

Detection of *leptospires* by RPA-NALFIA and CRISPR-Cas12a



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การตรวจหาเชื้อเลปโตสไปราด้วยเทคนิค RPA-NALFIA และ CRISPR-Cas12a



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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สิริวิษณุ จีรวรรณภรณ์ : การตรวจหาเชื้อเลปโตสไปราด้วยเทคนิค RPA-NALFIA และ CRISPR-Cas12a. (Detection of *leptospire*s by RPA-NALFIA and CRISPR-Cas12a)
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อุปสรรคของการวินิจฉัยโรคเลปโตสไปโรซิสคือการขาดแคลนวิธีการตรวจเชื้อ ณ จุดดูแลผู้ป่วยที่ดีพอ ดังนั้นในการศึกษานี้จึงมีวัตถุประสงค์เพื่อพัฒนาวิธีการตรวจหาสารพันธุกรรมของเชื้อเลปโตสไปราด้วย nucleic acid lateral flow immunoassay (NALFIA) และ clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 12a (CRISPR/Cas12a) ที่ทำงานร่วมกับ recombinase polymerase amplification (RPA) ซึ่งเป็นวิธีที่ใช้อุณหภูมิเดียวในการทำปฏิกิริยา พร้อมทั้งทดสอบการใช้งานจริงกับตัวอย่างคนไข้ ในการศึกษานี้ได้ทำการพัฒนาการตรวจหา ยีน *LipL32*, *SecY* และ *lfb1* ของเชื้อเลปโตสไปราก่อโรค ด้วยวิธี RPA-NALFIA และ RPA-CRISPR/Cas12a จากการทดลองพบว่าการตรวจหา ยีน *LipL32* ด้วยวิธี RPA-NALFIA สามารถตรวจเจอเชื้อน้อยที่สุด 10^5 สำเนาของ DNA ต่อปฏิกิริยา และการตรวจหา ยีน *LipL32* และ *SecY* ด้วยวิธี RPA-CRISPR/Cas12a สามารถตรวจเจอเชื้อน้อยที่สุด 100 สำเนาของ DNA ต่อปฏิกิริยาในขณะที่การตรวจด้วยยีน *lfb1* ไม่สามารถตรวจหาเชื้อได้ และจากการนำการตรวจหา ยีน *LipL32* ด้วยวิธี RPA-CRISPR/Cas12a มาตรวจตัวอย่างดินเอนเอที่สกัดจากเลือดคนไข้จำนวน 110 ตัวอย่างและเปรียบเทียบผลกับวิธีมาตรฐาน qPCR พบว่ามีความไว, ความจำเพาะ, และความถูกต้องที่ 85.2%, 100% และ 92.7% ตามลำดับ นอกจากนี้ยังได้มีการพัฒนา lateral flow detection assay (LFDA) ที่สามารถใช้ร่วมกับ RPA-CRISPR/Cas12a เพื่อทำให้วิธีการตรวจสามารถใช้งานได้สะดวกขึ้น อ่านผลง่ายขึ้นและพึ่งพาเครื่องมือน้อยลง ผลการทดลองพบว่าปริมาณเชื้อที่น้อยที่สุดที่ตรวจเจอคือ 100 สำเนา DNA ต่อปฏิกิริยา และจากการศึกษานำร่องกับตัวอย่างคนไข้พบว่าวิธี RPA-CRISPR/Cas12a-LFDA สามารถตรวจเชื้อได้อย่างแม่นยำ การตรวจหาเชื้อเลปโตสไปราจากยีน *LipL32* ด้วยเทคนิค RPA-CRISPR/Cas12a มีความไวที่ยอมรับได้และมีความจำเพาะที่ดีเยี่ยม ดังนั้นวิธีการตรวจเชื้อที่พัฒนาขึ้นมาใหม่นี้อาจจะใช้ในการคัดกรองโรคเลปโตสไปโรซิสชนิดเฉียบพลันในพื้นที่ที่เครื่องมือที่จำกัดได้



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The key barrier in leptospirosis diagnosis is a lack of available sensitive point-of-care testing. Therefore, we aimed to develop and validate nucleic acid lateral flow immunoassay (NALFIA) and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 12a (CRISPR/Cas12a) platform combined with isothermal amplification to detect leptospire from extracted patients' DNA samples. A recombinase polymerase amplification (RPA)-NALFIA and RPA-CRISPR/Cas12a assay was designed to detect the *LipL32*, *SecY* and *lfb1* genes of pathogenic *Leptospira* spp. The RPA-NALFIA targeting *LipL32* observed the LOD at 10^5 copies/reaction. In comparison, the RPA-CRISPR/Cas12a targeting *LipL32* and *SecY* demonstrated a limit of detection (LOD) of 100 copies/reaction, with no cross-reactivity against other acute febrile illnesses. However, RPA-CRISPR/Cas12a targeting *lfb1* failed to detect the *leptospira* spp. The clinical performance of the RPA-CRISPR/Cas12a assay targeting *LipL32* was validated with DNA extracted from 110 clinical specimens and then compared with qPCR detection of *Leptospira* spp. Relative to the qPCR detection, the RPA-CRISPR/Cas12a assay showed 85.2% sensitivity, 100% specificity, and 92.7% accuracy. We also developed a lateral flow detection assay (LFDA) combined with RPA-CRISPR/Cas12a to make this test more accessible for use and easier to read. The combined LFDA showed a similar LOD of 100 copies/reaction could correctly distinguish between known positive and negative clinical samples in a pilot study. The RPA-NALFIA targeting *LipL32* demonstrated acceptable sensitivity and excellent specificity for leptospire detection. This assay might be an appropriate test for acute leptospirosis screening in limited-resource settings.

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CHAPTER I INTRODUCTION

1. Background and rationale

Leptospirosis is a zoonotic disease that affects global health, with over a million cases per year and 58,900 deaths (1). The disease is caused by pathogenic Gram-negative spirochete *Leptospira spp.*, which can adapt to a broad spectrum of mammalian hosts and environments (2, 3). The clinical signs and symptoms of leptospirosis share similarities with various other infectious diseases, such as dengue, sepsis, and malaria (4-7), making it difficult to diagnose.

One of the key barriers to reduce the impact of leptospirosis is the lack of sensitive diagnostic tools currently available. There are three primary standard methods recommended by the WHO (8). The first is the microscopic agglutination test (MAT), a serological-based diagnosis method. Although the MAT is accurate, it requires a skilled technician, well-equipped laboratory, and is time-consuming. The second is dark field microscope diagnosis from sample cultures collected from the patient's blood at the early stage of *Leptospira spp.* infection. However, *Leptospira spp.* is a slow-growing bacterium, so it might take several weeks to get the results. Lastly, is quantitative polymerase chain reaction (qPCR), a nucleic acid detection method that is faster, accurate, and has been widely used as the primary diagnostic method. However, the real-time qPCR equipment is expensive and not available in every hospital, especially in rural areas (5, 9). Moreover, most leptospirosis cases are often admitted to hospitals in rural areas without proper laboratory equipment. Therefore, we need a better diagnostic tool (10).

Recombinase polymerase amplification (RPA) is an isothermal nucleic acid amplification technology that can be operated in the field due to its low resource

requirements. The RPA system utilizes three enzymes: recombinase, single-stranded DNA-binding protein (SSB), and strand-displacing polymerase (11). The recombinase can pair oligonucleotide primers with homologous sequences in the target DNA. Then, the SSB binds to the replaced strand of DNA and protects the dissociation of primers. After that, the strand displacing polymerase starts DNA synthesis. Amplification of the target DNA sequence by RPA can be accomplished at a constant temperature in less than 20 min. Moreover, the RPA can work with nucleic acid Lateral flow Immunoassay (RPA-NALFIA), and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas12a that has shown promising results in nucleic acid detection (12-14). The CRISPR/Cas12a system relies on a single guide RNA (sgRNA), which acts as a targeting system for the effector function of the Cas12a enzyme to recognize and cleave specific DNA targets. After CRISPR/Cas12a detects its target and cleaves it, the collateral cleavage activity is activated resulting in the fluorescent reporter being cleaved from the quencher and so the release of the detectable fluorescent signal (15). For this reason, the RPA preamplification combined with the CRISPR/Cas12a detection system can be used for diagnostic screening in a limited-resource setting without the need for specialized instruments. This study aimed to develop a new early leptospirosis diagnostic tool using the RPA combined with CRISPR/Cas12a targeting *LipL32*, *SecYIV* and *lfb1* genes which have been proven to be good target for pathogenic *Leptospira spp.* detection in a human's blood.

1.2 Research questions

Can *LipL32*, *SecY*, and *lfb1* RPA-NALFIA and CRISPR/Cas12a based detection system be able detect *Leptospire*s infection at the same or better specificity and sensitivity as qPCR?

1.3 Objectives

To develop a point-of-care diagnostic test of pathogenic *Leptospire*s using RPA-NALFIA and CRISPR/Cas12a detection system targeting *LipL32*, *SecY*, and *lfb1* genes.

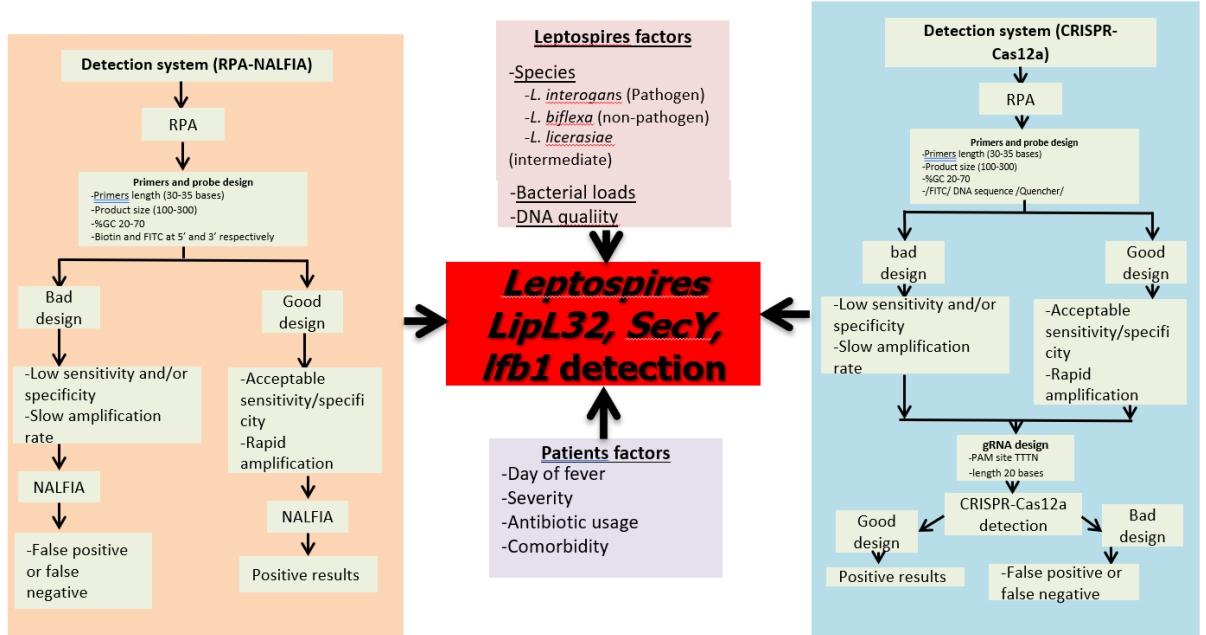
1.4 Hypothesis

LipL32, *SecY*, and *lfb1* RPA-NALFIA and CRISPR/Cas12a based detection system might be able to detect *Leptospire*s infection as high specificity and sensitivity as qPCR.

1.5 Research design

Research and development, clinical samples validation.

1.6 Conceptual frameworks



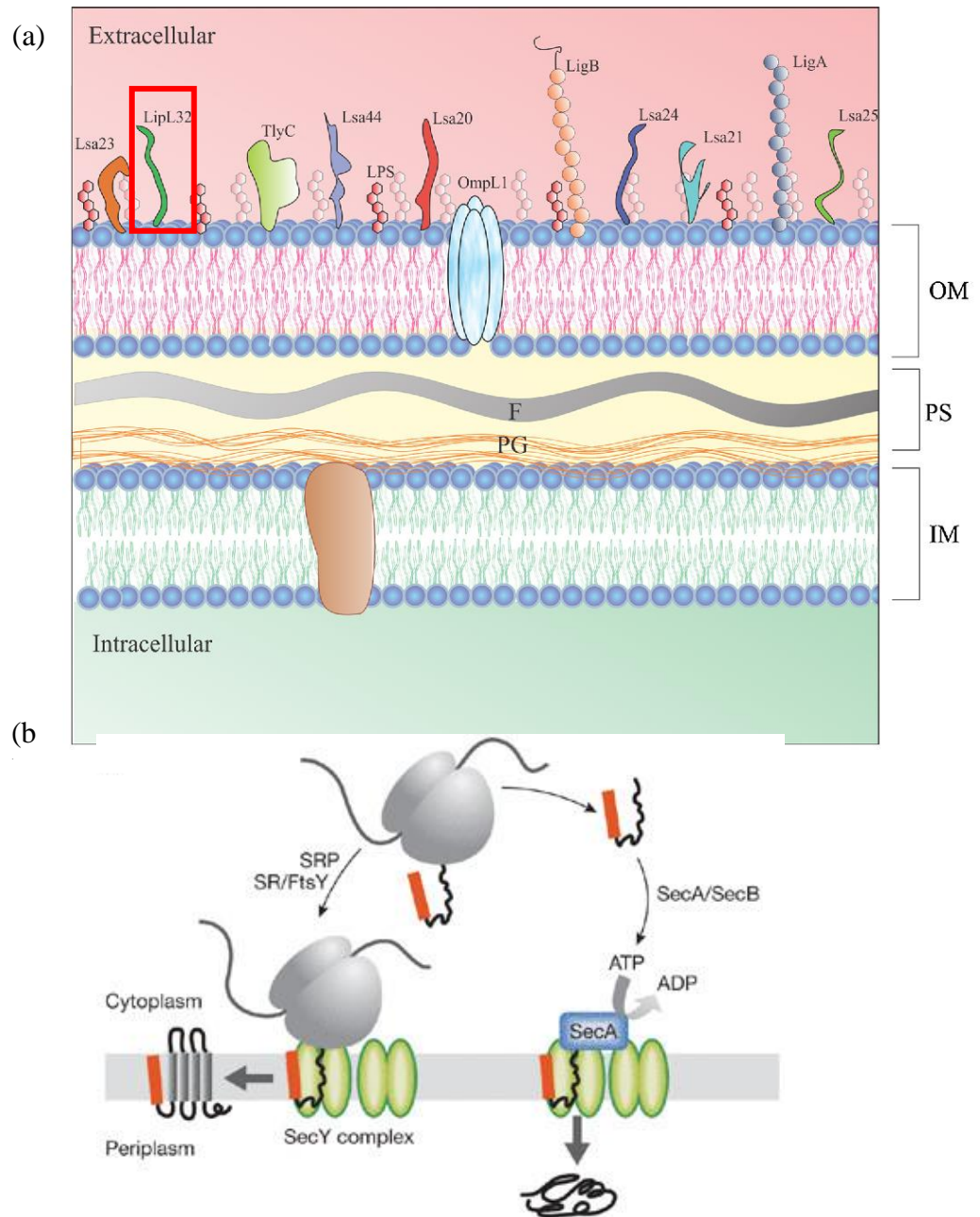
CHAPTER II LITERATURE REVIEW

1. Leptospirosis

Leptospirosis is a zoonotic disease caused by pathogenic gram-negative spirochete *Leptospira*. The *leptospira* genus was divided into three types which are pathogenic (*leptospira interrogans*), non-pathogenic or saprophytic (such as *leptospira biflexa*), and intermediate pathogenic (such as *leptospira broomii*) (2, 16, 17). Serological analysis has been used for classification by agglutination of cross-absorption with homologous antigen. There are over 200 serovars of *leptospira interrogans*, and over 60 serovars of *leptospira biflexa*. Moreover, serovars with antigenically similarity considered as the same serogroup which is useful for epidemiological understanding. Moreover, serological classification can also use for Microscopic Agglutination Test (MAT). (2)

Humans can be infected with *Leptospira* from direct and indirect exposure to the water contaminated with infected animals' urine. Contract with infected animal tissue or digesting contaminated water can also lead to the infection (18). The host immune response is the most likely primary cause of the pathogenic mechanism. The outer membrane of *leptospira* is composed of various outer membrane proteins (OMPs) and lipoproteins such as *Lip132*, *LipL21*, and *LipL41* (19) (Figure 1). These OMPs might play an important role in pathogenesis by immune responses, host-immune manipulation or targets for antibodies and receptors.(16, 19, 20). Moreover, *Lip132* can be found only in pathogenic *leptospira* has been proven as a promising target in various laboratory diagnostic tests such as Real-Time PCR. (21) In addition, *SecY* is one of the essential genes involving protein translocation (Figure 1b) (22), and

the *lfb1* gene plays a vital role in fibronectin-binding protein involving host tissue attachment (Figure 1c) (23). Both *SecY* and *lfb1* have also been a good target for *leptospira* by qPCR. (4, 24, 25)



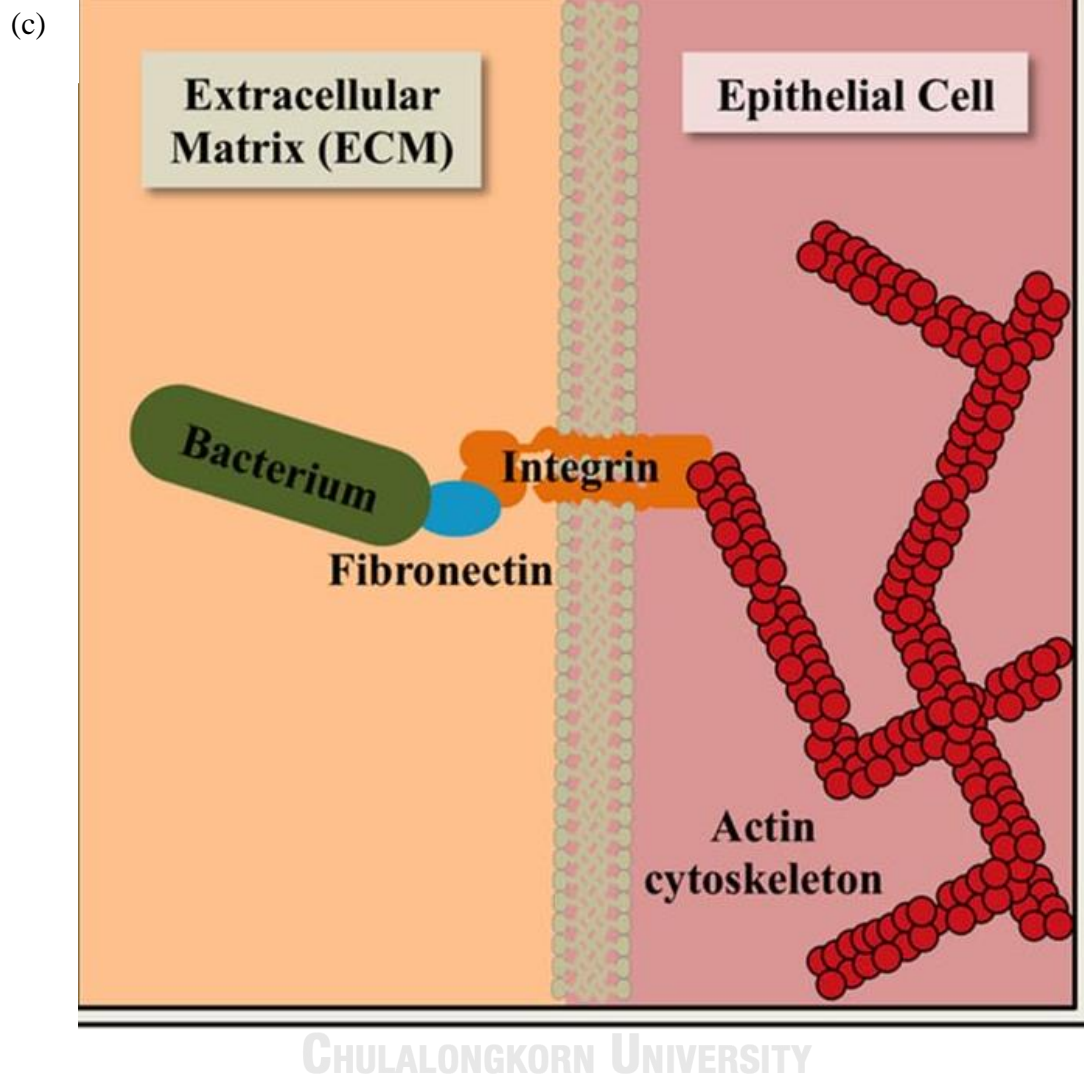


Figure 1. Genes found only on pathogenic *leptospira spp.* (a) Structure of the outer membrane *Leptospira* cell wall composition and *Lipl32*. (b) *SecY* gene function in protein translocation (c) *lfb1* gene function in fibronectin binding.

Leptospirosis clinical manifestation may be presented, ranging from mild "flu-like" to severe symptoms. These signs and symptoms may share many similarities to other infectious diseases.

- A mild, influenza-like illness;
- Weil's syndrome is characterized by jaundice, renal failure, hemorrhage, and myocarditis with arrhythmias;
- Meningitis/meningoencephalitis;
- Pulmonary hemorrhage with respiratory failure.

Therefore, the laboratory diagnosis is important for the diagnosis of pathogenic *Leptospira* infection. (6, 8, 9, 26)

2. Laboratory Diagnosis

Laboratory diagnosis is important because the clinical manifestation of leptospirosis is complicated and shares many similarities to other infectious diseases. According to WHO, there are three gold-standard diagnostic methods which are MAT, dark field microscope from culture, and real-time PCR any positive means *leptospira* positive. (8)

2.1 Dark Field Microscope

Dark field microscope is a direct diagnostic method used to detect *leptospira* in the samples such as blood, urine or from culture. Culturing *leptospira spp* required special medium such as oleic acid-albumin, Ellinghausm-McCullough-Johnson-Harris (EMJH). The leptospira spp is a slow-growing bacteria, so it might take at least 2 weeks to months to diagnose with a dark field microscope.

Leptospire can be seen under the dark field microscope as thin, spiral shape, bright, and moving with rapid spinning. (8, 9, 27)

2.2 Polymerase Chain Reaction (PCR)

PCR is also considered a direct diagnostic method. Real-time PCR is a gold standard and very popular to detect *leptospire*, with a successfully detecting DNA in urine and serum samples. However, real-time PCR is expensive, required a real-time PCR machine. (9, 21)

2.3 Microscopic Agglutination Test (MAT)

MAT is a gold standard serological reference and indirect diagnostic method using live *leptospire*. MAT test can detect *leptospire* by the agglutination of specific IgG and IgM antibodies found in a patient's serum with leptospire antigen and is observed under a dark field microscope. However, MAT required references *leptospire* of various serovars. (6, 8, 9)

2.4 The enzyme-linked immunosorbent assay (ELISA)

ELISAs are widely used and have various assays/commercial kits available. Similar to MAT, ELISA can detect IgG and IgM in a patient's serum. However, ELISA test does not indicate the serovar of *leptospire*, unlike MAT test. Moreover, ELISA test needs MAT test confirmation. (6, 8, 9, 26)

The window period of leptospirosis can be divided into 2 phases. The first phase is septicemia, or acute stage 3-10 days after infection, and *leptospire*s can be detected in serum until day 15. *Leptospire*s are detectable in urine from day 7-15. The second phase is the immune stage after the second week of infection which the antibody is detectable (Figure 2) (2, 20, 28).

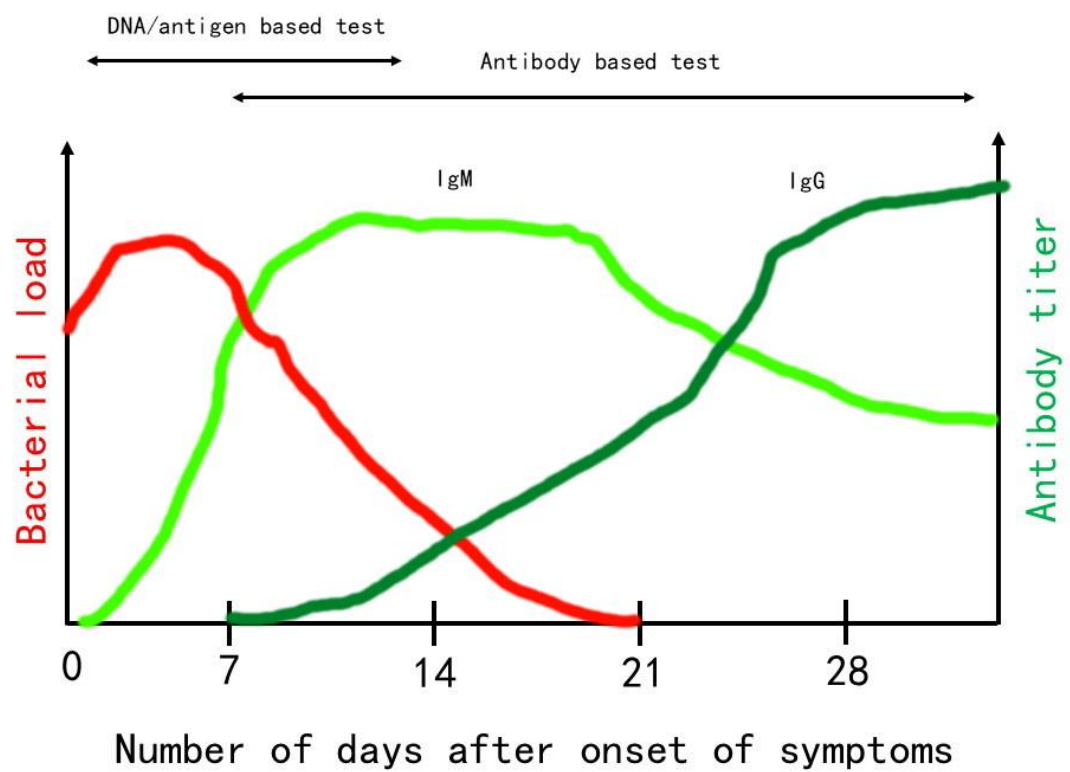


Figure 2. Phases of leptospirosis (adapted from Picardeau et al., 2013)

Table 1. A summary of advantages and disadvantages of each diagnostic tests for detection of *leptospire*s. (6, 8, 26)

Test	Advantages	Disadvantages	Window of positivity	Equipments	Processing time
Dark Field Microscope (DFM)	- Visualization of <i>leptospire</i> s, shape and motility.	-Low sensitivity and specificity.	1 st week blood 2 nd week urine	Dark field Microscope	1 hr
Polymerase Chain Reaction (PCR)	-Good sensitivity and specificity. -Can detect <i>leptospire</i> s in both urine and serum.	-Required expensive equipment, and skilled personnel. - Cannot identify the serovar.	1 st week blood Day 7-15 urine	-PCR/Real-time PCR machine. - Laboratory	4 hrs
Microscopic Agglutination Test (MAT)	-Gold standard -Identify the serovar	-Very difficult -Laboratory with reference <i>leptospire</i> s only.	From day 10-12	-Reference <i>leptospire</i> s -DFM	Days to weeks
The enzyme-linked immunosorbent assay (ELISA)	-Widely used. -Rapid test available.	-IgM cannot be detected during early stages. -Antibody can be detectable in blood for months caused false positive.	From day 6-8 (IgM)	-Plate reader -no equipment (rapid test)	30 mins (rapid test) 2-4 hrs (normal test)

Table 1 indicated that there is still no rapid test for the early phase of infection, so the development of a new early phase point-of-care test is ideal.

2.3 Recombinase Polymerase Amplification

Recombinase Polymerase Amplification (RPA) is an isothermal nucleic acid amplification platform developed by TwistDX, UK, based on three proteins. Recombinase will capture the oligonucleotide primers then recombine to the double-stranded by forming D-loop from non-complementary strand displacement. Then single-stranded binding protein (SSB) will bind to the displaced strand to prevent reannealing. The final step has occurred when strand-displacing polymerase making a new copy of target DNA from 5' to 3' (Figure 3) (11).



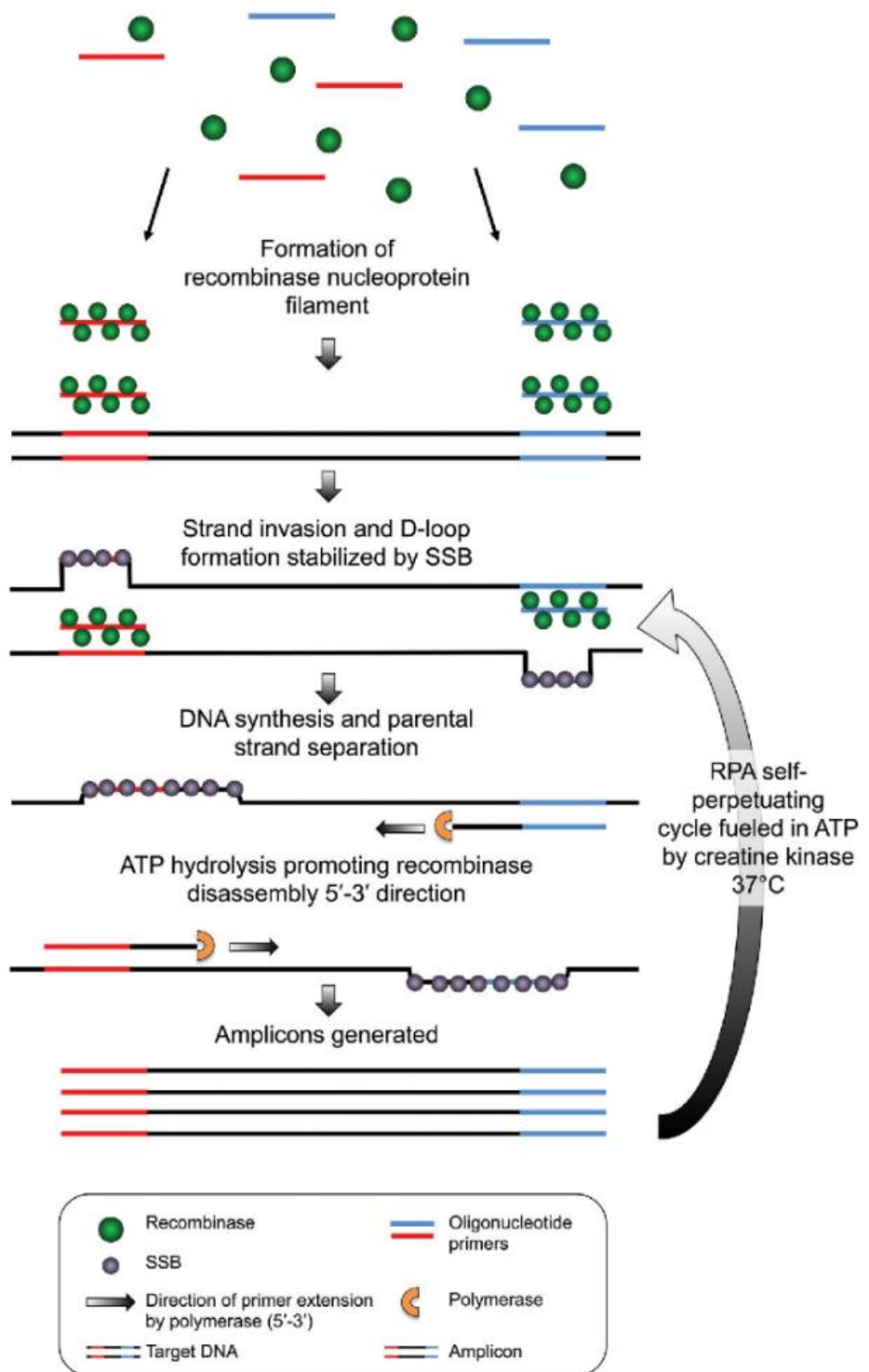


Figure 3. RPA cycle

RPA does not need denaturing and annealing temperatures; only extension temperature is needed between 37°C and 42°C. Thus, no expensive equipment is needed, ideal for point-of-care diagnosis. Not only the isothermal, amplifying the nucleic acid with RPA consume less time comparing with conventional PCR, and real-time PCR (11, 29).

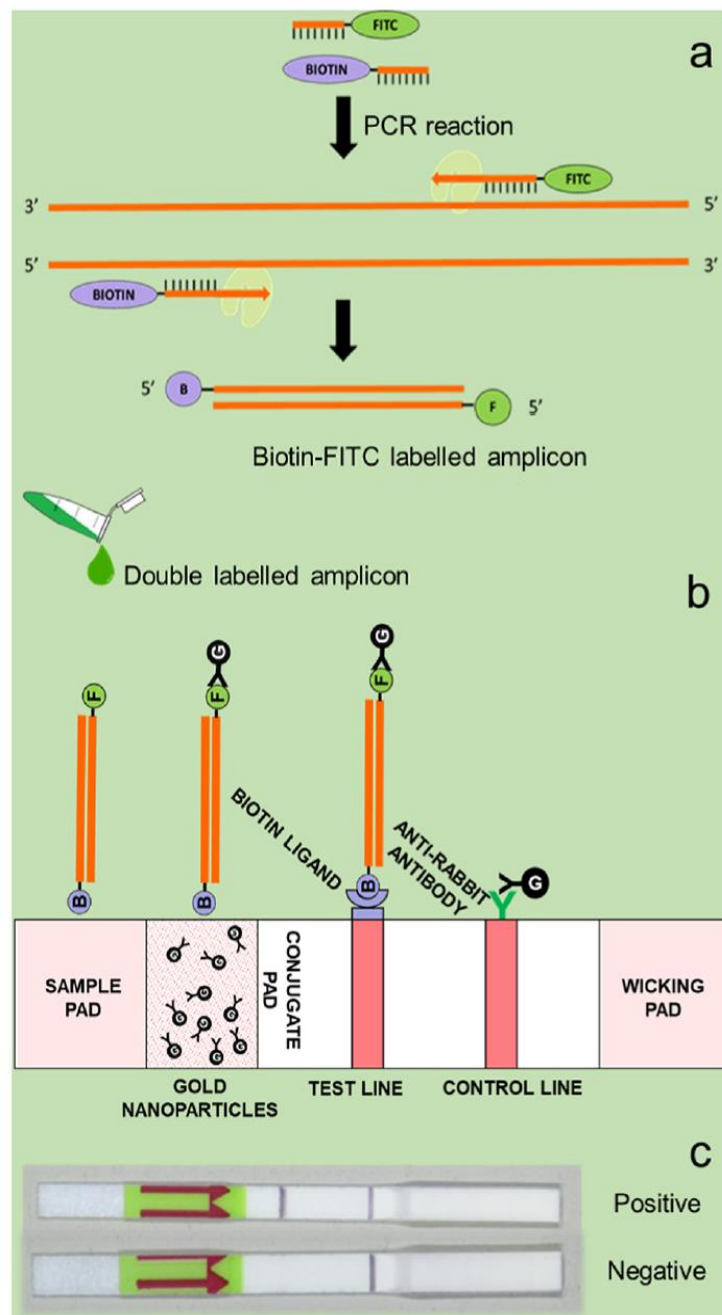
RPA can also work with nucleic acid lateral flow immunoassay (NALFIA) and CRISPR/Cas that will allow us to read the result with naked eyes (12, 30, 31).

2.4 Lateral Flow Assay

The Lateral flow assay (LFA) is a diagnostic platform based on paper. LFA was developed from the latex agglutination test used for serological detection of rheumatoid arthritis in 1956 by Singer and Plotz. Then in 1984, Unipath was released the pregnancy test using urine-based LFA. After that, LFA was developed to be the point-of-care (POC) diagnostic platform with various applications (32).

Nucleic acid Lateral flow Immunoassay (NALFIA) is one of the applications from LFA. It has an uncomplicated principle: the sample containing the analyte of interest will be amplified with the specific forward and reverse primers tagged with 5'-fluorescein isothiocyanate (FITC) 5'-Biotin, respectively. After that the amplified product in the liquid phase will flow in one direction with the help of capillary action. Along the way, the amplified product will be passing through the conjugate zone that contains the gold nanoparticles with the anti-FITC antibody that will bind to FITC. Then the flow will continue to the first control line that has immobilized biotin-ligand, which will capture the biotin (Figure 4). If there is an amplified product, the gold nanoparticle accumulation will generate the red line to read the result with the

naked eye. Finally, the sample that contains the gold nanoparticle with anti-FITC will flow to the control line that contains the immobilized anti-rabbit antibody, which will bind to the anti-FITC of the gold nanoparticle to generate the red line at the control line (Figure 4a). The negative result with no amplification will has



only one red line at control line (Figure 4b) (30-33)

Figure 4. Mechanism of NALFIA

NALFIA is simple, easy-to-use and can be applied to the point-of-care diagnostic system, especially combined with RPA, the isothermal point-of-care nucleic acid amplification platform.

2.5 CRISPR/Cas detection platform

CRISPR/Cas system was first discovered in 2013, and Cas9 was first recognized for its gene-editing ability. In 2015, Cas12a (Cpf1), and Cas13a (C2c2) was identified and later in late 2016, the collateral cleavage activity was discovered (34).

CRISPR-Cas system relies on pre-CRISPR RNA (crRNA) transcribed from the CRISPR array then processing to be the mature crRNA which acted as a targeting system for the effector function of the Cas enzyme. Knowing the CRISPR/Cas mechanism, we can manipulate and design guide RNA to target the DNA/RNA sequence of interest. The collateral cleavage activity of Cas12a, Cas13a and Cas14 are useful for nucleic acid detection platforms. After CRISPR-Cas detected its target and cleaved, CRISPR/Cas will activate the collateral cleavage then cleave the RNA/DNA (Figure 5) (35)

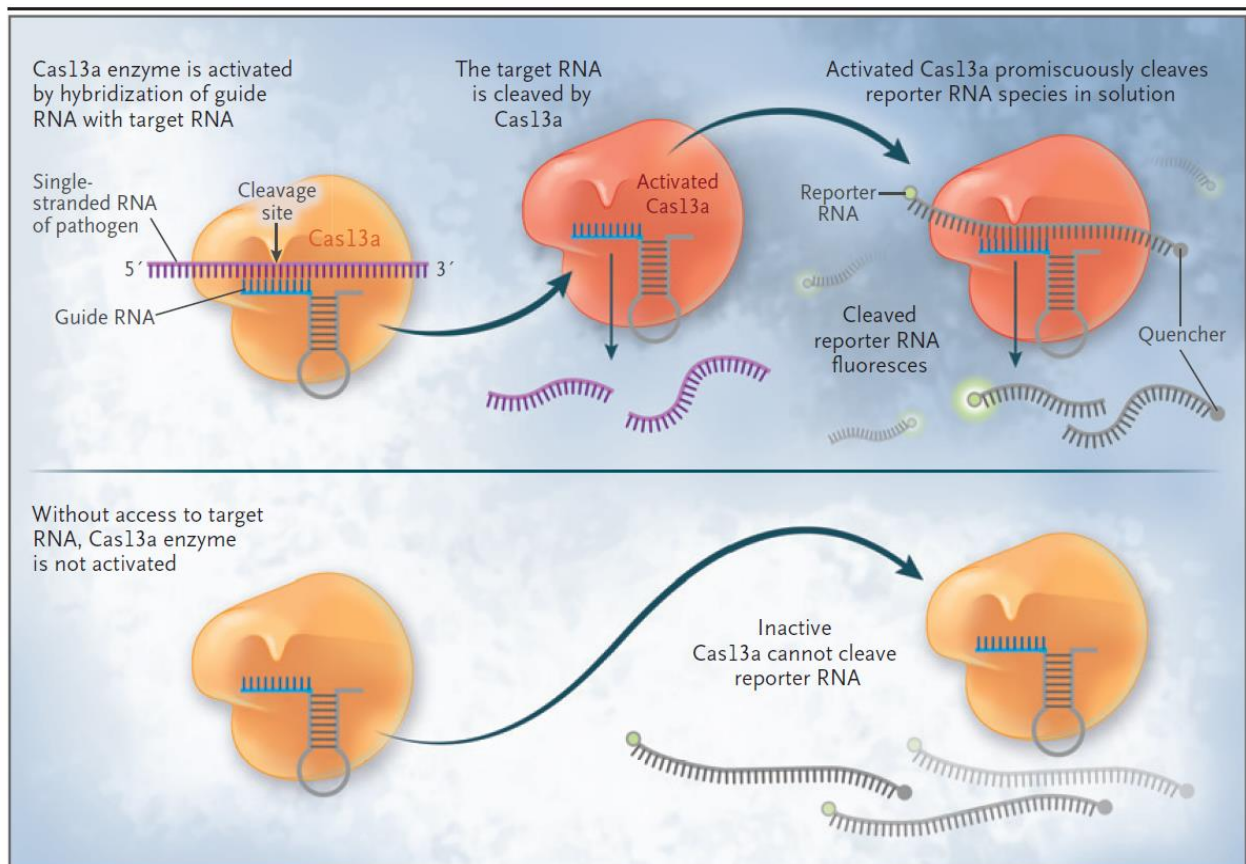


Figure 5. Mechanism of CRISPR-Cas13a collateral cleavage. (35)

Cas12 and Cas13 are mainly used in nucleic acid detection platforms. Both have collateral cleavage; however, the target type is different. Cas12a target is ssDNA and dsDNA with the requirement of PAM site, but Cas13a has no PAM site requirement and detects ssRNA only. (Figure 6) (36)

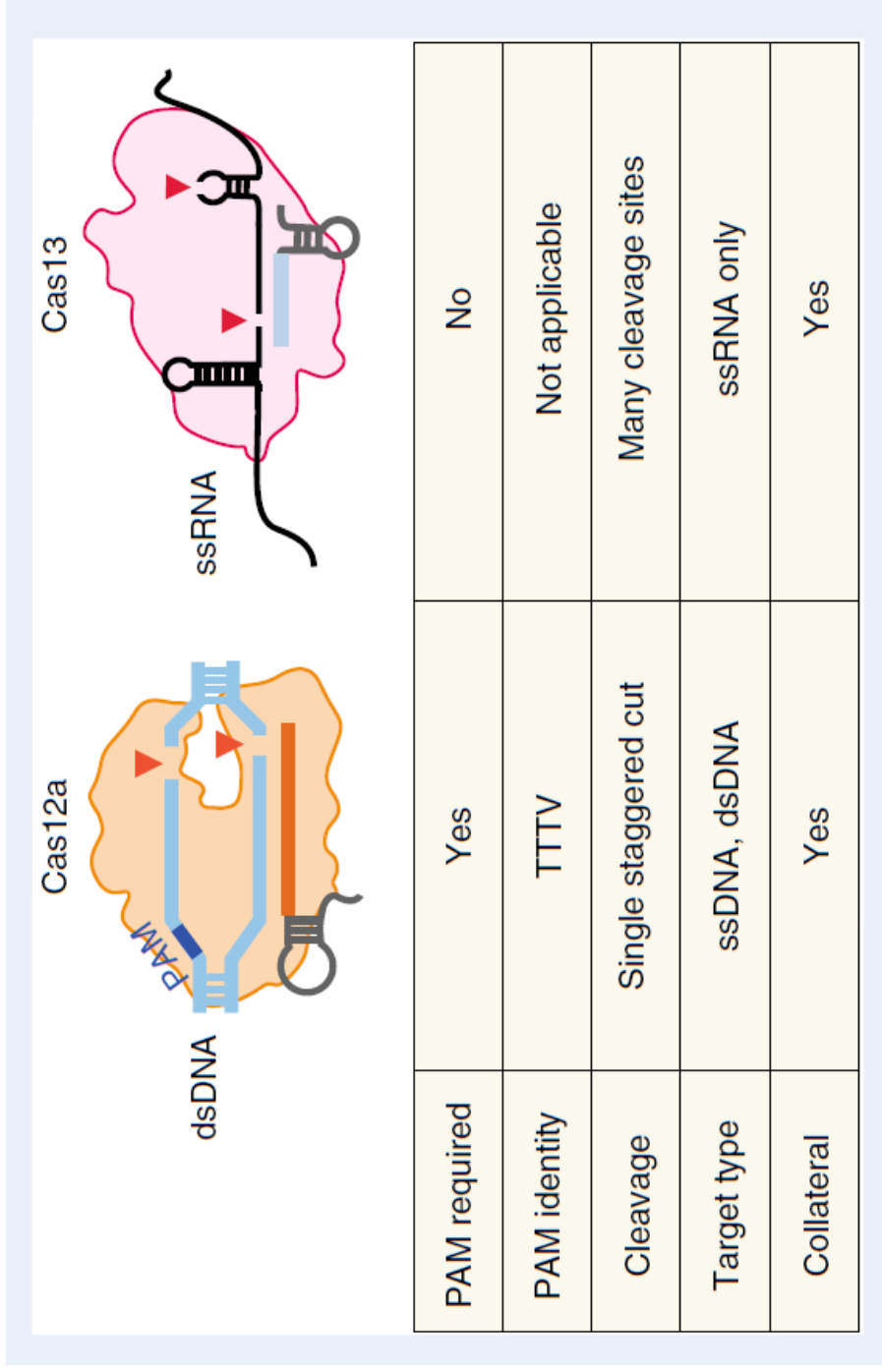


Figure 6. Comparison of Cas12a and Cas13 (36)

CHAPTER III MATERIALS AND METHODS

1. Ethics statement

The study protocol was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No.655/63). Furthermore, the study was performed under the international guidelines for human research protection of the Declaration of Helsinki, The Belmont Report, CIOMS Guideline, and International Conference on Harmonization in Good Clinical Practice.

2. Patients and study design

In this study, we tested the performance of the RPA-CRISPR/Cas12a targeting *LipL32*, *SecY*, and *lfb1* genes using blood samples from participants of a known leptospirosis status (infected or non-infected) from previous studies accomplished in 15 hospitals in Sisaket province, Thailand. The samples were collected between December 2015 to November 2016. The inclusion criteria were that all subjects must (i) be older than 18 years old and admitted to participating hospitals; (ii) have presented with clinical suspicion of leptospirosis, high fever (body temperature higher than 38 °C), severe myalgia; and (iii) a history of exposure to reservoir animals. The exclusion criteria were patients who suffered from other known infectious diseases or has history of using antibiotic drugs. The samples were stored at -80 °C until further analysis. The blood samples from the first day of enrollment were selected and used as a blind test.

Multicenter prospective observational study at 15 hospitals in Sisaket

- 1.Sisaket hospital
- 2.Rasi Salai hospital
- 3.Yang Chum Noi hospital
- 4.Uthumphon Phisai hospital
- 5.Huai Thap Than hospital
- 6.Prong Ku hospital
- 7.Pha Yu hospital
- 8.Nam Kliang hospital
- 9.Si Rattana hospital
- 10.Non Khun hospital
- 11.Phu Sing hospital
- 12.Phrai Bueng hospital
- 13.Khu Khan hospital
- 14.Khun Han hospital
- 15.Kantharalak hospital



3. Sample size

The minimum sample size was calculated considering the non-inferiority test in term of sensitivity and specificity between the two new methods compared to the standard method when the sensitivity or specificity value of the standard method (qPCR) was known which is 86% and 100% respectively (21).

The formula for sample size calculation related to the objective above is:

$$n = \frac{(z_{1-\alpha} + z_{1-\beta})^2}{(\varepsilon - \delta)^2} (p_{new} (1 - p_{new}))$$

Where $z_{1-\alpha}$ and $z_{1-\beta}$ are the $(1 - \alpha)^{th}$ and $(1 - \beta)^{th}$ percentiles from standard normal distribution

δ is margin of non-inferiority between the new method and the standard method (qPCR) $\varepsilon = p_{standard} - p_{new}$ is true difference between the sensitivity (or specificity) of the new method and the standard method which $p_{standard}$ which represent the sensitivity (or specificity) of the standard method was known.

For significant level of 0.05 ($\alpha = 0.05$), $z_{1-\alpha} = z_{0.95} = 1.645$. For power of the test of 0.90 ($\beta = 0.10$), $z_{1-\beta} = z_{0.95} = 0.842$.

For **sensitivity**, the estimated sensitivity of the new method is still unknown; however, we estimated the sensitivity about 90%. Therefore, the sample size is calculated as below.

For margin of non-inferiority 5% and the estimated sensitivity of the new method is at 90%

$$n = \frac{(1.645 + 0.842)^2 (0.90)(1 - 0.90)}{((0.86 - 0.90) - 0.05)^2} = 69$$

For **specificity**, the estimated specificity of the new method is 99%. Therefore, the sample size is calculated as below.

For margin of non-inferiority 5% and the estimated specificity of new method is

$$n = \frac{(1.645 + 0.842)^2 (0.99)(1 - 0.99)}{((1 - 0.99) - 0.05)^2} = 39$$

Therefore, we will select the total samples from the cohort study 70 samples known positive when test with qPCR, and 40 negative samples.

4. Culturing *Leptospira* spp.

For the direct culture of *Leptospira*, 1 mL of whole fresh blood was added into 4 mL of Ellinghausen, McCullough, Johnson, and Harris (EMJH) medium and incubated at 30 °C for two weeks. The culture was examined using dark field microscopy to confirm the existence of *Leptospira* (37, 38).

5. DNA extraction

Total DNA was extracted from 200 µL of whole blood samples or *Leptospira* cultures using the High Pure PCR Template Preparation Kit (Roche, USA) according to the manufacturer's instructions. The concentration and quality of the extracted DNA were determined using a NanoDrop 2000 (Thermo Scientific, USA). The extracted DNA was stored at -80 °C until further analysis.

6. Detection by qPCR assay

Each positive sample based on the qPCR assay was defined as a leptospirosis confirmed case. The qPCR targeting the *LipL32* gene was performed as previously described (39) with minor modification. Briefly, 242 base pair products were amplified and detected using the primers and *Taqman* probe in table 2. The qPCR mixture consisted of 5 μ L of extracted DNA, 10 μ L of SsoAdvanced Universal Probe Supermix (Bio-Rad Laboratories, USA), 1 μ L of each primer (10 μ M), 0.4 μ L of *Taqman* probe (10 μ M), and 2.6 μ L of nuclease-free water in a final volume of 20 μ L. The qPCR reactions were performed in duplicate. A no-template control (NTC) with all the above reagents was used as the negative control. Amplification and fluorescence detection were conducted in the StepOnePlus Real-Time PCR Systems (Applied Biosystems, USA). The amplification protocol consisted of 10 min at 95 °C, followed by 45 cycles of 15s at 95 °C and 1 min at 60 °C. A negative result was considered with the threshold cycle (Ct) value higher than 40 cycles.

7. The RPA

The *LipL32*, *SecY*, and *lfb1* genes amplification were performed using the TwistAmp® Basic Kit (TwistDx, United Kingdom) with the primers in table 2. In brief, lyophilized RPA was resuspended in rehydration buffer and mixed with 480 nM of each primer. Then, 14 mM of magnesium acetate (final concentration) and 1 μ L of extracted DNA were added to the reaction mixture. The genes were amplified by incubating at 39 °C for 40 min, followed by heat inactivation at 75 °C for 5 min.

8. The RPA-NALFIA

The RPA reaction was performed as previously described above for 15 min using the *LipL32* forward and reverse primers labelled with 5'-FitC and 5'-Biotin,

respectively in Table 2. Then the RPA product was mixed with 100 μ L of running buffer and pipetted into the commercial lateral flow strip test (Kestrelbioscience, Thailand). The amplicons of the *LipL32* gene were captured at the first detection line (test line), whereas the negative results were not generating the band at the test line. Therefore, the visible band at the control line indicated that the test is valid.

9. CRISPR RNA preparation

Using *in-silico* analysis with the basic local alignment search tool (BLAST), we designed the CRISPR RNA (crRNA) to specifically detect the *LipL32*, *SecY*, and *lfbI* genes using the crRNA sequence in table 2, which was synthesized by HiScribe T7 High Yield RNA Synthesis Kit (New England Biolabs, UK). For the preparation of crRNA, synthetic oligonucleotides were ordered as ultramer DNA (Macrogen, South Korea) with an appended T7 promoter sequence. Oligonucleotides for crRNA (1 μ M) were annealed to a short T7 primer (final concentration of 10 μ M each) and incubated with T7 polymerase at 37 $^{\circ}$ C for 2 h. The crRNA was then purified using a Monarch RNA Cleanup Kit (New England Biolabs, UK). The concentration of purified crRNA product was measured using a QubitTM miRNA assay kit and QubitTM 4 Fluorometer (Thermo Scientific, USA) and stored at -80 $^{\circ}$ C until further use.

10. CRISPR/Cas12a-fluorescent-based detection assay (FBDA)

The CRISPR/Cas12a-FBDA was performed as described previously with minor modifications (40, 41). The CRISPR/Cas12a reaction was composed of 30 nM of crRNA, 330 nM of EnGen *Lba* Cas12a (Cpf1) (New England Biolabs, USA), 600 nM of the fluorescent probe in Table 2, 1X of NEBuffer 2.0 (New England Biolabs, USA), and 1 μ L of RPA amplicons in a total reaction volume of 15 μ L. The CRISPR/Cas12a reaction was incubated at 39 $^{\circ}$ C for 20 min. The fluorescent signal

was then observed with the naked eye using a BluePAD Dual LED Blue/White Light Transilluminator (BIO-HELIX, Taiwan) at 470 nm wavelength. Each test was observed by three certified laboratory technicians who were instructed to identify the qualitative test outcome as "positive" or "negative". The tests were considered positive if at least two of the three technicians read the results as positive



Table 2. crRNA, primers, and probes design

Materials	Design	References
<i>LipL32crRNA</i>	5'-UAAUUUCUACUAAAGUGUAGAUUUUCUGAGCGAGGACACAAUC-3'	This study
<i>SecYIVcrRNA</i>	5'-UAAUUUCUACUAAAGUGUAGAUAAA <u>AAUACGGUGGUUCAU</u> -3'	This study
<i>lfbI</i> crRNA	5'-UAAUUUCUACUAAAGUGUAGAUUU <u>CCCCAAUCUGUCAAAAG</u> -3'	This study
<i>LipL32</i> ForwardPrimer	5'-AAGCATTACCGCTTGTGGTG-3'	(21)
<i>LipL3290b</i> ForwardPrimer	5'-AAAACTTTTAGTAAGAGGTCTTTACAGAAAT-3'	(30)
<i>LipL32</i> ForwardPrimerFitC	5'-FITC-AAGCATTACCGCTTGTGGTG-3'	(21), this study
<i>SecY</i> ForwardPrimer	5'-GCGATTTCAGTTTAAATCCTGC-3'	(42)
<i>lfbI</i> ForwardPrimer	5'- CATTTCATGTTTCGAATCATTTCAAA-3'	(43)
<i>LipL32</i> ReversePrimer	5'-GAACTCCCATTTACGCGATT-3'	(21)
<i>LipL3290b</i> ReversePrimer	5'-AGACCAACAGATGCAACGAAAGATCCTTTTCAC-3'	(30)
<i>LipL32</i> ReversePrimerBiotin	5'-Biotin- GAAC TCCCA TTT CAGCGATT-3'	(21), this study
<i>SecY</i> ReversePrimer	5'- GAGTTAGAGCTCAAATCTAAG-3'	(42)
<i>lfbI</i> ReversePrimer	5'-GGCCCCAAGTTCCTTCTAA AAG-3'	(43)
Taqman probe	5'-/56-FAM/AAAGCCAGGACAAGCGCCG/3IABkFQ/3'	(13)
Fluorescent probe	5'-FAM-TTATTATT-BHQ1-3'	(40)
lateral flow probe	5'- FITC-AGGACCCGTATTCCCA-BIOTIN-3'	This study

11. Limit of detection (LOD) and cross-reactivity testing

The analytic sensitivity of the assay was determined using genomic DNA isolated from *Leptospira* cultures. The DNA was quantified using the NanoDrop 2000 (Thermo Scientific, USA), and genome equivalents per microliter of the purified DNA were calculated. Serial dilutions of genomic DNA were made from 10^8 copies/ μL down to 1 copy/ μL . The LOD was determined from the detection of the fluorescent signal in the tube with the lowest genomic DNA concentration. The specimens obtained from patients with an acute febrile illness, including acute viral hepatitis, cellulitis, scrub typhus, systemic bacterial infection, acute cystitis, influenza, *Escherichia coli* septicemia, and dengue hemorrhagic fever, were tested to establish the analytical specificity of the RPA-CRISPR/Cas12a-FBDA and RPA-NALFIA.

12. RPA-CRISPR/Cas12a combined with a lateral flow detection assay (LFDA) pilot study

A lateral flow test strip was developed to improve the RPA-CRISPR/Cas12a test and make it easier to use and read. The FITC-biotin reporter molecule and lateral flow strips were designed to capture labelled nucleic acids. The lateral flow probe in Table 2 was used at 12 nM instead of the fluorescence probe at 600 nM under the otherwise same condition as the FBDA above. The reaction was incubated at 39 °C for 30 min. The reaction was then mixed with 100 μL of running buffer and pipetted into the commercial lateral flow strip test (Kestrelbioscience, Thailand). Uncleaved reporter molecules are captured at the first detection line (test line), whereas the indiscriminate ssDNA cleavage activity of CRISPR/Cas12a will not generate a signal at the first detection line but only a signal at the second line (control line).

13. Rapid diagnostic testing

The analytic sensitivity of the RPA-CRISPR/Cas12a detection system was compared with a commercial rapid diagnostic test (RDT). For this, 96 blood samples were tested with the RDT from the Medical Science Public Health (Department of Medical Sciences, Ministry of Public Health, Thailand). The RDT kit was designed to detect anti-*Leptospira* IgM antibodies and was used according to the manufacturer's instructions. First, the blood sample was thawed at room temperature and added to the sample well without air bubbles. Next, the assay diluent was added to the diluent well. The results were read at the end of 15 min by three trained technicians. The tests were considered positive if at least two of three technicians read the results as positive.

14. Statistical analysis

Continuous variables are shown as the mean \pm one standard deviation (SD) in case of a normal distribution and as a median and interquartile range (IQR) in case of non-normally distributed variables. The Student's t-test or Mann-Whitney test was used to analyze the differences between two continuous variables. Categorical variables were presented as numbers with percentages and were compared using the Chi-square test. The performance of the RPA-CRISPR/Cas12a targeting the *LipL32* gene detection system was expressed by calculating the sensitivity, specificity, accuracy, and positive and negative predictive values compared to the qPCR analysis of the same samples with the formulas shown in figure 7 (44). All statistical analyses were performed using the SPSS Version 22 software (SPSS, Chicago, IL).

		True Class		Measures
		Positive	Negative	
Predicted Class	Positive	True Positive <i>TP</i>	False Positive <i>FP</i>	Positive Predictive Value (PPV) $\frac{TP}{TP+FP}$
	Negative	False Negative <i>FN</i>	True Negative <i>TN</i>	Negative Predictive Value (NPV) $\frac{TN}{FN+TN}$
Measures		Sensitivity $\frac{TP}{TP+FN}$	Specificity $\frac{TN}{FP+TN}$	Accuracy $\frac{TP+TN}{TP+FP+FN+TN}$

Figure 7. The formulas used to calculate the sensitivity, specificity, accuracy, negative predictive value (NPV), and positive predictive value (PPV).

15. Expected Benefits and Applications

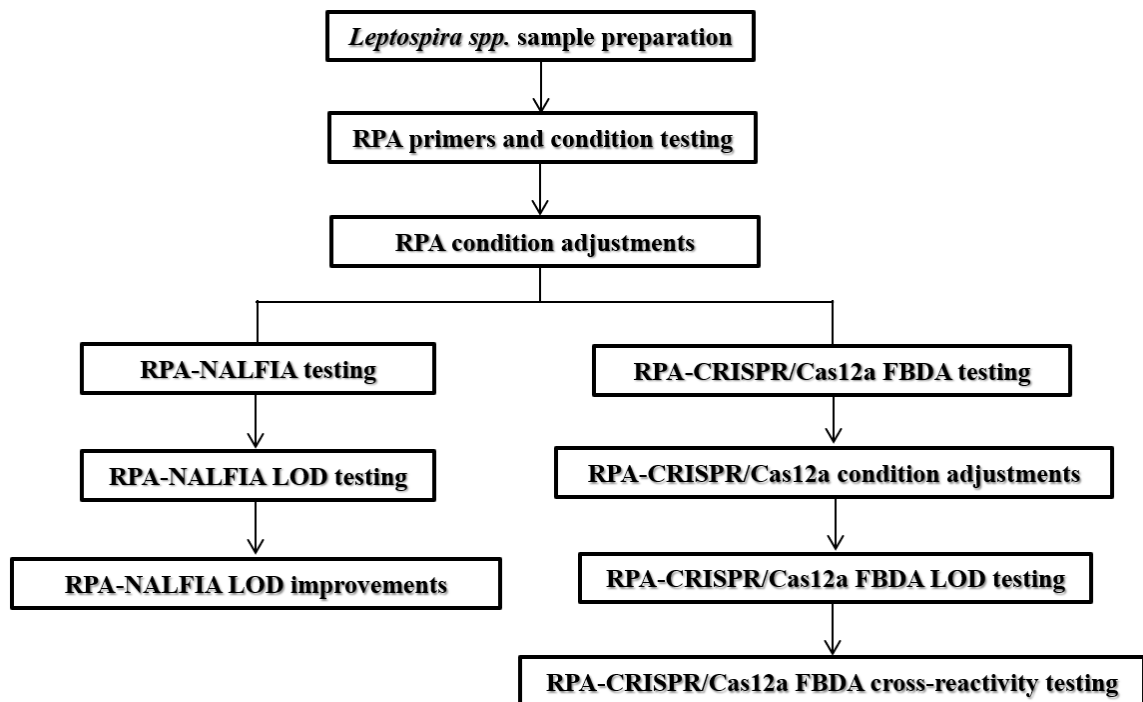
Detection of *leptospire*s with RPA-NALFIA and CRISPR/Cas12a will be useful in the field or the urban area hospitals because these two new tools are effective, simple, portable, rapid and cost effective.



CHAPTER IV RESULTS

Results

Part I. Research and Developments



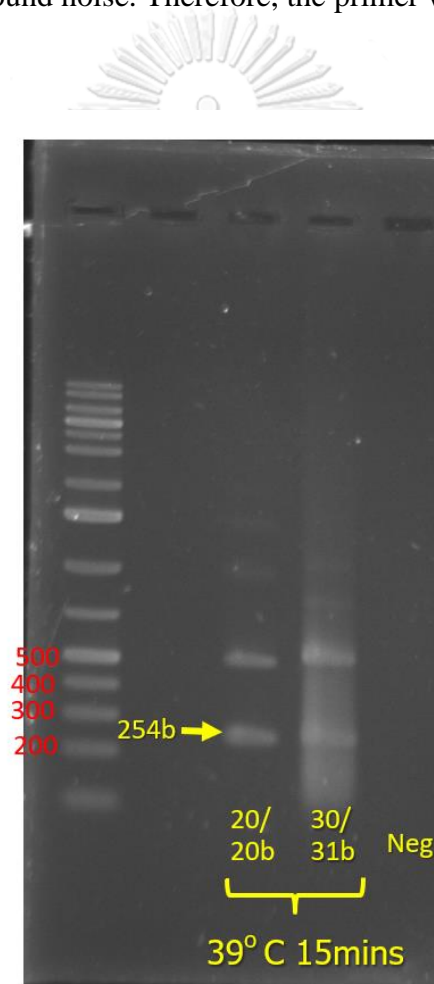
CHULALONGKORN UNIVERSITY

Figure 8. Overview of part I. research and developments of RPA-NALFIA and RPA-CRISPR/Cas12a FBDA

1. RPA amplification

1.1 RPA primers testing

To investigate the primers performance, the RPA reaction was performed at 39° C for 15 minutes, followed by the product purification then gel electrophoresis. The result revealed that the primer with the length of 20 bases showed a better target band with the product size of 241 base-pair (bp) whereas, the 30 bases primer length showed visible background noise. Therefore, the primer with 20 bases length was



selected for the next experiment (Figure 9).

Figure 9. RPA primers testing with 39° C, 15 minutes.

1.2 RPA optimum reaction time

The RPA was performed at 39° C with the variation in reaction time to find the optimum reaction time. The results showed that 15 minutes of reaction time provided the target band at 241bp without background noise or unspecific band. The unspecific amplification band with the size below 100bp increased with reaction time. Therefore, 15 minutes RPA reaction time was chosen for the next experiment (Figure 10).

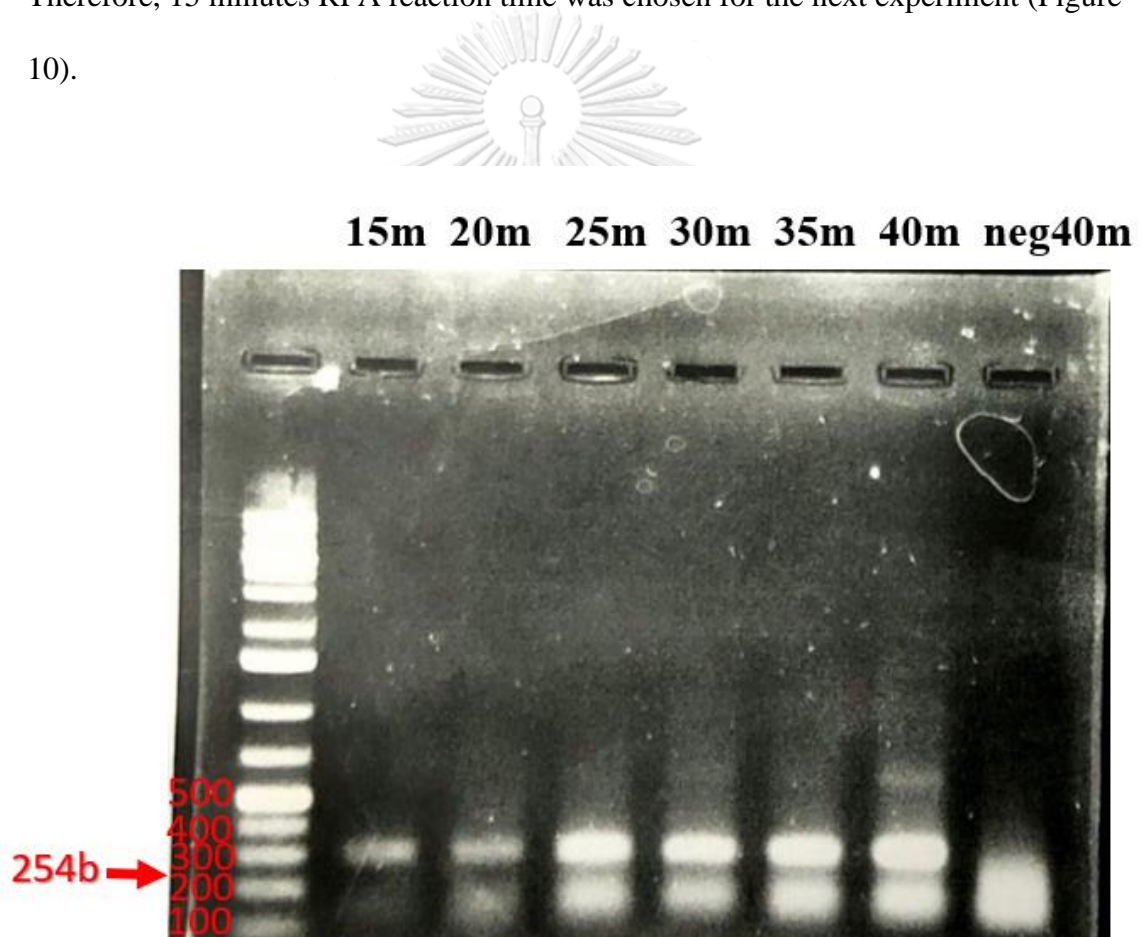
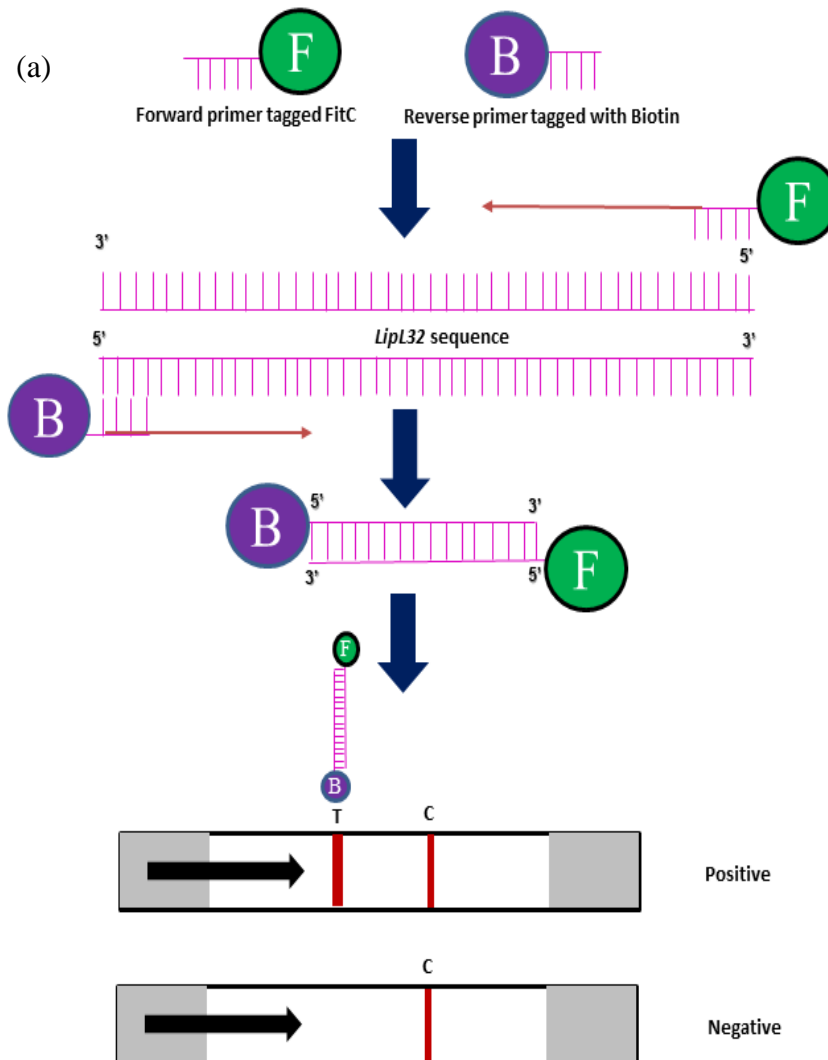


Figure 10. RPA reaction time variation at 39° C.

1.3 Testing RPA with NALFIA

The RPA forward primer and reverse primer amplicons of the reaction at 39° C 15 minutes, were mixed with 100 µL of running buffer and pipetted into the commercial lateral flow strip test (Kestrelbioscience, Thailand). The workflow of RPA-NALFIA showed in figure 11a. The test detected *leptospira spp.* at 10⁷ copies/reaction with two visible bands, whereas negative control without template showed one visible band (Figure 11b).





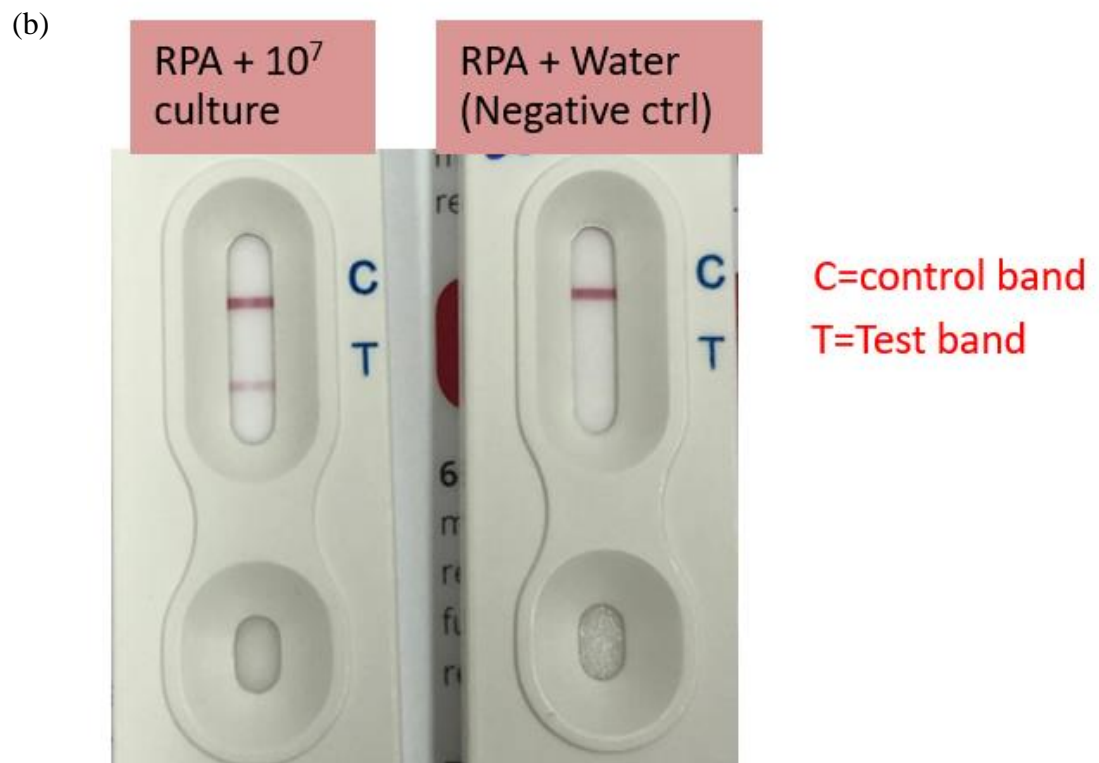


Figure 11 (a) Schematic representation of the RPA-NALFIA workflow. (b) RPA-NALFIA testing result.

1.4 The LOD of RPA-NALFIA

To investigate the LOD of the RPA-NALFIA, DNA was extracted from a *Leptospira* culture then serially diluted from 10^8 to 1 copies/ μ L. Next, the diluted DNA was amplified using RPA, followed by NALFIA. The LOD at 10^5 copies/ μ L was observed with the faded band at the test line (Figure 12a).

In order to achieve the acceptable LOD of the RPA-NALFIA, the RPA amplification time variation was performed ranging from 15 minutes to 40 minutes with the extracted DNA of a *Leptospira* culture at 10^7 copies/ μ L. In addition, the RPA amplicons were purified, and gel electrophoresis was performed. Unfortunately, the non-specific amplicon at under 100bp was found at the amplification time over 15 minutes which caused a false-positive of RPA-NALFIA (Figure 12b). The RPA-NALFIA at 40 minutes reaction time result showed in Figure 12c.

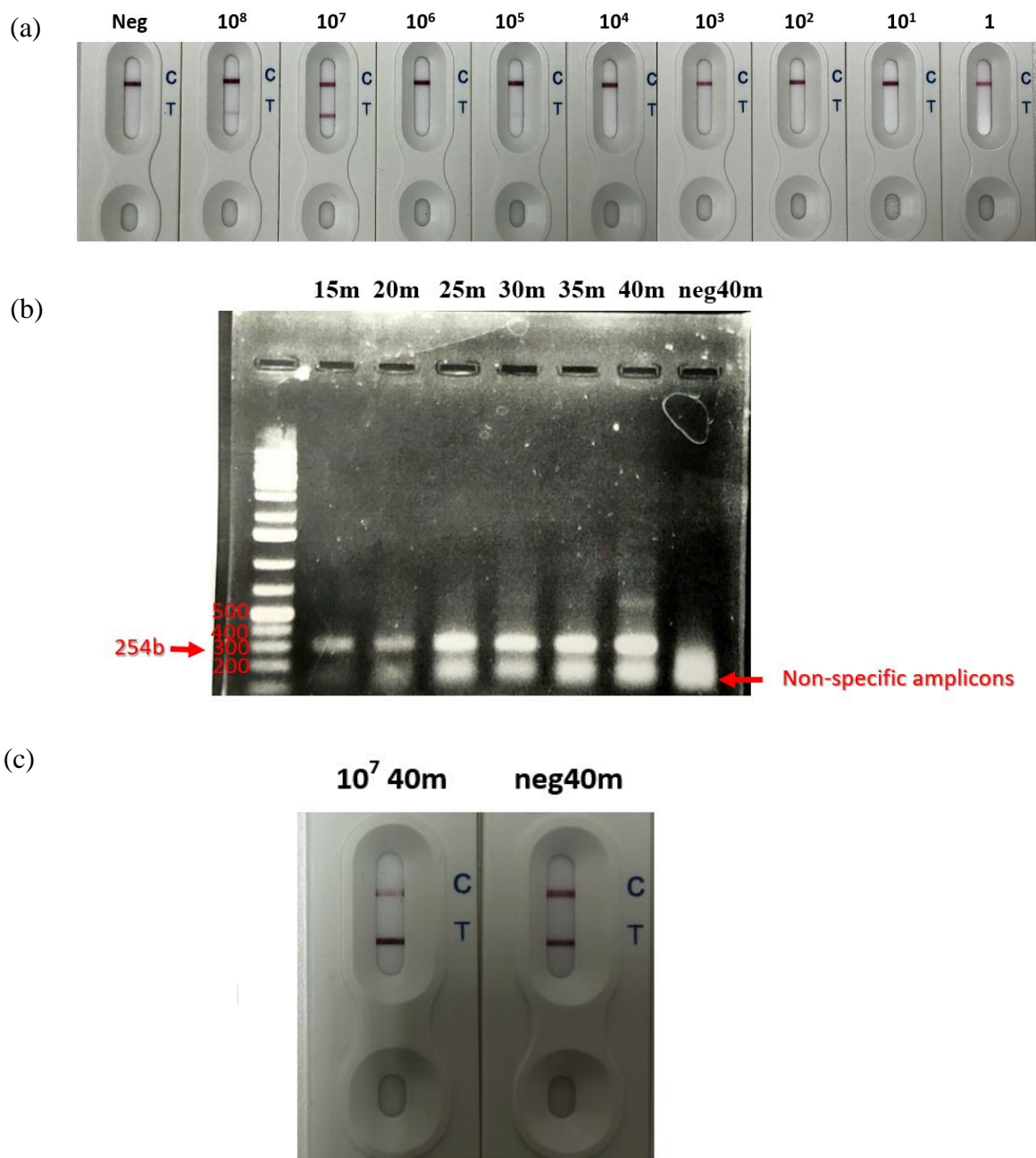
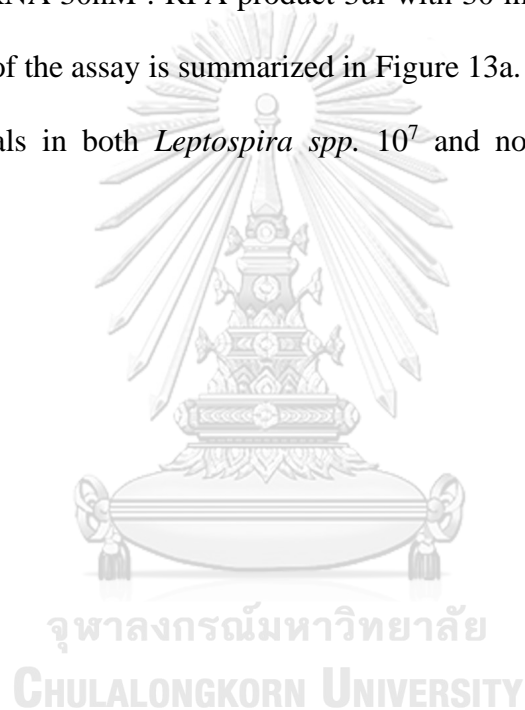


Figure 12. Detection of leptospirosis using the RPA-NALFIA. (a) LOD (b) Gel electrophoresis result of RPA time variation. (c) The RPA-NALFIA at 40 minutes reaction time.

2. RPA-CRISPR/Cas12a

2.1 RPA-CRISPR/Cas12a crRNA testing

The crRNA synthesis from the oligo-DNA template 3 μ L in total reaction volume 30 μ L was performed overnight (16hrs) followed by DNA template elimination by DNaseI, then purification and concentration measurement. The RPA-CRISPR/Cas FBDA was performed with the following condition Cas12a 30nM : Probe 400nM : gRNA 30nM : RPA product 3ul with 30 minutes reaction time at 39 $^{\circ}$ C. The workflow of the assay is summarized in Figure 13a. The results showed visible fluorescence signals in both *Leptospira spp.* 10 7 and non-template control (NTC) (Figure 13b).



(a)

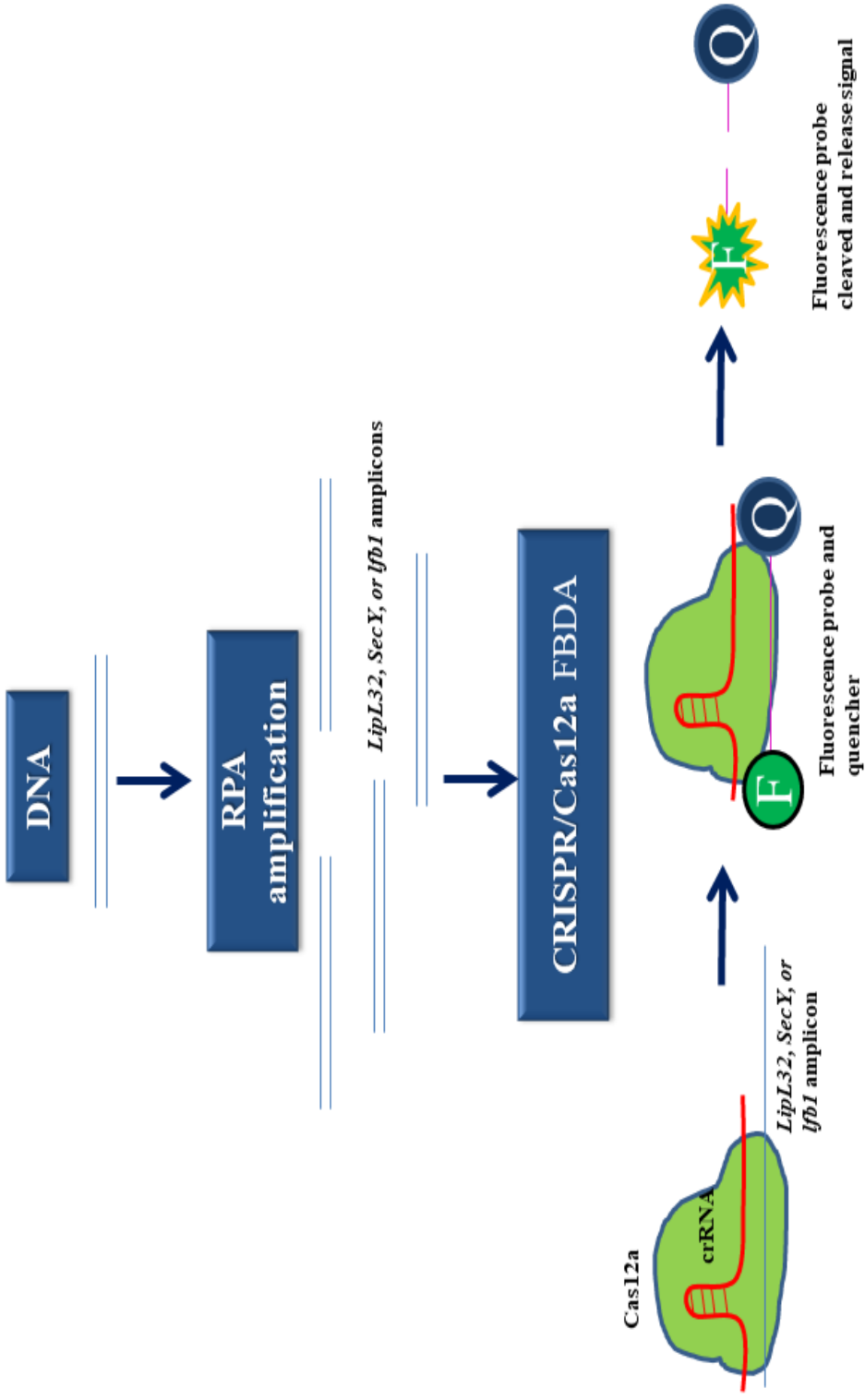




Figure 13. RPA-CRISPR/Cas12a crRNA testing. (a) Schematic representation of the RPA-CRISPR/Cas12-FBDA's workflow. (b) The fluorescence signal with UV gel-dock.

2.2 crRNA transcription adjustments

To eliminate the false-positive of RPA-CRISPR/Cas12a FBDA, the oligo-DNA template volume was decreased from 3 μ L to 1 μ L, followed by increased DNase I concentration from 0.067 U/mL to 0.13 U/mL and reaction time of template elimination from 20 minutes to 30 minutes. The RPA-CRISPR/Cas12a FBDA was tested with new adjustments, and the results showed no false-positive indicated that false-positive came from incompleting digestion of DNA template during the crRNA synthesis (Figure 14).

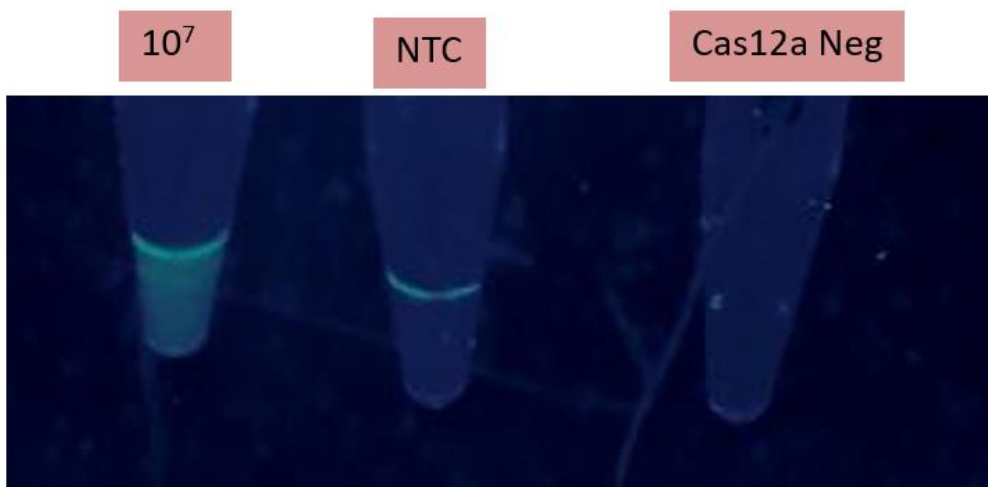
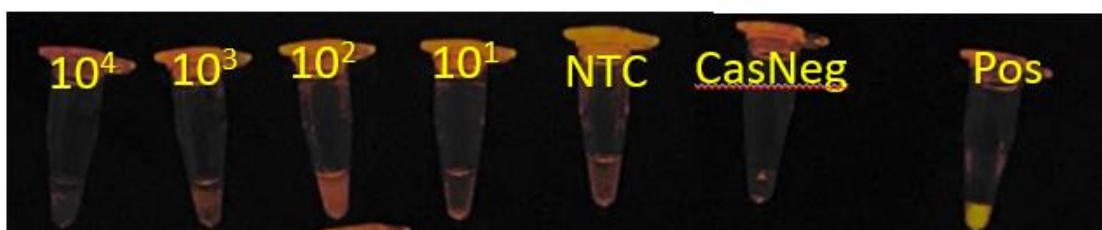


Figure 14. RPA-CRISPR/Cas12a crRNA transcription adjustments testing.

2.3 RPA-CRISPR/Cas12a limit of detection (LOD) testing

In order to test the LOD of the RPA-CRISPR/Cas12a-FBDA, the extracted DNA from a *Leptospira* culture was serially diluted from 10^4 to 10 copies/ μ L. The diluted DNA was amplified using RPA, followed by the CRISPR/Cas12a-FBDA. The



test was unable to detect the visible signal at 10^4 copies/reaction or lower (Figure 15).

Figure 15. The RPA-CRISPR/Cas12a FBDA LOD investigation.

2.4 RPA-CRISPR/Cas12a condition adjustments

The CRISPR/Cas12a detection step was adjusted from Cas12a 30nM : Probe 400nM : gRNA 30nM : RPA product 3ul to Cas12a 30nM : Probe 800nM : crRNA 330nM : RPA product 1ul with 30 minutes reaction time at 39° C. The results showed that the LOD was at 100 copies/reaction (Figure 16).

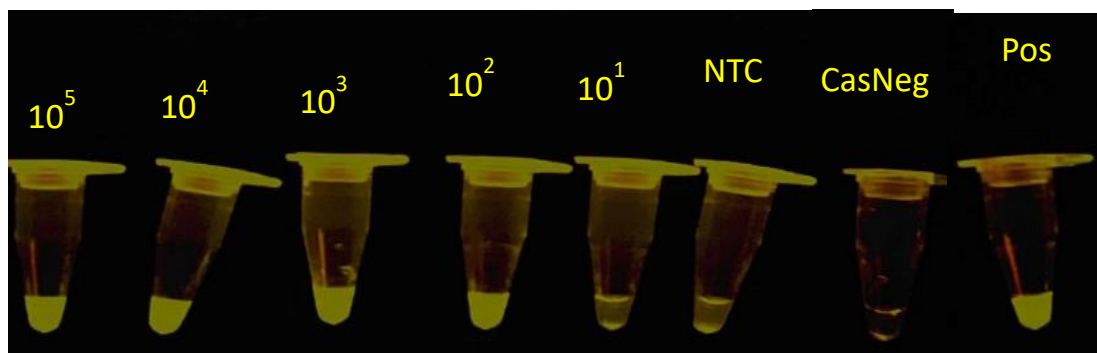


Figure 16. The RPA-CRISPR/Cas12a FBDA LOD of new condition.

2.5 RPA-CRISPR/Cas12a optimum reaction time investigation

To investigate the optimum reaction time of the assay, the CRISPR/Cas12a detection step reaction was varied from 5 minutes to 40 minutes. The results showed the visible signal at 5 minutes. However, there was high background noise at negative control (Figure 17).

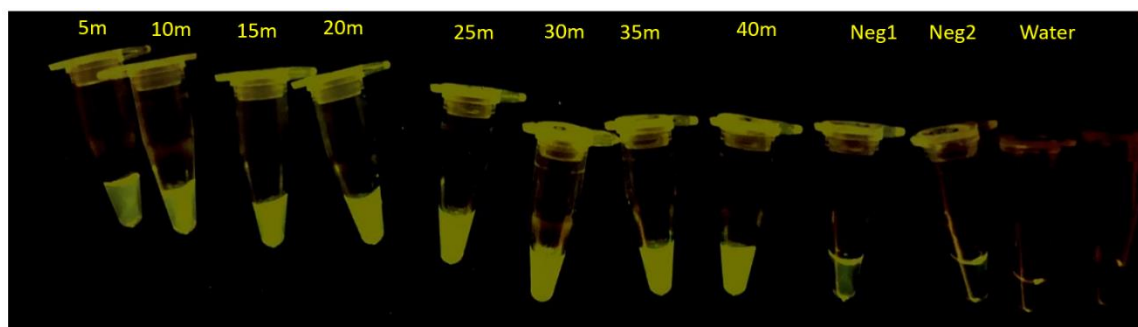














Figure 17. RPA-CRISPR/Cas12a optimum reaction time investigation

2.6 RPA-CRISPR/Cas12a probe and volume adjustments

The RPA product volume in CRISPR/Cas12a reaction was varied, ranging from 1 μ l to 5 μ l, and the probe concentration was compared between 200nM and 600nM. The results manifested that the best RPA product volume was 1 μ l with probe

RPA product	1ul	2ul	3ul	4ul	5ul	Negative
Probe 200nM						
Probe 600nM						

concentration at 600nM without background noise (Figure 18).











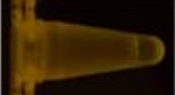
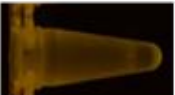







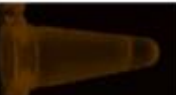








Figure 18. RPA-CRISPR/Cas12a probe and volume adjustments.

2.7 The LOD and cross-reactivity of the RPA-CRISPR/Cas12a-FBDA.

The LOD was tested with the new working condition; the results revealed the LOD to be 100 copies/reaction with *LipL32* 241b, and *SecY*. However, for *LipL32* 90b and *lfb1* were no fluorescence signal (Figure 19a). In addition, eight specimens from patients with other acute febrile illnesses, including acute viral hepatitis, cellulitis, scrub typhus, systemic bacterial infection, acute cystitis, influenza, *Escherichia coli* septicemia, and dengue hemorrhagic fever, were tested to explore the potential cross-reactivity. The results showed no cross-reactivity with the specimens obtained from these other diseases (Figure 19b).



(a)

	10^6	10^3	10^2	10^1	10^0	RPA Neg	Cas12a Neg
<i>LipI32 90b</i>							
<i>LipI32 241b</i>							
<i>LFB1</i>							
<i>SecYIV</i>							

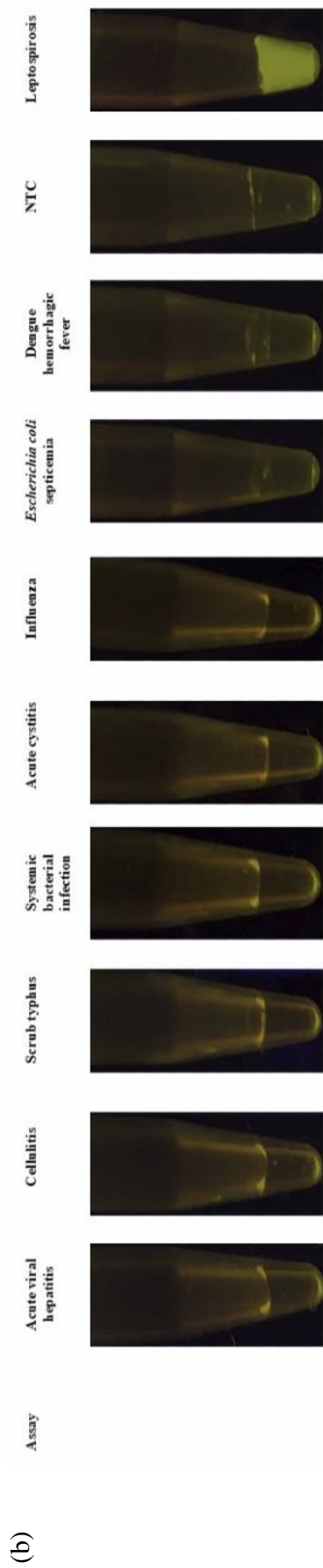
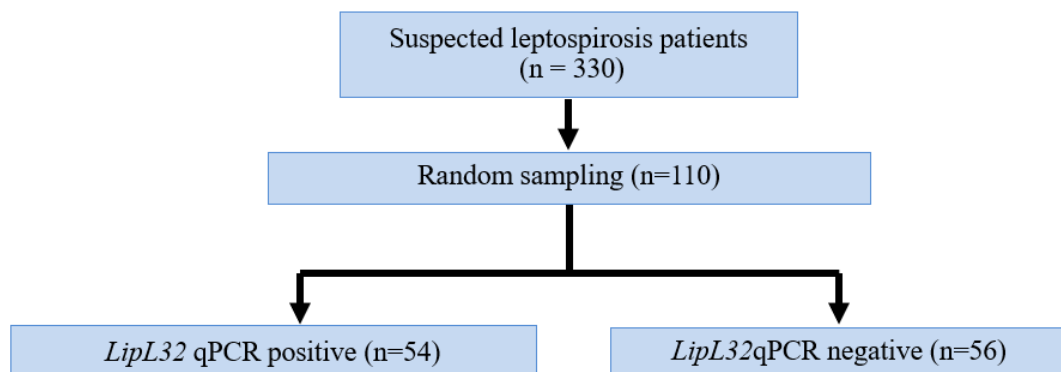


Figure 19. Detection of leptospirosis using the RPA-CRISPR/Cas12a-FBDA. The (b) LOD and (c) cross-reactivity of the RPA-CRISPR/Cas12a targeting *LipL32* gene against several infectious diseases with similar clinical manifestations as leptospirosis.

Part II. Clinical Samples validation

1.1 Study population

The performance of the RPA-CRISPR/Cas12a-FBDA was validated with 110 blood samples from clinically suspected leptospirosis patients. Among those, 54 (49.1%) were leptospirosis confirmed cases (positive by qPCR), and 56 (50.9%) were non-leptospirosis confirmed cases (negative by qPCR) (Figure 20). The clinical characteristics of the enrolled patients are shown in Table 3. Compared with non-leptospirosis, leptospirosis patients had significantly higher ($P < 0.05$) serum levels of white blood cells, creatinine, total bilirubin, direct bilirubin, and potassium, but a lower systolic blood pressure. In addition, there was a significant difference ($P = 0.01$) in terms of days of fever until enrollment between the groups. Other relevant



laboratory investigations were not found to be significantly different between the two groups.

Figure 20. Schematic flowchart of the participants

Table 3. Characteristics of the qPCR positive and negative groups with the patients' clinical and laboratory data.

Characteristic	Leptospirosis (N = 54)		Non-leptospirosis (N = 56)		Total (N = 110)	P-value
	Mean (SD)	Median (IQR)	Mean (SD)	Median (IQR)		
Male gender, n (%)	44 (81.48%)		45 (80.36%)		89 (80.91%)	0.88
Age, years, Mean (SD)	50.78 (16.71)		51.75 (15.93)		51.25 (16.26)	0.82
Days of fever until enrollment, Median (IQR)	3 (3, 5)		3 (2, 4)		3 (2, 4)	*0.01
Exposure to flood waters, n (%)	47 (85.45%)		40 (81.63%)		87 (79.09%)	0.18
Exposure to animals, n (%)	8 (14.55%)		9 (18.37%)		17 (15.45%)	0.73
Body temperature, Mean (SD)	38.14 (1.20)		38.16 (1.24)		38.15 (1.21)	0.97
SBP, mm Hg, Median (IQR)	109.00 (96.00, 121.50)		120.00 (101.00, 129.50)		111.00 (100.00, 126.00)	*0.02
DBP, mm Hg, Median (IQR)	61.00 (58.00, 73.25)		68.00 (60.00, 76.75)		64.00 (60.00, 74.00)	0.10
Platelet (x 10 ³ /μL), Median (IQR)	94500.00 (59500.00, 213250.00)		132000.00 (68500, 194750.00)		118500.00 (63000.00, 204000.00)	0.70
*WBC (x 10 ³ /μL), Median (IQR)	10950.00 (8525.00, 14025.00)		8600.00 (5375.00, 12250.00)		10500.00 (6350.00, 13375.00)	*0.02
Creatinine, mg/dL, Median (IQR)	1.33 (1.00, 2.90)		1.10 (0.86, 1.27)		1.12 (0.94, 1.96)	*0.01
*TB, g/dL, Median (IQR)	1.40 (0.82, 3.30)		0.90 (0.50, 2.35)		1.18 (0.70, 2.90)	*0.02
*DB, g/dL, Median (IQR)	0.90 (0.46, 1.97)		0.50 (0.24, 1.55)		0.70 (0.30, 1.80)	*0.03
*SGOT, U/L, Median (IQR)	63.00 (41.50, 147.00)		64.50 (39.50, 170.00)		63.00 (41.00, 164.00)	0.91
*SGPT, U/L, Median (IQR)	59.00 (31.50, 103.50)		60.00 (33.00, 84.75)		60.00 (32.50, 96.00)	0.77
Na, mEq/L, Median (IQR)	135.00 (132.00, 138.00)		135.00 (131.45, 139.00)		135.00 (131.70, 139.00)	0.71
K, mEq/L, Median (IQR)	3.77 (3.40, 4.26)		3.50 (3.09, 3.90)		3.63 (3.26, 4.01)	*0.01
HCO ₃ , mEq/L, Median (IQR)	24.00 (20.15, 25.75)		25.00 (22.25, 26.45)		24.55 (21.73, 26.00)	0.11

Abbreviations: WBC: white blood cell, TB: total bilirubin, DB: direct bilirubin, SGOT: serum glutamic oxaloacetic transaminase, SGPT: serum glutamic pyruvic transaminase, Na: sodium, K: potassium, HCO₃: bicarbonate. Continuous data are expressed as the mean (SD) or median (IQR). Categorical variables are expressed as numbers (%); * represents $P < 0.05$.

1.2. Diagnostic performance of the RPA-CRISPR/Cas12a-FBDA.







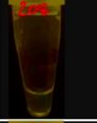
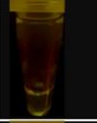
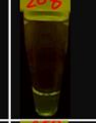
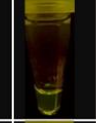







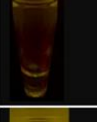




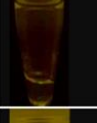






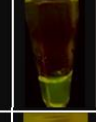

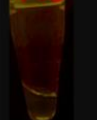

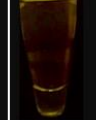

To evaluate the diagnostic performance of the RPA-CRISPR/Cas12a-FBDA, 33 DNA samples from leptospirosis and non-leptospirosis confirmed cases were tested, and the results were compared the qPCR results. Compared to the qPCR, the RPA-CRISPR/Cas12 targeting *LipL32* assay 71.43% specificity, 33.33% sensitivity, and 40.00% accuracy, with a positive predictive value (PPV) and a negative predictive value (NPV) of 84.62% and 18.52%, respectively, (Table 4). For RPA-CRISPR/Cas12 targeting *SecY*, the results showed 5.26% sensitivity, 100% specificity, 45.45%, and accuracy after 33 DNA samples were performed. Therefore, the researchers decided to discontinue the RPA-CRISPR/Cas12 targeting *SecY*.

Table 4. Performance of the RPA-CRISPR/Cas12a-FBD relative to qPCR detection.

Assay	<i>LipL32</i> qPCR	
	Positive	Negative
<i>LipL32</i> RPA/Cas12a	11	2
	22	5
Sensitivity	33.33%	
Specificity	71.43%	
Positive Predictive Value (PPV)	84.62%	
Negative Predictive Value (NPV)	18.52%	
Accuracy	40.00%	

1.3. RPA-CRISPR/Cas12a-FBDA performance improvement.

To improve the performance of RPA-CRISPR/Cas12a-FBDA, the factors reducing the assay's performance were investigated. The factors were the operator's technique, the Cas12a enzyme activity, and probe. Figure 21 showed that the Cas12a

ID	Orn's	Nick's Lab Cas+Lab probe	Nick's Lab Cas+Orn's probe	Nick's Orn Cas+Lab probe	Nick's Orn Cas+Orr probe	qPCR
194						Neg
208						29.35
150						34.47
243						Neg
140						Neg
10 ⁵						23.57
Neg						Neg

Clinical samples

Positive control

enzyme activity was the cause of the low test sensitivity.

Figure 21. RPA-CRISPR/Cas12a-FBDA performance improvement. The factors reducing the assay's performance were compared.

1.4 Diagnostic performance of the RPA-CRISPR/Cas12a-FBDA after adjustments.

To re-investigate the diagnostic performance of the RPA-CRISPR/Cas12a-FBDA with the new adjustments, 110 DNA samples from leptospirosis and non-leptospirosis confirmed cases were tested results were compared to the qPCR results. Compared to the qPCR, the RPA-CRISPR/Cas12 targeting *LipL32* assay yielded 100% specificity, 85.2% sensitivity, and 92.7% accuracy, with a positive predictive value (PPV) and a negative predictive value (NPV) of 100% and 87.50%, respectively, (Table 5).



Table 5. Performance of the RPA-CRISPR/Cas12a-FBDD relative to qPCR detection.

Assay	<i>LipL32</i> qPCR	
	Positive	Negative
<i>LipL32</i> RPA/Cas12a	46	0
	8	56
Sensitivity	85.19%	
Specificity	100%	
Positive Predictive Value (PPV)	100%	
Negative Predictive Value (NPV)	88.89%	
Accuracy	93.22%	

1.5. Diagnosis accuracy at different days after the fever onset

To evaluate the change in sensitivity and specificity of the assay with time after fever onset, the patients were categorized into three groups based on the time since the onset of fever (at the first day of enrollment); as within 3 d after fever onset (n = 69), within 4–6 d from fever onset (n = 19), and 7 d or longer after the onset of fever (n = 17). We found that the sensitivity and accuracy of RPA-CRISPR/Cas12a-FBDA targeting *LipL32* were increased on days 4–6 and decreased after day 7. In contrast, the specificity was consistent for every day after the onset of fever Table 6 and Figure 22.

We also compared the diagnostic accuracy of our assay with a commercial RDT based on the detection of anti-*Leptospira* IgM antibodies. We found that the commercial RDT assay yielded a lower sensitivity, specificity, and accuracy than the RPA-CRISPR/Cas12a-FBDA every day after the onset of fever table 7 and Figure 22.

RPA-CRISPR/Cas12a-FBDA	Day After Onset of Fever	
	≤3 (n=69)	4-6 (n=19)
Sensitivity	83.87%	100%
Specificity	100.00%	100%
Accuracy	92.75%	100%

Table 6. RPA-CRISPR/Cas12a-FBDA diagnosis accuracy at different days after the fever onset.

RDT	Day After Onset of Fever	
	≤3 (n=56)	4-6 (n=16)
Sensitivity	56.67%	73%
Specificity	27.78%	40%
Accuracy	40.91%	63%

Table 7. RDT diagnosis accuracy at different days after the fever onset.

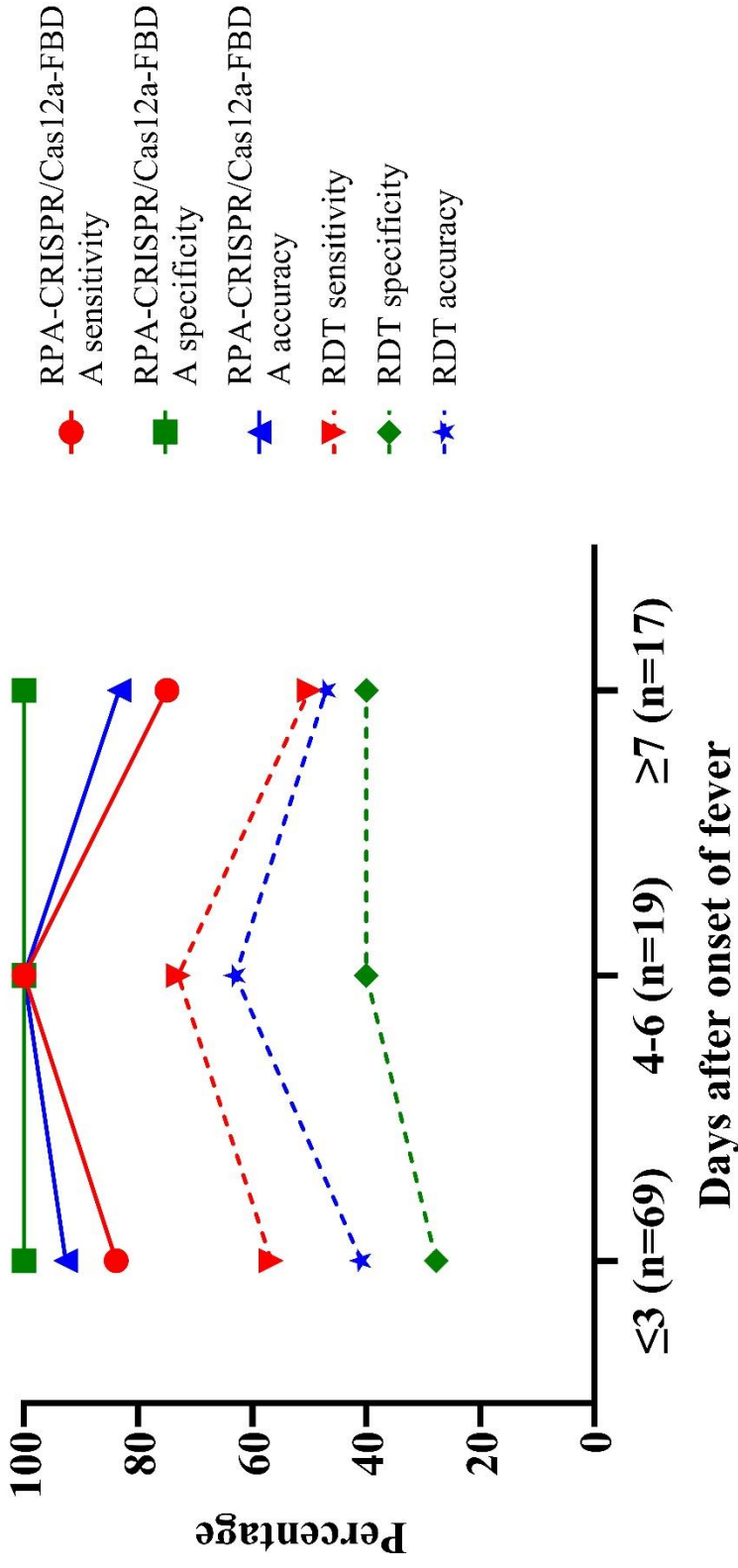


Figure 22. Sensitivity, specificity, and accuracy of the RPA-CRISPR/Cas12a-fluorescence-based detection assay (solid line) and RDT (dashed line) at 3 d (red), 4–6 d (green), and ≥ 7 d (blue) after the onset of fever.

1.6 Inter-observer variability

The sensitivity, specificity, and accuracy were calculated separately to investigate each observer's variability, with the results summarized in Table 4. The data revealed no significant difference between observers in the ability to identify the fluorescent signal.

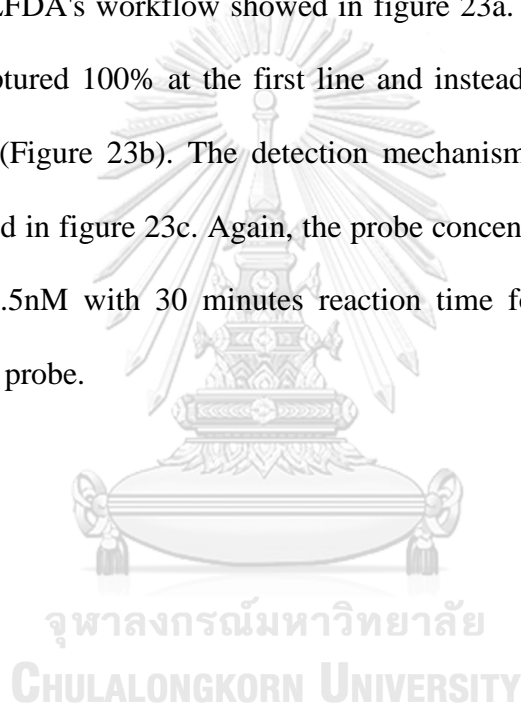


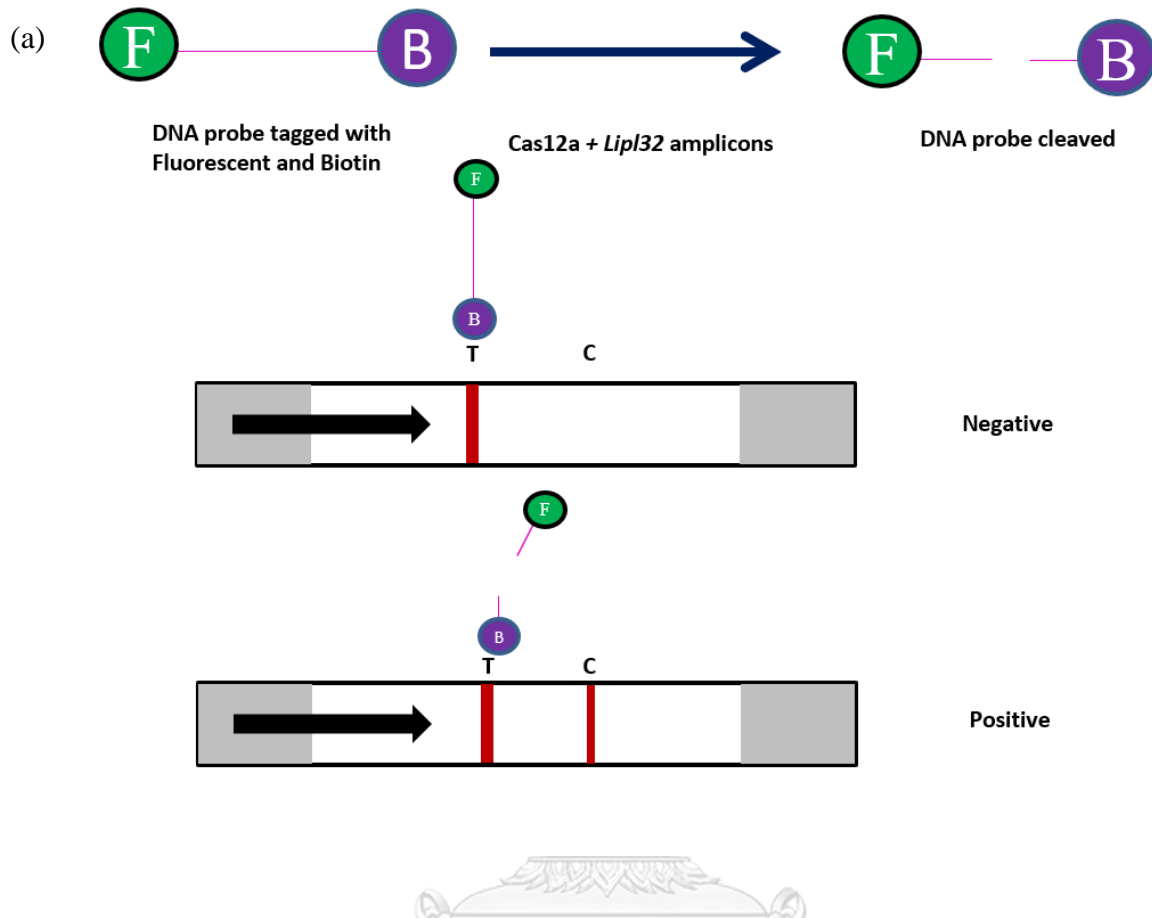
Parameter	Observer 1	Observer 2	Observer 3
Sensitivity (%)	87.04	87.04	85.19
Specificity (%)	100	100	100
Accuracy (%)	93.64	93.64	92.73

Table 8. Inter-observer comparison.

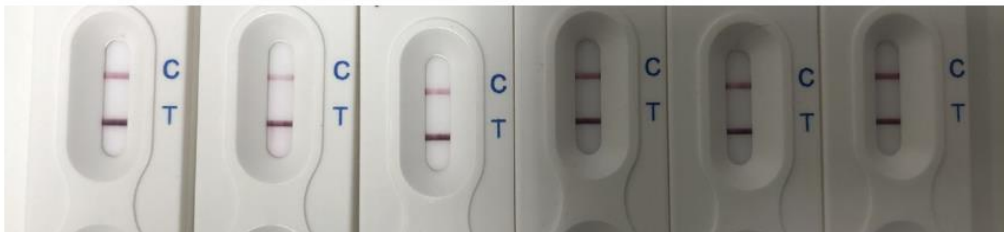
1.7 The RPA-CRISPR/Cas12a LFDA development

To improve this test and make it more accessible for general use and easier to read. The lateral flow detection assay was developed. The probe concentration was varied, ranging from 600nM to 18.75nM, to find the optimum probe concentration captured 100% at the first line of the lateral flow strip test. The RPA-CRISPR/Cas12a-LFDA's workflow showed in figure 23a. Every probe concentration unsuccessfully captured 100% at the first line and instead formed two visible lines: the false-positive (Figure 23b). The detection mechanism of lateral flow was then changed, as showed in figure 23c. Again, the probe concentration was varied, ranging from 50nM to 12.5nM with 30 minutes reaction time for the CRISPR/Cas12a to completely cut the probe.





(b) 600nM 300nM 150nM 75nM 37.5nM 18.75nM



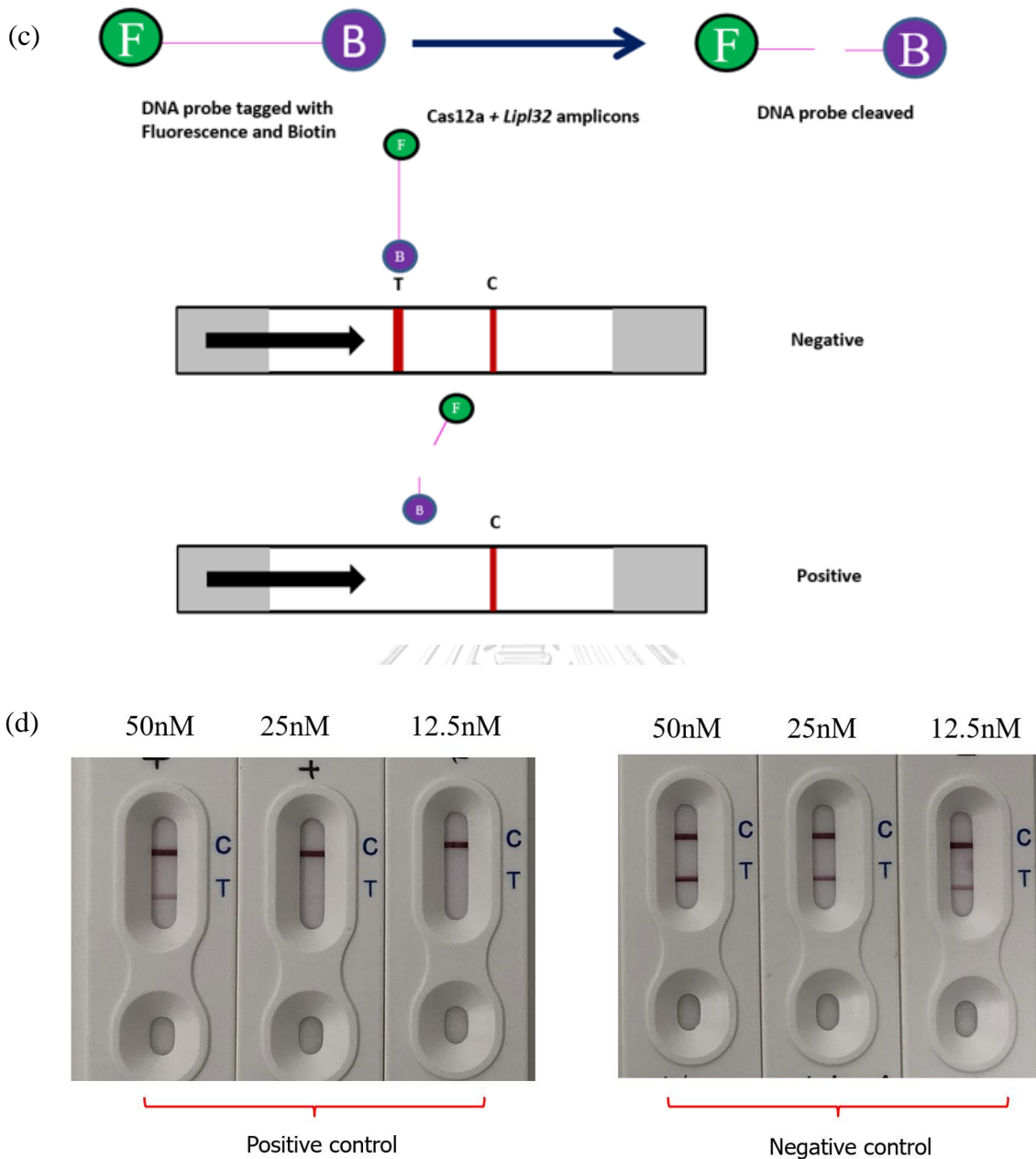


Figure 23. The RPA-CRISPR/Cas12a LFDA development. (a) The RPA-CRISPR/Cas12a-LFDA's workflow. (b) The variation of probe concentration. (c) The new schematic of RPA-CRISPR/Cas12a-LFDA. (d) The variation of probe concentration of the second method.

1.8 The RPA-CRISPR/Cas12a LFDA pilot study

The pilot study of RPA-CRISPR/Cas12a-LFDA was performed. The LOD of this LFDA was similar to that for the FBDA, at 100 copies/reaction, (Figure 24a). Moreover, nine DNA samples from leptospirosis confirmed cases ($n = 5$) at a Ct between 27–37 and non-leptospirosis cases ($n = 4$) were tested in the pilot study. The results showed that the LFDA could reliably distinguish between the known positive and negative clinical samples (Figure 24b).



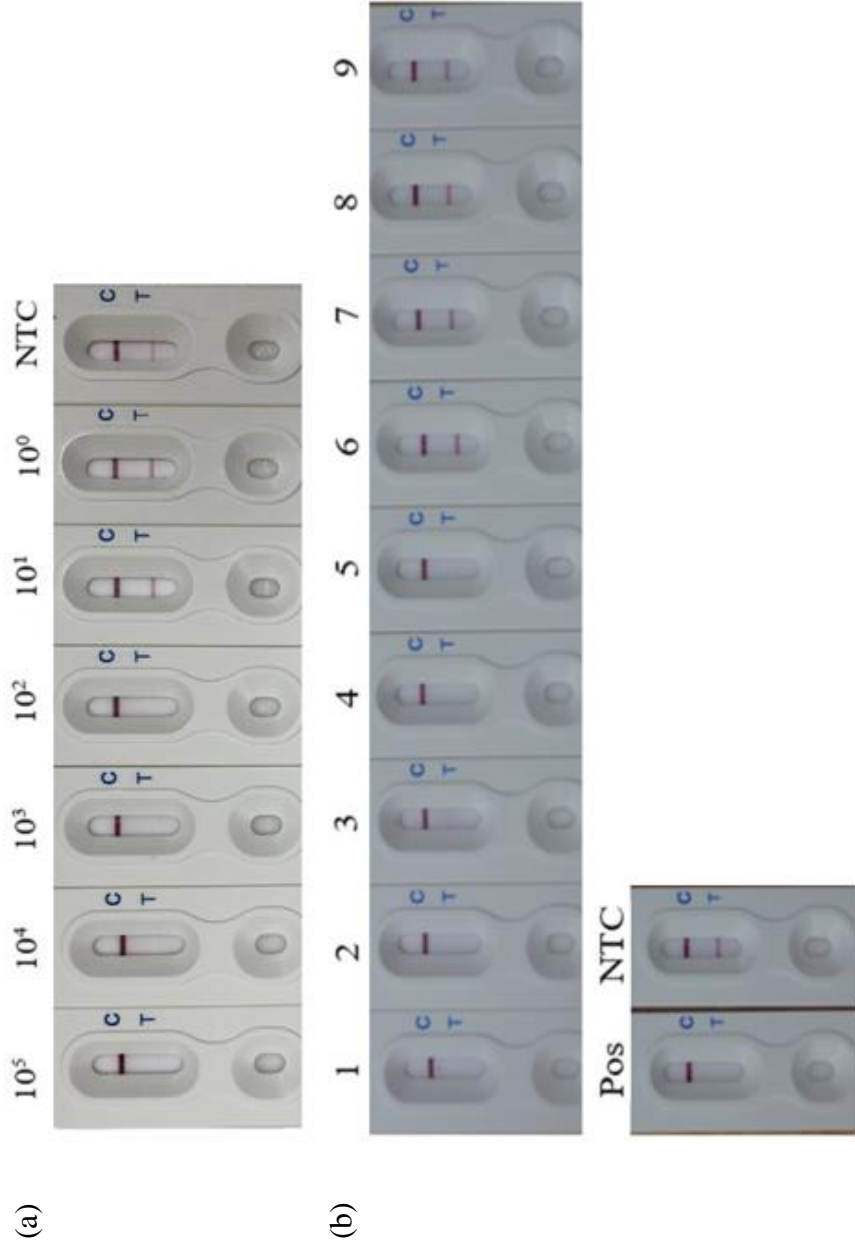


Figure 24. The RPA-CRISPR/Cas12a-LFDA. (a) LOD (b) clinical sample validation [1–5 and 6–9 are known positive and negative samples, respectively, while NTC and Pos are the no-template negative and positive control, respectively]

CHAPTER V DISCUSSION

The RPA-NALFIA and RPA-CRISPR/Cas12a-FBDA is a new nucleic acid detection platform used to diagnose many infectious diseases (12, 34-36, 40, 45, 46). This study is the first report for leptospires detection using the RPA-CRISPR/Cas12aFBDA assay targeting the *LipL32*, *SecY*, and *lfb1* genes. The RPA-CRISPR/Cas12aFBDA targeting *LipL32* demonstrated acceptable sensitivity (85%) and excellent specificity (100%) of leptospirosis detection compared to qPCR as the reference test, whereas the RPA-CRISPR/Cas12 targeting *SecY* showed 5.26% sensitivity, 100% specificity, 45.45%, and accuracy after 33 DNA samples were performed. However, the RPA-NALFIA targeting *LipL32* failed to achieve the acceptable LOD. Therefore, further experiments were discontinued. The RPA is an isothermal nucleic acid amplification platform, which is less time-consuming than conventional PCR and qPCR (11, 29).

A previous study revealed that PCR inhibitors in clinical samples can affect the qPCR's performance in detecting *LipL32* (47). However, the RPA is more tolerant of PCR inhibitors, so it is an ideal amplification platform in this study (48). The qPCR system provides a highly specific and sensitive tool for detecting and quantifying *Leptospira* (39). However, due to its higher cost than other diagnostic methods and its requirements for specialized instruments, it has not been widely used as an early diagnostic tool at the point of care.

According to our study, the RPA-CRISPR/Cas12a-based detection system targeting *LipL32* could be used to detect *Leptospira* with an acceptable sensitivity and high specificity. A previous study reported that more than 1,000 copies *Leptospira* /mL was associated with severe leptospirosis (49). It is, therefore, notably that our

assay was sensitive enough to detect *Leptospira* in the patient's blood and administered treatment before the pathogen level and so before disease symptoms become severe.

Moreover, the specificity of the RPA-CRISPR/Cas12a-FBDA was found to be consistent for all three tested periods of time after the onset of fever, while the sensitivity increased to 100% on days 4–6 after fever onset and decreased after day 7, which may reflect that the serum *Leptospirosis spp.* peaked at days 4–6 after fever onset (28) and decreased after day 7. We compared the assay performance with a commercial RDT designed for detection of anti-*Leptospira spp.* IgM antibodies. The RDT performance was similar to that previously reported (50), but had a lower sensitivity, specificity, and accuracy than the RPA-CRISPR/Cas12a-FBDA developed here. The window of positivity for the RPA-CRISPR/Cas12a platform was from the first week of infection, whereas that for the RDT was from days 6–8 (5, 28). Therefore, using this RPA-CRISPR/Cas12a-FBDA targeting *LipL32* combined with RDTs would expand the window of positivity and enhance the accuracy of the leptospirosis diagnosis. We also developed the RPA-CRISPR/Cas12a-LFDA to improve the test by making it more accessible for use and easier to read. The preliminary result showed that the RPA-CRISPR/Cas12a-LFDA could reliably distinguish between known positive and negative *Leptospira spp.* from clinical samples in a pilot study.

The RPA-NALFIA unsuccessfully reach an acceptable LoD. Moreover, RPA condition adjustment to reach better LoD has caused the false positive due to unspecific amplification. Several studies have also encountered a similar problem (51-

53). The possible solution is to design RPA primers with self-avoiding molecular recognition systems (SAMRS) (54).

The RPA-CRISPR/Cas12a-LFDA targeting *SecY* and *lfbI* were also failed to detect the *leptospira spp.* especially in clinical samples. The crRNA target sequences analysis found that there is a single nucleotide mismatch among the *leptospira interrogans* serovar. A single mismatch could lead to the detection ability of the assay due to reduced affinity in target sequence binding activity of Cas12a resulted in a decreased rate of the enzyme cleavage activity (55).

This study had several strengths. First, we compared the test sensitivity, specificity, and accuracy with the day of fever. The results showed that the time window after the onset of infection is a vital factor in the detection of positive infections in the different types of assays. Second, our study was performed blind and with three different observers to give less biased results. Third, we also developed a LFDA for a more comfortable result reading, and the pilot study achieved a similar LOD as with the FBDA. Fourth, we performed a bioinformatics analysis to investigate nucleotide variation that would affect the test's sensitivity (10, 56, 57). The result showed no variation in the *LipL32* targeted by crRNA of CRISPR/Cas12a among serovars found in Thailand (data not shown). Finally, the RPA-CRISPR/Cas12a-based detection system is ideal for rural hospitals, as it has less expensive laboratory equipment requirements, such as avoiding the use of real-time qPCR machines. We only need a heat box for the isothermal reaction.

However, our study was not without limitations. First, we only performed the blood sample test from the first day of admission. Hence, we could not compare the test sensitivity and specificity in different types of samples. Second, only a small

number of samples from patients at more than 7 d after the onset of fever were included ($n = 12$), because most patients visit the hospital early after fever onset. This could alter the test's accuracy. Third, the use of more than one target gene may enhance the efficiency of the test. We have developed *SecY* and *lfb1*. Unfortunately, both genes were unable to reach our goal. Fourth, the clinical samples were collected and had been stored for at least four years which could be the caused of reduced test sensitivity. Finally, the clinical samples were collected from a single province in Thailand, which might limit the universalization results.



CHAPTER VI CONCLUSION

In conclusion, detecting *leptospires* with the RPA-CRISPR/Cas12a-FBDA targeting *LipL32* provided a satisfactory LOD, sensitivity, and specificity. It is suitable for use in the field, especially in rural hospitals with limited resources. Only a heat box is required to perform the isothermal reaction. Furthermore, with the LFDA, we can further decrease the use of equipment. Therefore, it is practical, simple, portable, and rapid.



Appendix



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Appendix Table 1. Raw data of CRISPR/Cas12a assay

No.	ID	qPCR	CRISPR/Cas12a LipL32	Observer 1 (N)	Observer 2 (J)	Observer 3 (B)	CRISPR/Cas12a SecY	DoF	MAT	IgM strip test
1	RLSS-066	Negative	Negative	0	0	0	Negative	1	Negative	Negative
2	RLSS-193	37.03	Positive	1	1	0	Negative	3	Negative	Positive
3	RLSS-211	Negative	Negative	0	0	0	Negative	14	Negative	Negative
4	RLSS-008	34.55	Positive	1	1	1	Negative	7	Negative	Positive
5	RLSS-185	35.91	Positive	1	1	1	Negative	5	Negative	Positive
6	RLSS-182	Negative	Negative	0	0	0	Negative	4	Negative	Negative
7	RLSS-207	Negative	Negative	0	0	0	Negative	3	Negative	Positive
8	RLSS-206	Negative	Negative	0	0	0	Negative	7	Negative	Positive
9	RLSS-180	Negative	Negative	0	0	0	Negative	2	Negative	Negative
10	RLSS-219	37.95	Negative	0	0	0	Negative	9	Positive	Positive
11	RLSS-065	34.88	Positive	2	2	2	Negative	1	Negative	Positive
12	RLSS-228	Negative	Negative	0	0	0	Negative	3	Positive	Positive
13	RLSS-075	31.47	Positive	2	2	2	Negative	2	Positive	Negative
14	RLSS-056	31.96	Positive	2	2	2	Negative	3	Negative	Positive
15	RLSS-244	Negative	Negative	0	0	0	Negative	4	Positive	Positive
16	RLSS-230	32.98	Positive	2	2	2	Negative	3	Negative	Negative
17	RLSS-186	33.93	Positive	2	2	2	Negative	5	Negative	Positive
18	RLSS-091	35.89	Positive	0	1	1	Negative	7	Negative	Positive
19	RLSS-122	Negative	Negative	0	0	0	Negative	3	Negative	Negative
20	RLSS-042	32.73	Positive	2	2	2	Positive	3	Negative	Negative
21	RLSS-129	Negative	Negative	0	0	0	Negative	4	Negative	Negative
22	RLSS-049	Negative	Negative	0	0	0	Negative	7	Negative	Negative
23	RLSS-231	32.91	Positive	2	2	2	Negative	7	Negative	Positive
24	RLSS-048	37.47	Negative	1	0	0	Negative	7	Negative	Negative
25	RLSS-280	Negative	Negative	0	0	0	Negative	NA	Negative	Negative
26	RLSS-190	35.1	Positive	2	2	2	Negative	7	Negative	Positive
27	RLSS-198	Negative	Negative	0	0	0	Negative	7	Negative	Positive
28	RLSS-079	32.09	Positive	2	2	2	Negative	2	Positive	Positive
29	RLSS-025	33.88	Positive	2	2	2	Negative	1	Negative	Negative
30	RLSS-071	30.98	Positive	2	2	2	Negative	3	Negative	Negative
31	RLSS-023	32.83	Positive	2	2	2	Negative	3	Negative	Positive
32	RLSS-053	Negative	Negative	0	0	0	Negative	3	Negative	Positive
33	RLSS-054	34.57	Positive	2	2	2	Negative	3	Negative	Negative
34	RLSS-047	33.22	Positive	2	2	2		1	Positive	Positive
35	RLSS-074	30.05	Positive	2	2	2		2	Negative	Positive
36	RLSS-072	34.95	Positive	2	2	2		3	NA	Negative
37	RLSS-206	33.9	Negative	2	2	2		7	Negative	Negative
38	RLSS-245	31.69	Positive	2	2	2		4	Negative	Positive
39	RLSS-177	36.55	Positive	1	1	1		3	Negative	Positive
40	RLSS-140	Negative	Negative	0	0	0		6	Positive	No sample
41	RLSS-214	35.33	Negative	0	0	0		3	Negative	Positive
42	RLSS-168	36.08	Positive	2	2	2		3	Negative	Positive
43	RLSS-243	Negative	Negative	0	0	0		3	Positive	Positive
44	RLSS-208	29.35	Positive	2	2	2		4	Negative	Negative
45	RLSS-138	33.85	Positive	2	2	2		3	Negative	Positive
46	RLSS-194	Negative	Negative	0	0	0		3	Negative	Positive
47	RLSS-195	35.82	Positive	2	2	2		5	Positive	Positive
48	RLSS-144	Negative	Negative	0	0	0		4	Negative	Positive
49	RLSS-199	37.72	Negative	0	0	0		3	Positive	Positive
50	RLSS-150	33.07	Positive	2	2	2		3	Negative	Positive
51	RLSS-117	33.38	Positive	2	2	2		4	Positive	Positive
52	RLSS-246	35.23	Positive	2	2	2		11	Negative	Positive
53	RLSS-203	Negative	Negative	0	0	0		3	Positive	Negative
54	RLSS-112	32.48	Positive	2	2	2		4	Negative	Positive
55	RLSS-172	32.5	Positive	2	2	2		7	Positive	Negative
56	RLSS-055	35.17	Positive	2	2	2		7	Negative	Negative
57	RLSS-226	Negative	Negative	0	0	0		NA	Negative	Negative
58	RLSS-110	25.83	Positive	2	2	2		5	Negative	Positive
59	RLSS-225	Negative	Negative	0	0	0		NA	Negative	Positive
60	RLSS-070	35.14	Positive	2	2	2		3	Negative	No sample
61	RLSS-259	34.93	Positive	2	2	2		3	Negative	Positive
62	RLSS-043	34.93	Positive	2	2	2		2	Positive	Negative
63	RLSS-044	33.19	Positive	2	2	2		2	Negative	Negative
64	RLSS-069	34.85	Positive	2	2	2		1	Negative	Negative
65	RLSS-127	Negative	Negative	0	0	0		1	Negative	Negative
66	RLSS-258	33.99	Positive	2	2	2		4	Negative	Positive
67	RLSS-260	35.99	Negative	0	0	0		3	Negative	Negative
68	RLSS-064	35.2	Positive	2	2	2		2	Negative	Negative
69	RLSS-077	Negative	Negative	0	0	0		2	Negative	Negative
70	RLSS-219	36.01	Positive	2	2	2		9	Positive	Negative
71	RLSS-076	34.95	Positive	2	2	2		4	Positive	Positive
72	RLSS-251	32.95	Positive	2	2	2		5	Positive	Negative
73	RLSS-099	33.37	Positive	2	2	2		3	Negative	Positive
74	RLSS-068	Negative	Negative	0	0	0		1	Negative	Negative
75	RLSS-015	28.57	Positive	2	2	2		3	Negative	Negative
76	RLSS-279	Negative	Negative	0	0	0		NA	Negative	Positive
77	RLSS-012	37.65	Negative	0	0	0		1	Negative	Positive
78	RLSS-169	32.99	Positive	2	2	2		7	Negative	Negative
79	RLSS-292	Negative	Negative	0	0	0		2	Negative	Positive
80	RLSS-275	Negative	Negative	0	0	0		2	Negative	Negative
81	RLSS-257	Negative	Negative	0	0	0		1	Negative	Positive
82	RLSS-294	Negative	Negative	0	0	0		7	Negative	Positive
83	RLSS-276	Negative	Negative	0	0	0		2	Negative	Positive
84	RLSS-283	Negative	Negative	0	0	0		1	Negative	Positive
85	RLSS-220	Negative	Negative	0	0	0		1	Negative	Positive
86	RLSS-253	Negative	Negative	0	0	0		2	Negative	Positive
87	RLSS-265	Negative	Negative	0	0	0		1	Negative	Positive
88	RLSS-290	Negative	Negative	0	0	0		3	Negative	Negative
89	RLSS-288	Negative	Negative	0	0	0		2	Negative	Positive
90	RLSS-260	38.65	Negative	0	0	0		3	Negative	Positive
91	RLSS-244	Negative	Negative	0	0	0		4	Positive	Positive
92	RLSS-262	Negative	Negative	0	0	0		1	Negative	Positive
93	RLSS-255	Negative	Negative	0	0	0		1	Negative	Positive
94	RLSS-154	Negative	Negative	0	0	0		3	Negative	Positive
95	RLSS-232	Negative	Negative	0	0	0		3	Negative	No sample
96	RLSS-147	Negative	Negative	0	0	0		2	Negative	No sample
97	RLSS-146	Negative	Negative	0	0	0		4	Negative	No sample
98	RLSS-145	Negative	Negative	0	0	0		2	Negative	No sample
99	RLSS-254	Negative	Negative	0	0	0		3	Negative	No sample
100	RLSS-286	Negative	Negative	0	0	0		3	Negative	No sample
101	RLSS-284	Negative	Negative	0	0	0		3	Negative	No sample
102	RLSS-287	Negative	Negative	0	0	0		3	Negative	No sample
103	RLSS-249	Negative	Negative	0	0	0		3	Negative	No sample
104	RLSS-218	Negative	Negative	0	0	0		3	Negative	No sample
105	RLSS-241	Negative	Negative	0	0	0		7	Negative	No sample
106	RLSS-274	Negative	Negative	0	0	0		1	Negative	No sample
107	RLSS-239	Negative	Negative	0	0	0		1	Negative	No sample
108	RLSS-273	Negative	Negative	0	0	0		2	Negative	No sample
109	RLSS-248	Negative	Negative	0	0	0		4	Negative	No sample
110	RLSS-227	Negative	Negative	0	0	0		1	Negative	Negative

<i>Leptospira spp.</i>	crRNA target sequence (<i>LipL32 241b</i>)
<i>Leptospira interrogans</i> serovar Lai	TTTGTCTGAGCGAGGACACAATC
<i>Leptospira interrogans</i> serovar Australis	TTTGTCTGAGCGAGGACACAATC
<i>Leptospira interrogans</i> serovar Autumnalis	TTTGTCTGAGCGAGGACACAATC
<i>Leptospira interrogans</i> serovar Copenhageni	TTTGTCTGAGCGAGGACACAATC

<i>Leptospira spp.</i>	crRNA target sequence (<i>LipL32 90b</i>)
<i>Leptospira interrogans</i> serovar Lai	TTTCACTACCTATAAACCAGGTGA
<i>Leptospira interrogans</i> serovar Australis	TTTCACTACCTACAACCAGGTGA
<i>Leptospira interrogans</i> serovar Autumnalis	TTTCACTACCTACAACCAGGTGA
<i>Leptospira interrogans</i> serovar Copenhageni	TTTCACTACCTACAACCAGGTGA

<i>Leptospira spp.</i>	crRNA target sequence (<i>SecY</i>)
<i>Leptospira interrogans</i> serovar Lai	TTTGAAAAAATACGGTGGGTTTCAT
<i>Leptospira interrogans</i> serovar Australis	TTTGAAAGGAATAGATTGACTCTT
<i>Leptospira interrogans</i> serovar Autumnalis	TTTGAAAAAATACGGTGGGTTTCAT
<i>Leptospira interrogans</i> serovar Copenhageni	TTTGAAAAAATACGGTGGGTTTCAT

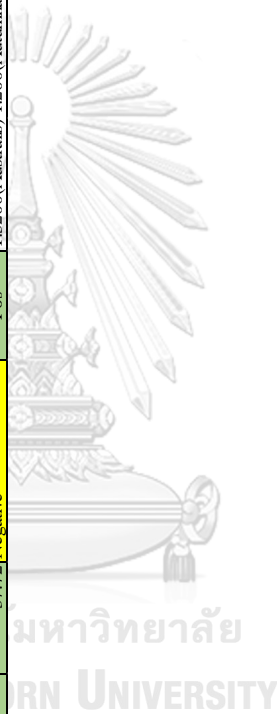
<i>Leptospira spp.</i>	crRNA target sequence (<i>fb1</i>)
<i>Leptospira interrogans</i> serovar Lai	TTTCTCCCAATCTACTGTCAAAG
<i>Leptospira interrogans</i> serovar Australis	TTTCTCCCAATCTACTGTCAAAG
<i>Leptospira interrogans</i> serovar Autumnalis	TTTCTCCCGAATCTAGAGAAAGAA
<i>Leptospira interrogans</i> serovar Copenhageni	TTTCTCCCAATCTACTGTCAAAG



Appendix Figure 1. The cDNA target sequence comparison between *leptospira interrogans*.

Appendix Table 2. The MAT results. The most common serovars found in this study by MAT (a single titer of $\geq 1:400$, or 4-fold rising titer) were Shermani, Autumnalis, Australis, Sejroe, Mini, Panama, Cynopteri and Louisiana.

No.	ID	DoF	MAT	qPCR	RPA-CRISPR/Cas12a	IgM RDT	MAT serology test (a single titer of $\geq 1:400$, or 4-fold rising titer)
13	RLSS-075	2	Positive	31.47	Positive	neg	1:100 (Autumnalis) 1:200 (Australis and Sejroe)
28	RLSS-079	2	Positive	32.09	Positive	Pos	1:100 (Autumnalis and Panama) 1:200 (Australis, Louisiana and Sejroe) 1:3200 (Shermani)
34	RLSS-047	1	Positive	33.22	Positive	Pos	1:400 (Shermani)
47	RLSS-195	5	Positive	35.82	Positive	Pos	1:200 (Autumnalis) 1:100 (Louisiana) 1:200 (Semarang)
51	RLSS-117	4	Positive	33.38	Positive	Pos	1:400 (Australis) 1:100 (Autumnalis) 1:200 (Djasiran) 1:800 (Shermani)
55	RLSS-172	7	Positive	32.5	Positive	Neg	1:3200 (Shermani)
62	RLSS-043	2	Positive	34.93	Positive	Neg	1:200 (Autumnalis) 1:400 (Louisiana, Mini, Panama and Semarang) 1:800 (Shermani) 1:3200 (Australis)
70	RLSS-219	9	Positive	36.01	Positive	neg	1:400 (Australis) 1:100 (Ballum) 1:800 (Shermani)
71	RLSS-076	4	Positive	34.95	Positive	Pos	1:100 (Ballum) 1:200 (Australis, Hebdomadis and Icterohaemorrhagiae) 1:3200 (Sejroe) 1:6400 (Shermani)
72	RLSS-251	5	Positive	32.95	Positive	neg	1:800 (Shermani) 1:1600 (Mini, Panama) 1:3200 (Cynopteri, Louisiana)
10	RLSS-219	9	Positive	37.95	Negative	Pos	1:400 (Australis) 1:100 (Ballum) 1:800 (Shermani)
49	RLSS-199	3	Positive	37.72	Negative	Pos	1:3200 (Australis) 1:200 (Autumnalis) 1:100 (Sejroe) 1:3200 (Shermani)



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