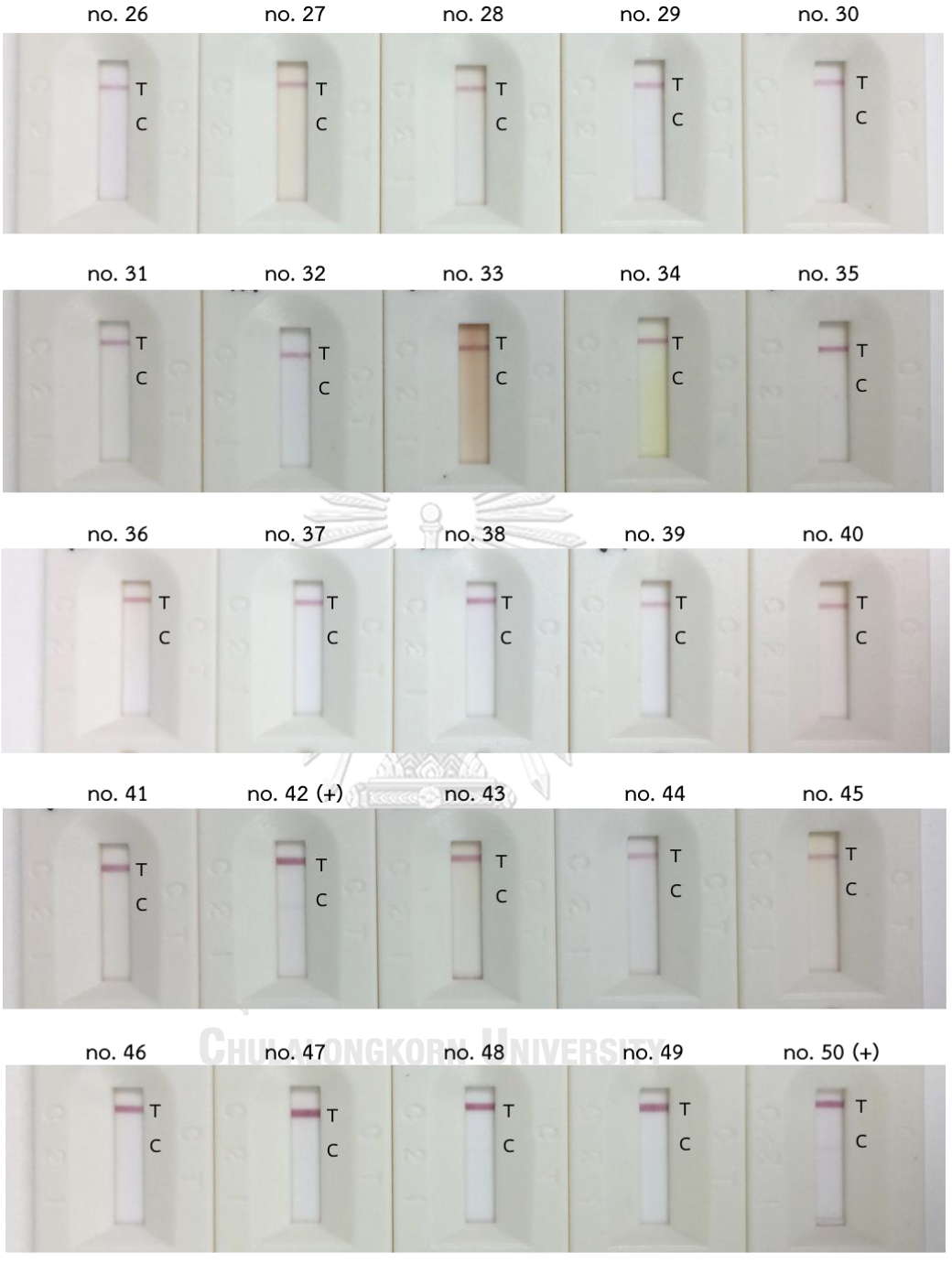

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