DEVELOPMENT OF REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION COMBINE WITH LATERAL FLOW DEVICE AS A DETECTION ASSAY FOR SWINE INFLUENZA VIRUS

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การพัฒนาวิธี REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ร่วมกับ LATERAL FLOW DEVICE เพื่อตรวจหาเชื้อไวรัสไข้หวัดใหญ่สุกร

น.ส.อัลบาริยาติ อุมมิ อะซีห์ -

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	DEVELOPMENT OF REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION COMBINE WITH LATERAL FLOW DEVICE AS A DETECTION ASSAY FOR SWINE INFLUENZA VIRUS
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อัลบาริยาติ อุมมิ อะซีห์ - : การพัฒนาวิธี REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ร่วมกับ LATERAL FLOW DEVICE เพื่อตรวจหาเชื้อไวรัสไข้หวัดใหญ่สุกร. (DEVELOPMENT OF REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION COMBINE WITH LATERAL FLOW DEVICE AS A DETECTION ASSAY FOR SWINE INFLUENZA VIRUS) อ.ที่ปรึกษาหลัก : อลงกร อมรศิลป์

เชื้อไข้หวัดใหญ่สุกรเป็นไวรัสที่มีอัตราการป่วยสูงแต่อัตราการตายต่ำในสุกร โรคไวรัสชนิดนี้แพร่กระจายอย่างรวดเร็วในสุกร ก่อให้เกิดปัญหาการโตช้าต้องใช้เวลาในการเลี้ยงนานขึ้น นอกจากนี้สุกรยังเป็น“mixing vessel”ของเชื้อไวรัสไข้หวัดใหญ่ชนิดอื่นๆ ทั้งเชื้อไวรัสไข้หวัดใหญ่จากสัตว์ปีก จากคน และจากสุกรเองสามารถเติบโตและแพร่กระจายได้อย่างดีในตัวสุกร ซึ่งทำให้ไวรัสไข้หวัดใหญ่สุกรไม่เพียงแต่ส่งผลกระทบทางเศรษฐกิจแต่ยังมีผลกระทบในทางสาธารณสุขอีกด้วย การตรวจวินิจฉัยไวรัสไข้หวัดใหญ่สุกรจึงควรทำอย่างรวดเร็วและถูกต้องแม่นยำเพื่อลดการแพร่กระจายในกลุ่มประชากรของสุกร อีกทั้งยังเป็นการลดโอกาสที่สุกรจะได้รับไวรัสไข้หวัดใหญ่ชนิดอื่นๆด้วย วัตถุประสงค์ของการศึกษาวิจัยนี้คือการพัฒนาวิธีการ Reverse Transcription Loop-Mediated Isothermal Amplification ร่วมกับ Lateral Flow Device (RT-LAMP-LFD) รวมถึงประเมินค่าความไวและความจำเพาะของวิธีการนี้ การศึกษาวิจัยถูกแบ่งออกเป็น 2 ระยะ โดยระยะแรกคือการพัฒนาวิธีการ RT-LAMP-LFD เพื่อตรวจหาไวรัสไข้หวัดใหญ่สุกรและระยะที่สองคือการประเมินค่าความไวและความจำเพาะของวิธีการ ผลการศึกษาวิจัยแสดงให้เห็นว่าวิธีการ RT-LAMP-LFD ใช้ไพรเมอร์ที่ออกแบบใหม่ โดยเฉพาะจำนวนหกคู่ โดยอุณหภูมิที่เหมาะสมต่อการทำงานอยู่ที่ 63 องศาเซลเซียล เป็นเวลา 30 นาที ความเข้มข้นของเชื้อที่วิธีการนี้สามารถตรวจวัดได้อย่างน้อยที่สุดคือ 1.14 pg/µl ซึ่งดีกว่าวิธีการ real time RT-PCR ถึง 10 เท่า อีกทั้งวิธีการ RT-LAMP-LFD ยังมีค่าความจำเพาะที่สูงมากโดย ไม่มีการทำปฏิกิริยากับไวรัสชนิดอื่นๆ ที่ก่อโรคในสุกร เช่น ไวรัสพีอาร์อาร์เอส เซอร์โคไวรัสชนิดที่สอง ไวรัสพิษสุนัขบ้าเทียม และไวรัสพีอีดี จากการศึกษาวิจัยพบว่าวิธีการ RT-LAMP-LFD นั้นมีค่าความไว 100% และค่าความจำเพาะ 100% อีกทั้งยังมีค่า kappa = 1 เมื่อเปรียบเทียบกับวิธีมาตรฐานอื่น เช่น virus isolation หรือ real time RT-PCR กล่าวโดยสรุปวิธีการ RT-LAMP-LFD เป็นวิธีการที่มีประสิทธิภาพในการตรวจหาไวรัสไข้หวัดใหญ่สุกรได้อย่างรวดเร็วและแม่นยำ อีกทั้งยังสามารถนำไปพัฒนาเป็นชุดตรวจในราคาประหยัดซึ่งใช้ตรวจในหน่วยปฏิบัติงานได้เป็นอย่างดี วิธีการนี้จึงเหมาะสมที่จะถูกนำไปใช้ในการรับมือกับการระบาดของไวรัสไข้หวัดสุกรเพื่อช่วยในการควบคุมและป้องกันโรคในฟาร์มสุกรต่อไป

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Albarriyati Ummi Asih - : DEVELOPMENT OF REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION COMBINE WITH LATERAL FLOW DEVICE AS A DETECTION ASSAY FOR SWINE INFLUENZA VIRUS. Advisor: Prof. ALONGKORN AMONSIN, D.V.M., Ph.D.

Swine influenza causes high morbidity and low mortality in pigs. The disease spreads rapidly in pig farms causing the delay of pig weight to reach market. Moreover, pigs play important role as “mixing vessel” of influenza viruses. Pigs can support infection and replication of influenza viruses from avian, human and swine origins. These lead to major concerns of the important of swine influenza which are not only the economic impacts but also the human health. There is the need of accurate and rapid diagnosis for swine influenza virus (SIV) to minimize further spreading of the viruses in pig population and to reduce the risk of multiple infections of influenza viruses. The objective of this study was to develop a Reverse Transcription Loop-Mediated Isothermal Amplification combine with Lateral Flow Device (RT-LAMP-LFD) assay for the detection of SIVs and to evaluate the performance, sensitivity and specificity of the assay. This study was conducted in two phases: the development of RT-LAMP-LFD assay for SIVs detection and the evaluation of the performance, sensitivity and specificity of the assay. The result showed that RT-LAMP-LFD assay was developed with six newly designed primers and with optimum condition at 63°C and 30 minutes. The detection limit of the assay was 1.14 pg/µl, which is 10-fold higher than real time RT-PCR assay, RT-LAMP-LFD assay is specific method for the detection of SIV, there were no cross reaction with other important viruses of pigs (PRRSV, PCV2, Pseudorabies virus and PEDV). From our result the newly developed RT-LAMP-LFD assay has good performance with 100% sensitivity, 100% specificity and perfect percentage of agreement (Kappa = 1) compared to reference assays (viral isolation and/or real time RT-PCR). In conclusion, this RT-LAMP–LFD assay has a potential for patent technology as a rapid diagnostic test for SIV detection and could be developed for low-cost or in house kits. The RT-LAMP-LFD assay could be implemented and applied at first point care of SIV outbreaks to help control and prevent the spread of disease in pig farms.

Field of Study:	Veterinary Science and technology	Student's Signature .....
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Albarriyati Ummi Asih -

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CHAPTER I
INTRODUCTION

Swine influenza virus (SIV) or swine-origin influenza virus (S-OIV) causes endemic respiratory disease in pigs. SIV is an influenza A virus of the family *Orthomyxoviridae*. There are three main SIV subtypes circulating in pigs worldwide including SIV-H1N1, H3N2 and H1N2. SIV infection in pigs causes high morbidity rate (probable 100%) while mortality rate is usually low. Clinical signs of SIV infection are mild respiratory problems, which lead to weight loss causing a delayed time needed to fulfill the market weight. These make SIV infection as one of the important hidden causes of economic loss in pig farms (OIE, 2018).

There is a major concern about risk of SIV for human health. Due to pigs play an important role as "a mixing vessel" of influenza viruses, pigs can support infection and replication of influenza viruses of avian, human and swine origins (Brown et al., 1997; Campitelli et al., 1997; Webby et al., 2000; Qi and Lu, 2006). Several studies showed SIV and swine-origin influenza virus (S-OIV) seropositivity in humans especially those who have close contact with pigs (Olsen et al., 2002; Myers et al., 2006; Myers et al., 2007). For example, the pandemic (H1N1) 2009 was first detected in human and it appeared to be a reassortant from at least three parent viruses (avian, pig, human) (Gibbs et al., 2009; Neumann et al., 2009).

There are several methods for influenza A virus detection such as reverse transcription-PCR (RT-PCR) (Ellis and Zambon, 2002), real time RT-PCR (Playford and Dwyer, 2002), multiplex RT-PCR assay (Wu et al., 2008), nucleic acid sequence-based amplification (NASBA) (Collins et al., 2003), mismatch amplification mutation assay (MAMA) (Hata et al., 2007), and DNA/RNA microarray (Dawson et al., 2007). However, those methods require expensive equipment and skilled technicians, thus they do not suitable in the field-setting or in laboratories of developing countries (Ge et al., 2013). Another disadvantage of PCR is a false positive due to DNA/RNA contamination during PCR procedure. Moreover, PCR assay need prior sequence data to design and synthesize the primers for PCR reaction (Smith and Osborn, 2009). Thus, the rapid and accurate assay and adaptable for field application for the detection of influenza A virus especially swine influenza is necessary.

Loop-mediated isothermal amplification (LAMP) has widely used and employed in isothermal amplification research (Parida et al., 2008). LAMP is a highly specific and sensitive method operated under constant temperature between 60 – 65° C, within 30-45 minutes. It is an efficient technique and can be process with less equipment (only water bath) (Notomi et al., 2000). Combined with reverse transcription reaction (RT), RT-LAMP assay has been used for detection of several influenza subtypes such as H5, H7, H9 and H10 (Poon et al., 2005; Imai et al., 2006; Ito et al., 2006; Chen et al., 2008; Zhang et al., 2013; Luo et al., 2015). The interpretation of RT-LAMP assay

can be performed in many ways, by measure the turbidity, by gel electrophoresis, by visual detection of fluorescence from the integration with SYBR Green intercalating dye or by lateral flow device (Mori et al., 2004; Chen et al., 2010a; Ge et al., 2013).

Lateral flow device (LFD) is one of the rapid and easy to interpret assays. Briefly, LFD dipstick can detect biotinylated LAMP-amplicons which hybridized with fluorescent amidite (FAM)-labeled DNA probe. Hybridized LAMP product and gold-labeled anti FAM antibody on conjugated pad of the LFD can form complexes as signal or color on test line. Non-target products could not form complexes, therefore signal or color could not be observed (Yongkiettrakul et al., 2014; Mallepaddi et al., 2018). Several studies have proven the combination of LAMP assay with LFD to be easy and field-capable (Ge et al., 2013; Khunthong et al., 2013; Yongkiettrakul et al., 2014). In this study, RT-LAMP-LFD assay will be developed for rapid and sensitive detection of swine influenza virus. Newly developed RT-LAMP-LFD will be a simple rapid assay and suitable for the use in-farm setting or field application in the future.

Objectives of study

1. To develop a RT-LAMP-LFD assay for the detection of swine influenza viruses.
2. To evaluate the performance, sensitivity and specificity of newly developed RT-LAMP-LFD for swine influenza virus detection.

CHAPTER II
LITERATURE REVIEW

Swine influenza virus (SIV)

Swine influenza virus (SIV) or swine-origin influenza virus (S-OIV) belongs to the genus influenza A virus (Alphainfluenza) of the family *Orthomyxoviridae*. Influenza A virus is a single stranded enveloped RNA virus with segmented genome. The viruses can be classified into subtypes based on two major surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA). Currently, there are 18 HA and 11 NA subtypes (Tong et al., 2013). The most common influenza A subtypes in pigs are H1N1, H1N2 and H3N2. Other subtypes that have been rarely found in pigs are H1N7, H3N1, H4N6 and H9N2 (Xu et al., 2004; Kothalawala et al., 2006).

It has been known that pig is a "mixing vessel" for influenza viruses. Since pig possess both receptors for avian influenza virus (α 2-3-linked sialic acids) and human influenza virus (α 2-6-linked sialic acids), pig can support infection and replication of influenza viruses of avian, human and swine origins (Brown et al., 1997; Campitelli et al., 1997; Webby et al., 2000; Qi and Lu, 2006; Peiris et al., 2009). For example, the pandemic (H1N1) 2009 was first detected in human and it appear to be a reassortant from at least three parent viruses (avian, pig, human) (Gibbs et al., 2009; Neumann et al., 2009). There are many reports showed that there are S-OIV and SIV seropositivity

in humans especially those who have close contact with pigs (Olsen et al., 2002; Myers et al., 2006; Myers et al., 2007).

Swine Influenza

Swine influenza is an endemic respiratory disease in pigs that highly contagious within the herd. The disease can quickly spread to all ages of pigs but rapid recovery (OIE, 2018). The clinical signs of swine influenza in pigs are fever, sneezing, coughing, lethargy, weight loss and some cases of abortion. However, the abortion appears as a result of high fever from the infection not as direct cause from the virus. If the infection is in enzootic form, the symptoms may be mild and often need early diagnostic testing (Janke, 2000). The morbidity rate due to SIV infection can reach to 100% but mortality rate is usually low. The main economy impact of SIV infection is loss weight gain resulting in a delay of times needed to fulfill the market weight (Kothalawala et al., 2006).

Detection assays for swine influenza

Swine influenza virus (SIV) causes concern to human health and impact in economic loss of swine industry. Early and accurate detection of SIV is necessary. There are several assays for the detection of influenza A viruses, such as PCR based detection:

reverse transcription-PCR (RT-PCR) (Ellis and Zambon, 2002), real time RT-PCR (Playford and Dwyer, 2002), multiplex RT-PCR assay (Wu et al., 2008), nucleic acid sequence-based amplification (NASBA) (Collins et al., 2003), mismatch amplification mutation assay (MAMA) (Hata et al., 2007), and DNA/RNA microarray (Dawson et al., 2007). Isothermal amplification such as Loop mediated isothermal amplification (LAMP) (Notomi et al., 2000), Recombinase Polymerase Amplification (RPA) (Piepenburg et al., 2006), and Polymerase cross-linking spiral reaction (PCLSR) (Wozniakowski et al., 2017). PCR based methods are highly sensitive which slightly laboratory contamination can lead to false positive in post-analysis handling. Nevertheless, PCR based methods are time consuming in post-visualization and need expensive equipment (Parida et al., 2008).

Real time RT-PCR is one of the methods that acknowledged by World Health Organization (WHO) for the detection of pandemic swine-origin H1N1 infection. This real time RT-PCR used probes and four sets of primers for the detection of universal influenza A, swine H1 (new H1N1) and swine influenza A. Real time RT-PCR is rapid, has high sensitivity and specificity. However real time RT-PCR has limitation due to its expensive primers, probes and equipment (Parida et al., 2011). Thus, make real time RT-PCR not suitable for routine use in the field and or in-farm setting. Therefore, it is needed to develop simple, economical and accurate detection assay for SIV.

Table 1. Comparison of detection methods

Criteria	Method				
	LAMP[a]	RPA[b,d]	PSR[c]	PCR[a]	Real time-qPCR[d]
Preferred amplicon	RNA, DNA	DNA	DNA	RNA, DNA	RNA, DNA
Amplicon type	Various sized amplicons	RNA, DNA	Spiral structure	DNA	DNA
Reaction	Isothermal reaction	Isothermal reaction	Isothermal reaction	Cyclic reaction	Cyclic reaction
Tool requirement: thermocycler	Doesn't require	Doesn't require	Doesn't require	Require	Require
Temperature (°C)	60-65	37-42	61-65	Variable temperature	Variable temperature
Pre-heating	Doesn't require	Doesn't require	Doesn't require	Require	Require
Primers	4-6	2	2	2	2
Amplification specificity	Higher (use 4/6 oligonucleotides)	Lower	Lower	Lower	Lower
Visualization	Visual color, agarose gel electrophoresis, turbidimeter, LFD	Agarose gel electrophoresis, LFD	Visual color, agarose gel electrophoresis	Agarose gel electrophoresis	Real time analysis

[a] LAMP: Loop-mediated isothermal amplification (Notomi et al., 2000; Nagamine et al., 2002)

[b] RPA: Recombinase polymerase amplification (Piepenburg et al., 2006)

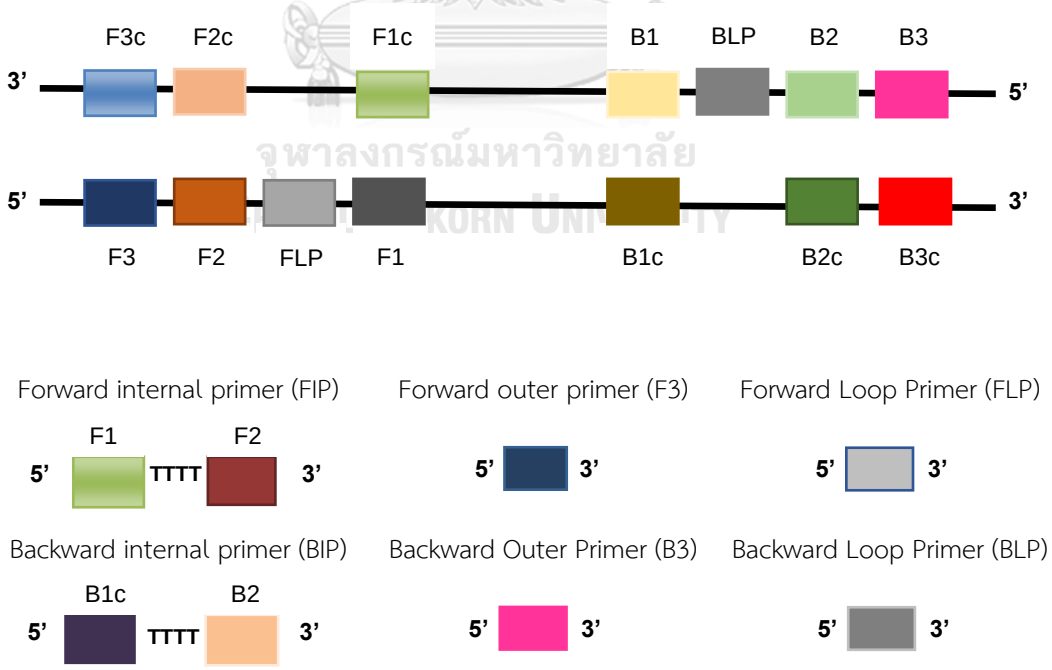
[c] PSR: Polymerase spiral reaction (Liu et al., 2015)

[d] (Aebischer et al., 2014)

Loop-mediated isothermal amplification (LAMP) for swine influenza

Loop-mediated isothermal amplification (LAMP) is a nucleic acid amplification method that widely used and employed in isothermal amplification research (Parida et al., 2008). Unlike PCR that used steps of temperature, LAMP conducts in constant temperature between 60° – 65°C, make it possible to apply in simple water bath (Ge et al., 2013). LAMP uses 4 – 6 primers that recognized 6 – 8 distinct regions of target gene (Figure 1). The primers consist of two internal primers (FIP and BIP), two outer primers (F3 and B3) and two additional primers (Loop-F and Loop-B) (Nagamine et al., 2002).

Figure 1. Schematic of 6 primers (FIP, BIP, F3, B3, FLP and BLP) for LAMP and their position spanning in target DNA sequence



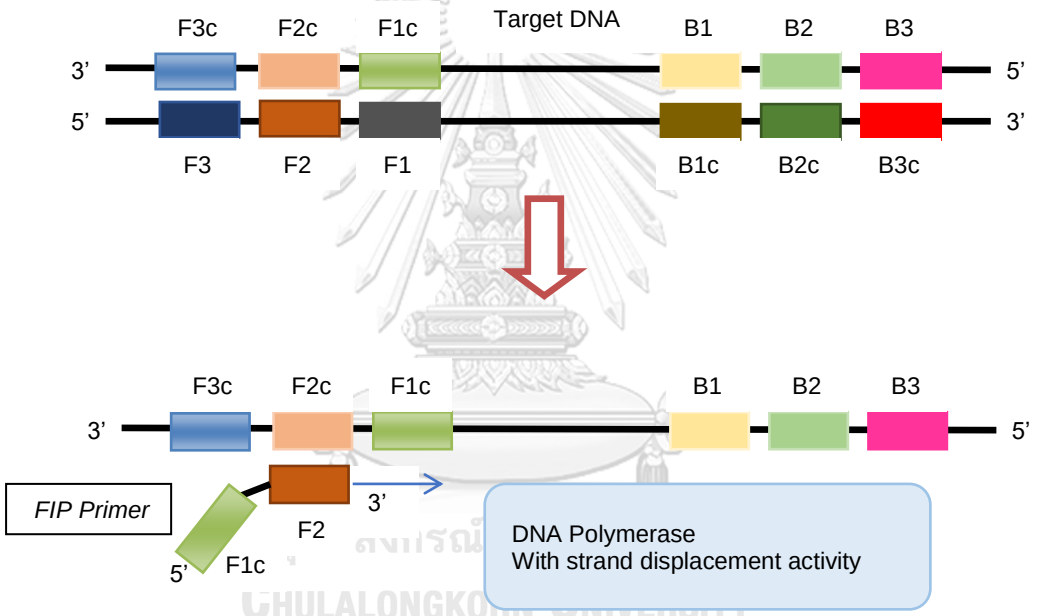
In Figure 1, the forward internal primer (FIP primer) consists of complementary sequence of F1 (F1c) and a sense sequence of F2, similar to the backward internal primer (BIP primer) consists of B1c and B2. During LAMP amplification process, the internal primers can develop loop structure. The forward loop primer (FLP) is a complementary sequence of region between F1 and F2 and backward loop primer (BLP) is a complementary sequence of region between B1 and B2. The position of loop primers add starting points of DNA synthesis which help to increase rapidity in LAMP amplification (Parida et al., 2008).

The principle of LAMP amplification is based on strand displacement activity of DNA polymerase enzyme at a constant temperature. LAMP amplification includes *Bst* DNA Polymerase and four to six primers (two internal primers, two outer primers and/or two loop primers). The LAMP assay can be conducted within 30 to 60 minutes (Notomi et al., 2000; Nagamine et al., 2002). LAMP amplification consists of two steps: non cyclic step and cyclic step (Parida et al., 2008).

A. Non cyclic step of LAMP amplification

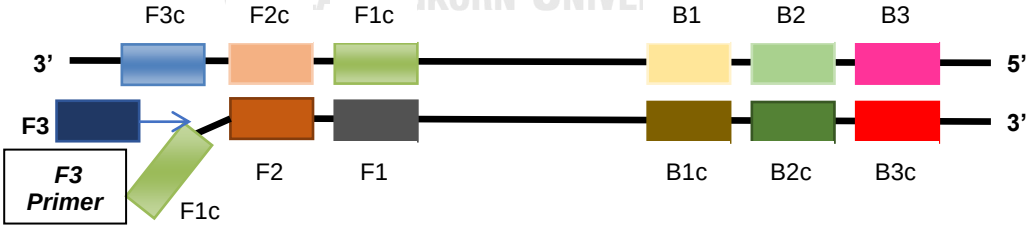
In non cyclic step, four specific primers (two internal primers and two outer primers) targeting six region of DNA target are annealing and then producing dumbbell-liked DNA-form. This dumbbell-liked DNA form acts as starting structure in next cyclic step amplification. In detail, when the DNA template and the reagents are incubated at a constant temperature between 60 – 65^{o} C, the following reaction steps proceed:

Step 1. In LAMP method, there is no need for heat denaturation of the double stranded One of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, then initiates DNA synthesis using DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA. The FIP primer then anneals to such released single stranded DNA.

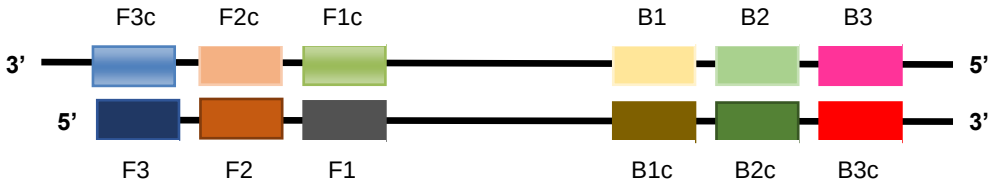


Step 2. Starting from the 3’ end of F2 region of FIP and with strand displacement activity of DNA polymerase, a DNA strand complementary to the template DNA is synthesized.

Step 3. The F3 primer anneals to the F3c region on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand. Step 3 will produce double strand (Step 4) and FIP-linked complementary strand (Step 5).

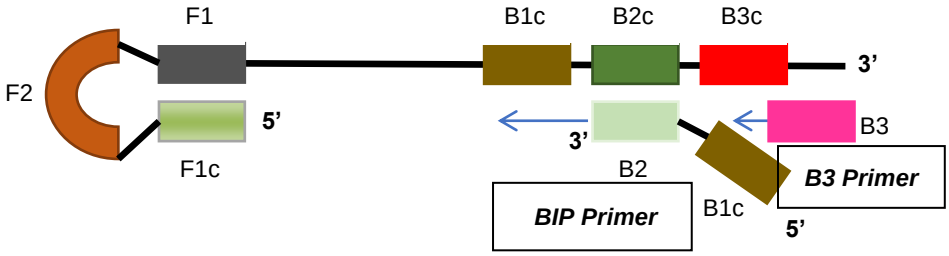


Step 4. From Step 3, a double strand is formed from the F3 primer and the template DNA strand.

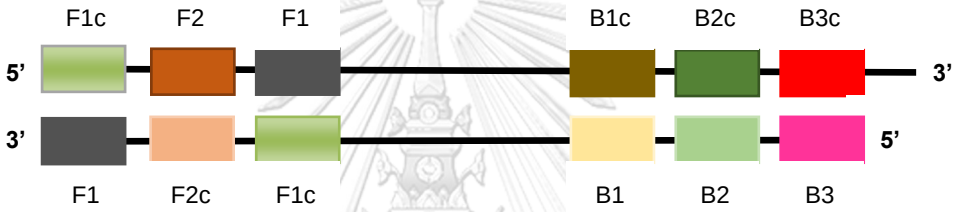


Step 5. The FIP-linked complementary strand release as a single strand from the displacement by the DNA strand synthesized from the F3 primer. Then, this released single strand can forms a stem-loop structure at 5' end because of the complementary F1c and F1 regions.

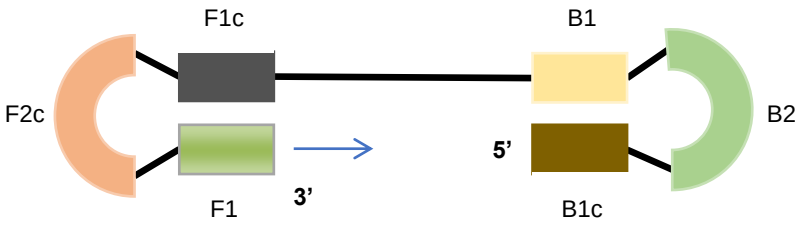
Step 6. The single strand DNA in Step 5 serves as a template for the BIP primer to initiates DNA synthesis. Starting from the 3' end of the BIP, synthesis of complementary DNA takes place. Through this process, the DNA reverts from a loop structure into a linear structure. The B3 Primer anneals to the outside of the BIP and then, through the activity of the DNA polymerase and starting at the 3' end, the DNA synthesized from the BIP is displaced and released as a single strand before DNA synthesis from the B3 Primer.



Step 7. Double stranded DNA is produced through the processes described in Step (6).



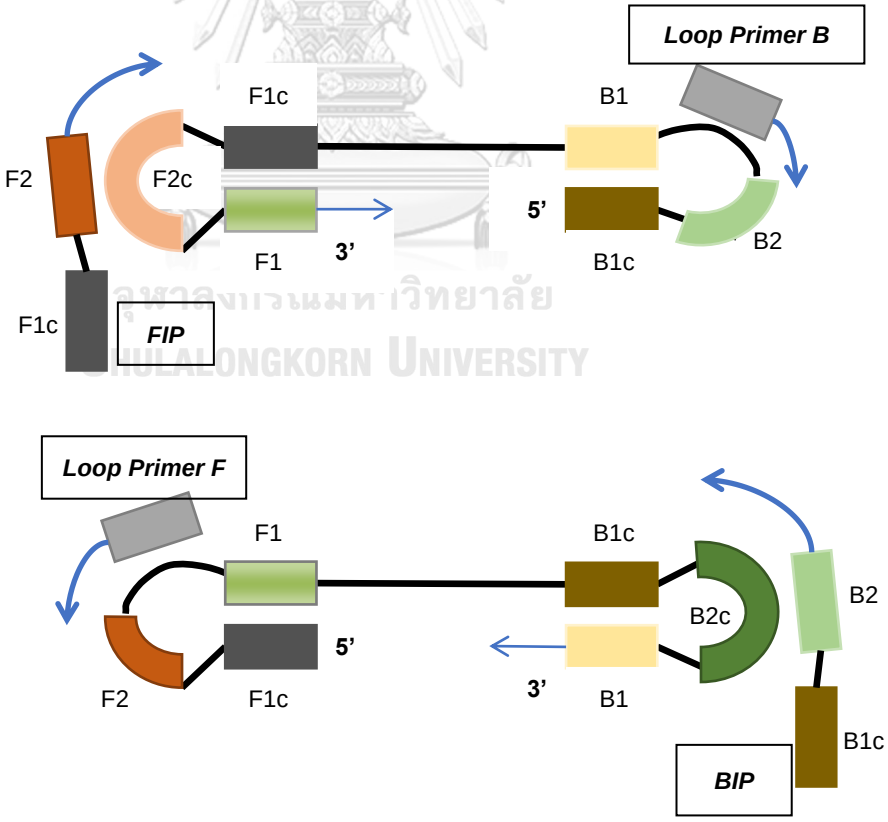
Step 8. The BIP-linked complementary strand displaced in Step (6) forms a structure with stem-loops at each end, which looks like a dumbbell structure. This structure serves as the starting structure for the amplification cycle in the LAMP method (cyclic step of LAMP amplification).



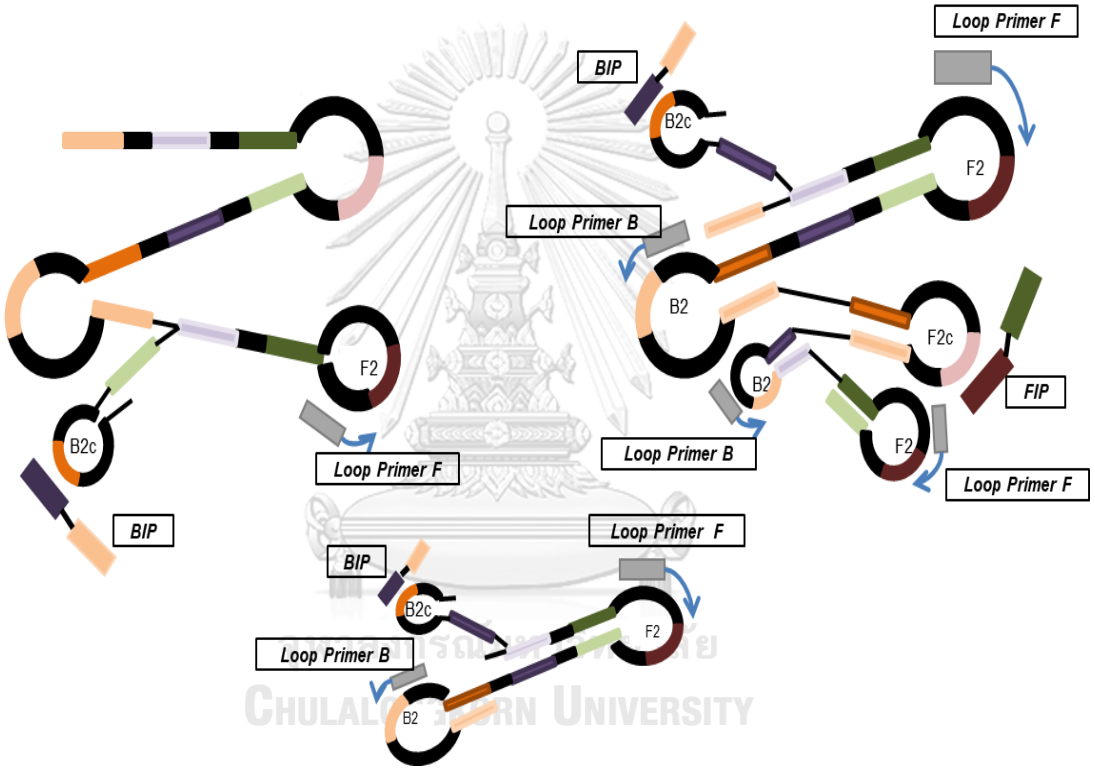
B. Cyclic step of LAMP amplification

The dumbbell-liked DNA-form continuously generates in cyclic step and follow with elongation step. Resulting in various sizes of LAMP amplicons and can be observed as a ladder in agarose gel electrophoresis or called “ladder pattern”.

Step 9. The Loop Primers (either Loop Primer F or Loop Primer B), containing sequences complementary to the single stranded loop region (either between the F1 and F2 regions, or between the B1 and B2 regions) on the 5' end of the dumbbell-like structure, provide an increased number of starting points for DNA synthesis.



Step 10. The loop primers (Loop F and Loop B primers) for the LAMP method are required for enhancing the speed and specificity of the LAMP reaction. As example in the following picture which there is an amplified product containing six loops. Through the use of Loop primers, all the single stranded loops can be used as starting points for DNA synthesis.



LAMP can be combined with reverse transcription enzyme for RNA amplification or called reverse transcription LAMP (RT-LAMP) (Notomi et al., 2000). RT-LAMP assay has been used for detection of several influenza virus subtypes H5, H7, H9 and H10 (Poon et al., 2005; Imai et al., 2006; Ito et al., 2006; Chen et al., 2008; Zhang et al., 2013; Luo et al., 2015). Comparing to real time RT-PCR, RT-LAMP has advantages in

simplicity regarding to the reaction process, and sensitivity in detection and cost of operation (Nagamine et al., 2002; Parida et al., 2011).

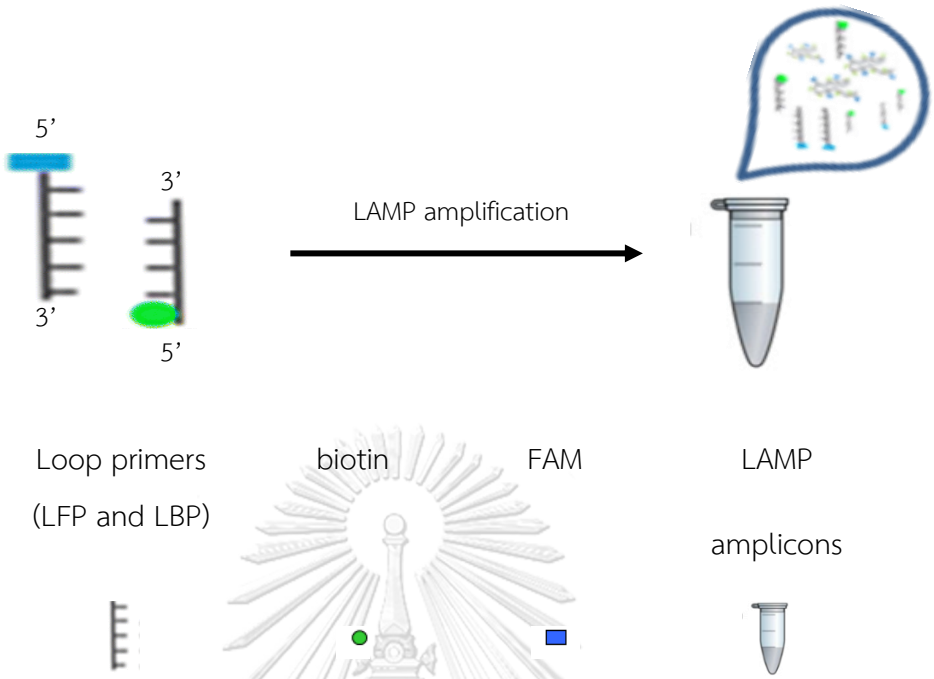
In previous study, RT-LAMP assay for SIV detection have been developed for H3 subtype (Gu et al., 2010), pandemic (H1N1) 2009 (Kubo et al., 2010), swine origin influenza A H1N1 (Parida et al., 2011), and classical swine fever (Chen et al., 2010b), The result showed that RT-LAMP assay has high sensitivity and specificity. Gu et al., in 2010 revealed that RT-LAMP have 100-fold more sensitive than conventional PCR in the detection of SIV-H3.

There are several ways to interpret RT-LAMP result: by gel electrophoresis staining with Redsafe™, by combine magnesium pyrophosphate with LAMP product resulting in white precipitates, or by using SYBR Green intercalating dye integrated with LAMP product resulting in fluorescence measurement (Notomi et al., 2000; Mori et al., 2001). Recently, lateral flow device (LFD) has been developed and can be combined with LAMP assay. LAMP-LFD visualization makes this method more applicable in field setting with excellent sensitivity and specificity (Ge et al., 2013; Yongkiettrakul et al., 2014). LAMP-LFD has advantages to speed up the total time needed for detection comparing to LAMP assay.

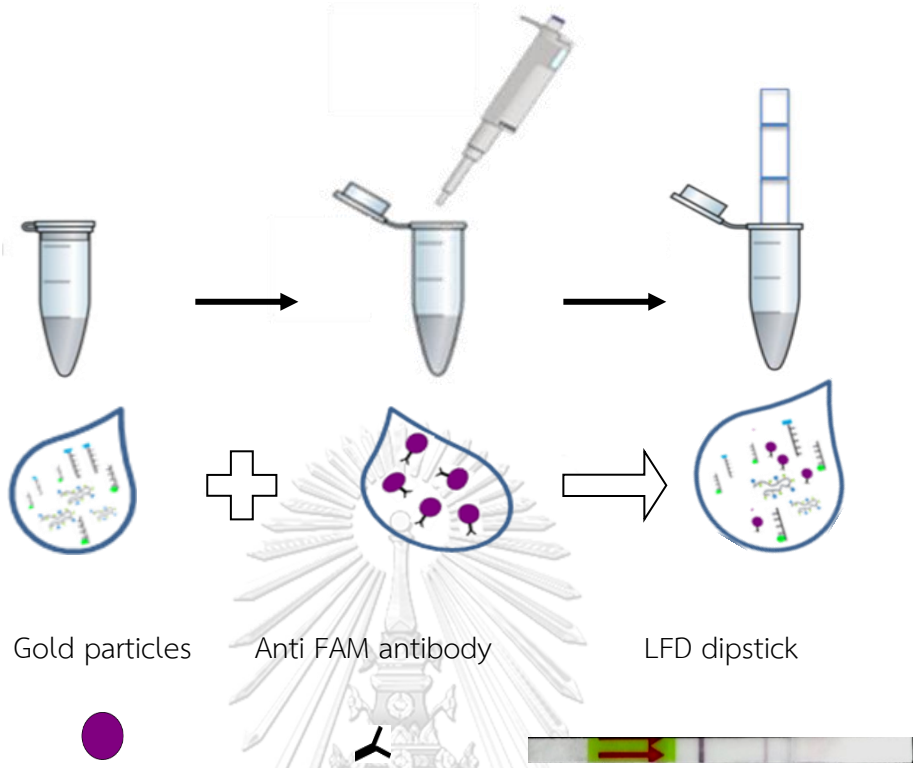
Detection of swine influenza virus by LAMP combined with lateral flow device (LFD)

The LFD dipstick (Milenia Genline HybriDetect) is developed based on lateral flow technology using gold particles. To combine this LAMP assay with LFD, first the LAMP amplicons containing two detectors labeled with fluorescein amidite (FAM) and biotin are developed (Mallepaddi et al., 2018). The integrated biotinylated LAMP-amplicons can hybridize with FAM-labeled DNA probes. This hybridized LAMP product and gold-labeled anti FAM antibody on conjugated pad of the LFD can form complexes as signal or color on test line. On the other hand, non-target LAMP products could not form complexes, thus signal or color on test line could not be observed (Yongkiettrakul et al., 2014; Mallepaddi et al., 2018). The principle of LAMP-LFD assay can be explains by the following steps:

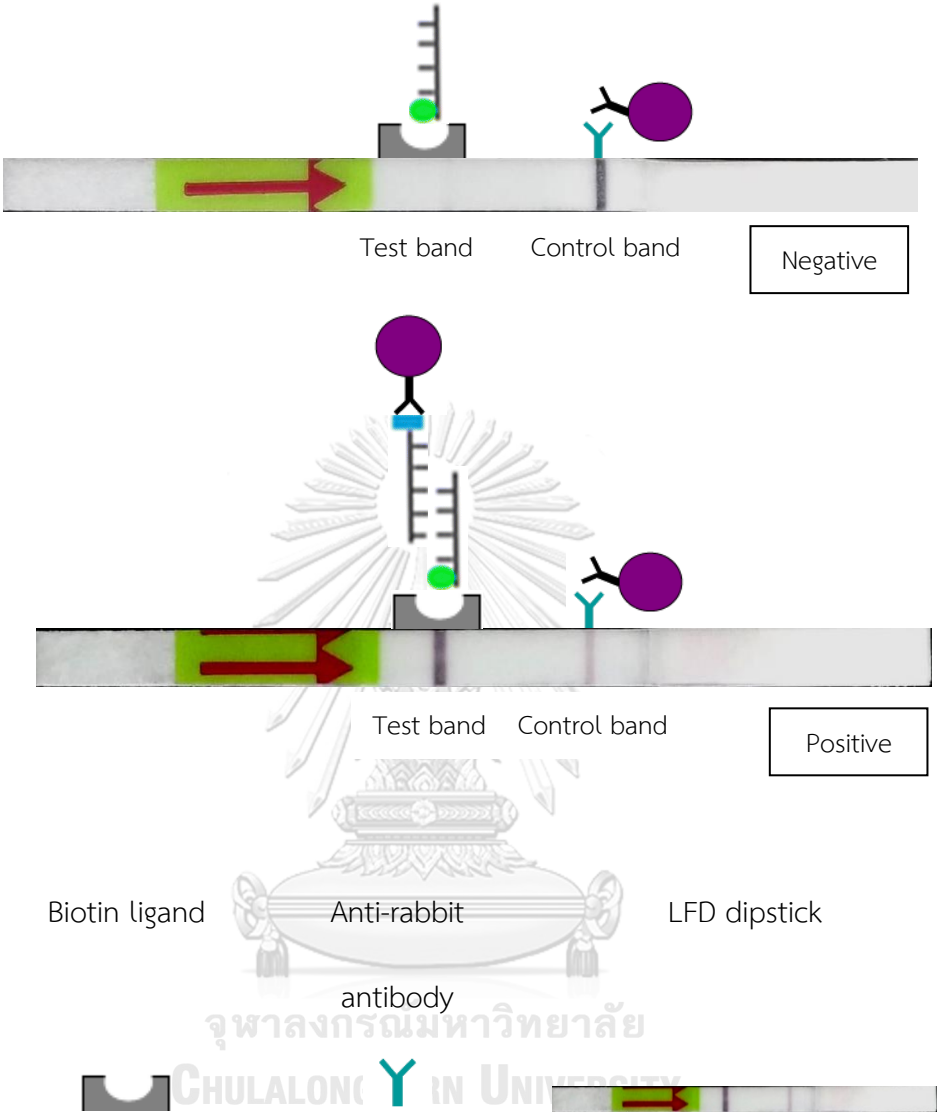
Step 1. To combine this LAMP assay with LFD, first the LAMP amplicons containing two detectors labeled with fluorescein amidite (FAM) and biotin needs to be developed. In this study, the two detectors are the loop primers (loop F and loop B). FAM and biotin are tagged at 5’ end of loop primers respectively. Through LAMP amplification, the primers will amplify the target sequences and produce LAMP amplicons labeled with FAM and biotin.



Step 2. Pipet 5-10 µl of LAMP amplicons into tube and add 100 µl of HybriDetect Assay Buffer. This buffer contains gold particles and anti FAM antibody. Gold particles have function in giving color as a visible line in LFD dipstick. Place the LFD dipstick with the sample application area into the solution and incubate for 5 – 15 minutes in an upright position.



Step 3. The LAMP amplicons, labeled with FAM and biotin, will bind first to the gold particles labeled with anti-FAM antibody. Then, the complexes diffuse over the membrane through capillary of LFD. Only the LAMP amplicons-gold particles complexes will bind with biotin ligand at test line and generate a red-blue line over the time. Not-captured gold particles flow over to the control band and will be captured by anti-rabbit antibodies. With increasing incubation time, the formation of an intensely colored control line will appear. In case of very high concentrations of hybridizations product, control line's intensity may be affected. Nevertheless, the control line should be still visible clearly.

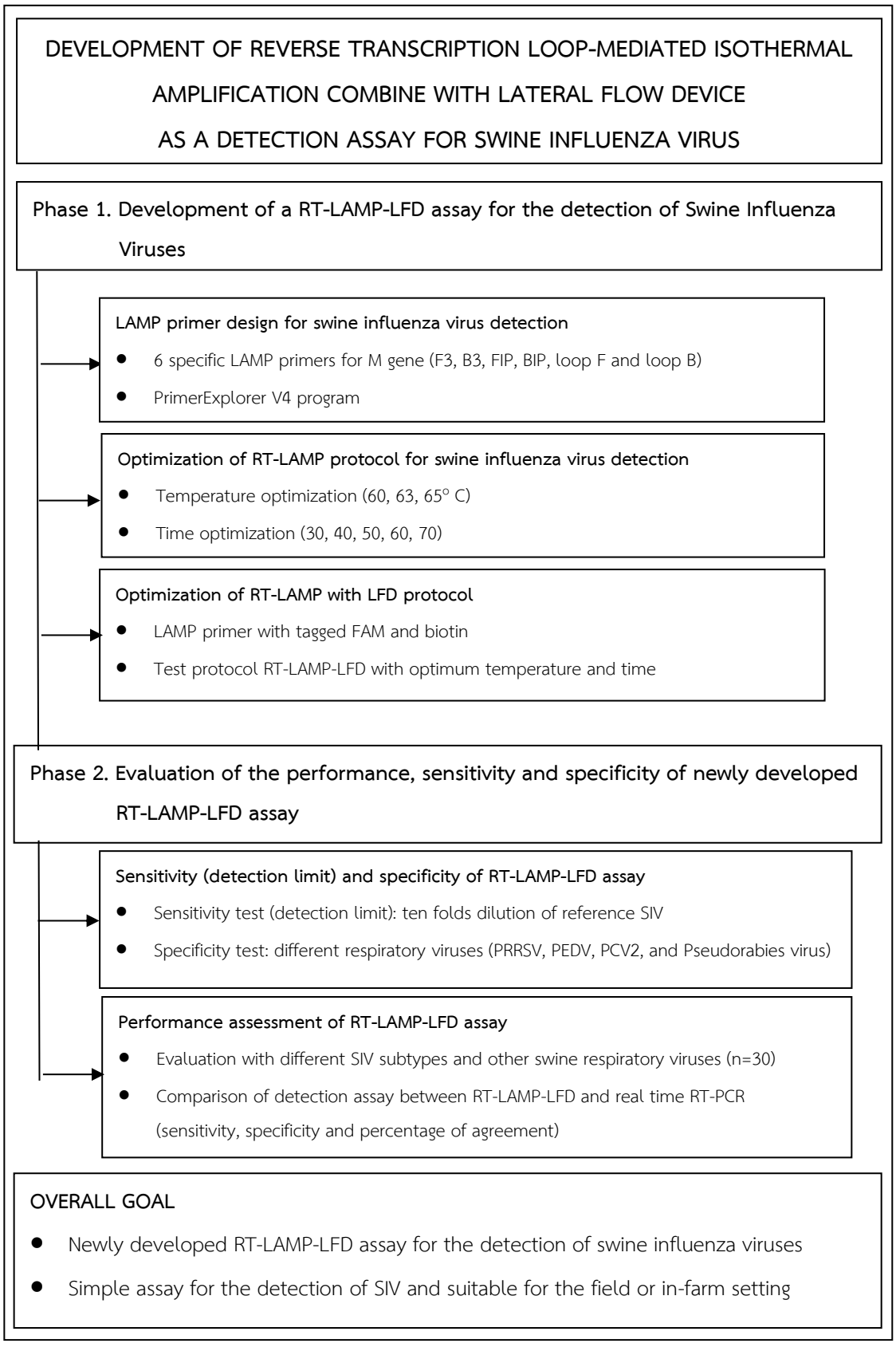


The combination of RT-LAMP and LFD have been developed for the detection of *Rhizoctonia solani* (Patel et al., 2015) and malaria (Mallepaddi et al., 2018). With the combination of RT-LAMP and LFD, a rapid, sensitive and accurate assay will be achieved without the need of expensive equipment. RT-LAMP-LFD is suitable and applicable in field or in-farm setting.

CHAPTER III
MATERIALS AND METHODS

This study consists of 2 phases including: Phase 1. Development of RT-LAMP-LFD assay for the detection of swine influenza viruses; Phase 2. Evaluation of the performance, sensitivity and specificity of newly developed RT-LAMP-LFD assay. The conceptual framework of this study is shown in Figure 2.

Figure 2. Conceptual framework of this study



Phase 1. Development of RT-LAMP-LFD assay for the detection of swine influenza viruses.

SIV-H1N1 was used as template in Phase 1. Template was received from previous study by the Laboratory of Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University and has previously identified by viral isolation (egg inoculation) and real time RT-PCR. The development of a RT-LAMP-LFD assay was performed as the following steps:

1.1 LAMP primer design for swine influenza virus detection

In this study, 6 primers (F3, B3, FIP, BIP, loop F, and loop B) were designed for specific detection of swine influenza virus based on M gene. In brief, reference sequences of M gene of SIVs were obtained from GenBank database to generate the consensus of M gene sequences suitable for primer design. All the nucleotide sequences were aligned by MegAlign program and conserved regions (approximately 200 base pairs) were determined to use as target for primer design.

The LAMP primers consist of six specific primers including two internal primers (FIP and BIP), two outer primers (F3 and B3) and two loop primers (loop F and loop B) were designed. The primer design was performed by using PrimerExplorer V4 program with criteria as following: the Tm of primers were about 57°C (55 - 59°C) for F3 and B3, about 63°C (60 – 65°C)

for loop F and loop B primers, about 80°C (78 - 82° C) for FIP and BIP primers. The free energy (ΔG) was -4 kcal/mol or less and the GC content was between 40 – 60%. In this study, the set of primers was selected and subjected to optimization in step 1.2.

1.2 Optimization of RT-LAMP protocol for swine influenza virus detection

1.2.1. Optimization of RT-LAMP protocol by temperature variation

The RT-LAMP assay was performed in a total of 25 µl of reaction mixture containing 10 µM each of the internal primers (FIP and BIP), 10 µM each of the outer primers (F3 and B3), 25 µM each of loop primers (Loop F and Loop B), 10X ThermoPol buffer, 0.4 mM dNTPs, 8 mM $MgSO_4$, 5M Betaine, 8U *Bst* DNA Polymerase, 10X AMV buffer, 10 U AMV reverse transcriptase, 5.15 µl H_2O and 2 µl of template RNA. Free nuclease water was used as negative control in this assay. Optimization of RT-LAMP protocol was conducted by variation of temperature. Temperature for RT-LAMP was conducted with three different values (60°C, 63°C, and 65°C) for 45 minutes and inactivates reaction at 80°C for 5 minutes.

1.2.2. Optimization of RT-LAMP protocol by time variation

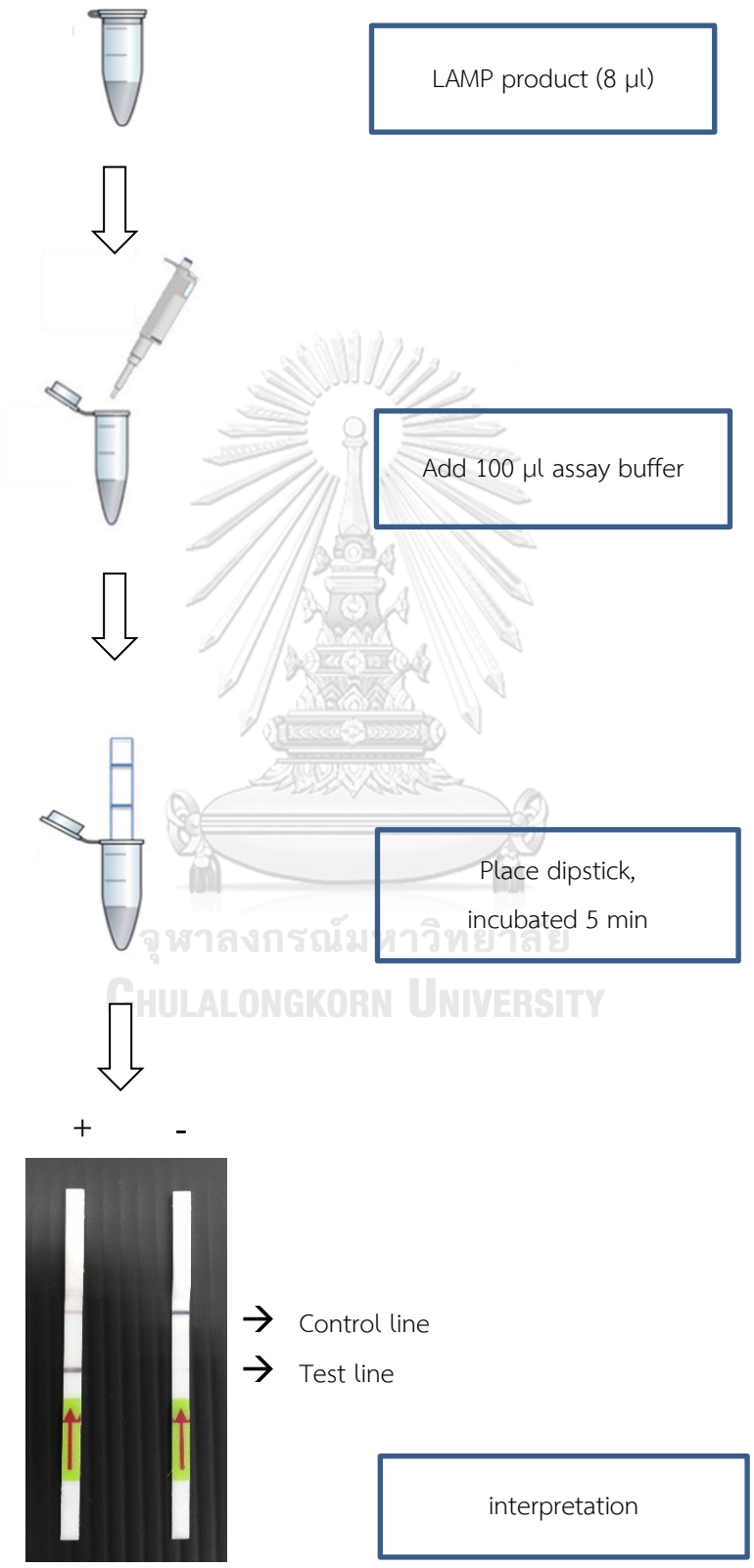
After the optimum temperature was obtained, time variation for RT-LAMP was conducted with 30, 40, 50, 60, 70 minutes respectively. The

visualization was done by gel electrophoresis with Redsafe™ stain. The best optimized temperature and time was selected for step 1.3.

1.3 Optimization of RT-LAMP with LFD protocol for swine influenza virus detection

In order to combine RT-LAMP with LFD, primers loop F and loop B were tagged with biotin and fluorescein amidite (FAM) at 5'end respectively. With this labeled loop F and B, the LAMP products could be detected by lateral flow device (LFD). RT-LAMP-LFD assay was performed with optimum temperature and time from step 1, 2. The LFD is commercially used acquired from Milenia® Genline, Germany. Briefly, the sample (8 µl) was mixed with 100 µl of HybriDetect Assay Buffer. Then the dipstick with the sample application area is placed into the solution and incubated for 5 – 10 minutes. At the end of incubation period, the dipstick could be removed from the assay solution and interpret the results immediately. For LFD interpretation positive result was formed two red lines (control line and test line) and negative result was shown only one red line (control line) (Figure 3).

Figure 3. LFD dipstick application and interpretation



Phase 2. Evaluation of the performance, sensitivity and specificity of newly developed RT-LAMP-LFD assay.

Evaluation of the performance, sensitivity and specificity of RT-LAMP-LFD was performed as following steps:

2.1 Evaluation of sensitivity and specificity of RT-LAMP-LFD assay

2.1.1 Sensitivity of RT-LAMP-LFD assay

Analytical sensitivity or another term “the detection limit” of RT-LAMP-LFD assay was assessed using tenfold serial dilution of reference SIV (H1N1 subtype). The viral RNA was diluted using RNAase free water. RT-LAMP-LFD assay was performed in each serial dilution. The result was interpreted as the strength of RT-LAMP-LFD assay to detect SIV. The sensitivity of RT-LAMP-LFD assay was compared with real time RT-PCR.

2.1.2 Specificity of RT-LAMP-LFD assay

The specificity of RT-LAMP-LFD assay was assessed against other respiratory swine viruses (PRRSV, PEDV, PCV2 and Pseudorabies virus). There was one isolates of each viruses and has been previously identified by PCR conventional and/or real time RT-PCR. The RT-LAMP-LFD assay was evaluated by testing RNA of

each virus. The result was interpreted as the specific of RT-LAMP-LFD assay to differentiate SIV from other swine viruses.

2.1 Performance assessment of RT-LAMP-LFD

To evaluate the performance of RT-LAMP-LFD assay or another term ‘diagnostic sensitivity and specificity”, the result of RT-LAMP-LFD was compared to the real time RT-PCR assay. In this study, 30 samples of different types of viruses (SIV, PRRSV, PEDV and Pseudorabies virus) were randomly blinded to the researchers. With details: 16 isolates of SIV, 5 isolates of PRRSV, 5 isolates of PEDV, 1 isolates of Pseudorabies virus and 3 RNAase free water. All samples have previously identified by viral isolation (egg inoculation) and/or real time RT-PCR. The samples were then tested with RT-LAMP-LFD and real time RT-PCR assay, to assess the performance of both assays. The results were un-blinded and compared by two-by-two table with gold standard test (Table 2).

Table 2. Two-by-two table used for diagnostic sensitivity and specificity calculation (Kanchanaraksa, 2008)

		Gold standard test result (as reference test)	
		True Positive	True Negative
RT-LAMP LFD result (developed test)	Positive	*a* *(tested positive)*	*b* *(false negative)*
	Negative	*c* *(false positive)*	*d* *(tested negative)*

Sensitivity and specificity of RT-LAMP-LFD were calculated with following formula:

$$\textit{Diagnostic sensitivity} = \frac{tested\ positive}{tested\ positive + false\ negative} = \frac{a}{a+c}$$

$$\textit{Diagnostic specificity} = \frac{tested\ negative}{tested\ negative + false\ positive} = \frac{d}{b+d}$$

The agreement of the RT-LAMP-LFD assay with gold standard was assessed by using Kappa value, with following calculation:

$$\textit{Kappa} = \frac{Observe\ agreement - Expected\ agreement}{1 - Expected\ agreement}$$

Where:

$$\textit{Observe agreement} = \frac{a+d}{a+b+c+d}$$

$$\textit{Expected agreement} = \frac{Expected(a)+Expected(d)}{a+b+c+d}$$

$$\textit{Expected (a)} = \frac{(a+b)X(a+c)}{a+b+c+d}$$

$$\textit{Expected (d)} = \frac{(b+d)X(c+d)}{a+b+c+d}$$

CHAPTER IV
RESULT

Phase 1. Development of RT-LAMP-LFD assay for the detection of swine influenza viruses

In this study, the RT-LAMP-LFD assay was developed by designing the primers and optimizing the protocol. Six primers were designed by using PrimerExplorer V4 program. The optimization of RT-LAMP assay was assessed by the variation of time and temperature. The details were as following:

1.1. LAMP primer design for swine influenza virus detection

A set of primers has been designed based on the specific target (M gene) of influenza virus. The set of primers was designed by the PrimerExplorer V4 program. There were six primers including two internal primers (FIP and BIP), two outer primers (F3 and B3) and two loop primers (Loop F and loop B) as shown in Table 3. The locations of each primers on the target sequence are shown in Figure 1.

Table 3. Properties and nucleotide sequence of primers for LAMP assay in this study

Primer	Position* (bp)	Length (mer)	Tm (°C)	GC content (%)	Sequence
F3	358 - 375	18	58.4	61.11	5'-GGCCAAGGAGGTGTCACT-3'
B3	530 - 549	20	56.4	45	5'-TGCCTGATTAGTGGATTGGT-3'
FIP:		42	79.9	50	
F1c	431 - 452	22			5'-CAGCTTCTGTGGTCACTGTTCC-3'
F2	380 - 399	20			5'-TATTCAACTGGTGCACTTGC-3'
BIP:		42	80.9	52.38	
B1c	458 - 479	22			5'-GGTCTAGTGTGTGCCACTTGTG-3'
B2	510 - 529	20			5'-GGTAGTAGCCATCTGTCTGT-3'
Loop F	400 - 421	22	62.1	50	5'-GTATATGAGGCCCATGCAACTG-3'
Loop B	489 - 509	23	62.9	47.83	5'-ATTGCTGATTCACAGCATCGGTC-3'

* nucleotide position of M gene (consensus sequence). GenBank: KJ162040.1

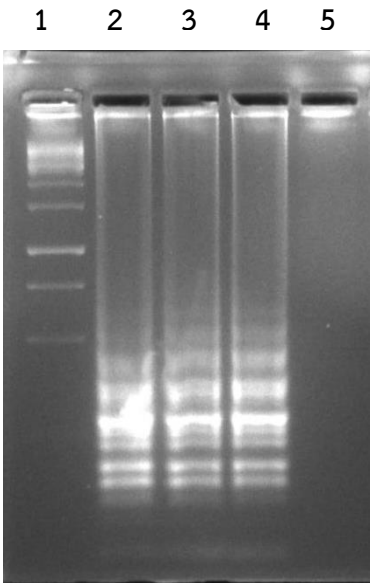
1.2. Optimum conditions of RT-LAMP assay for swine influenza virus detection

1.2.1. Optimization of RT-LAMP protocol by temperature variation

The result of RT-LAMP amplification was shown as "ladder pattern" in agarose gel electrophoresis. In this study, RT-LAMP protocol was assessed for optimum temperature for the detection of SIV. Figure 4 showed that RT-LAMP products can be visualized in all range of temperature 60° C, 63° C, and 65° C. In this study, temperature of 63°C was chosen for optimized RT-LAMP protocol.

Figure 4. Effect of temperature in RT-LAMP assay. Lane 1: marker, lane 2: 60° C, lane 3: 63° C, lane 4: 65° C and lane 5: negative control (no template)

Lane	Reagent	Temperature	Result
1	Marker	N/A	N/A
2	RT-LAMP product	60° C	+
3	RT-LAMP product	63° C	+
4	RT-LAMP product	65° C	+
5	Negative control	-	-

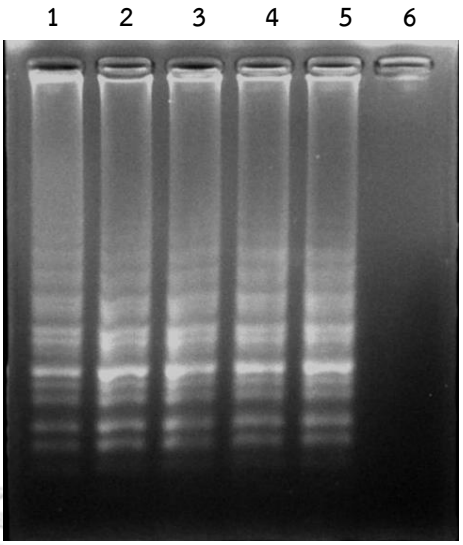


1.2.2. Optimization of RT-LAMP protocol by time variation

With the result from step 1.2.1, temperature of 63°C was chosen and used for optimization of RT-LAMP protocol by time variation. The variations of time were 30, 40, 50, 60 and 70 minutes. Figure 5 showed that RT-LAMP product can be visualized within the range of time variation. In this study, at temperature of 63°C for 30 minutes was chosen for optimized RT-LAMP protocol.

Figure 5. Effect of time in RT-LAMP assay. Lane 1: 30 minutes, lane 2: 40 minutes, lane 3: 50 minutes, lane 4: 60 minutes, lane 5: 70 minutes and line 6: negative control (no template)

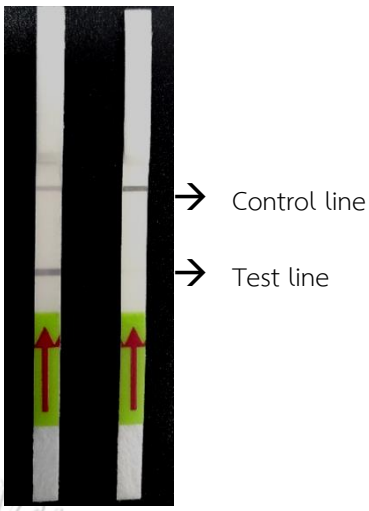
Lane	Reagent	Time (minutes)	Result
1	RT-LAMP product	30	+
2	RT-LAMP product	40	+
3	RT-LAMP product	50	+
4	RT-LAMP product	60	+
5	RT-LAMP product	70	+
6	Negative control	-	-



1.3. Optimization of RT-LAMP with LFD protocol

RT-LAMP with LFD assay was conducted with the optimized condition from step 1.2.1 and step 1.2.2. The optimized RT-LAMP condition at 63°C for 30 minutes was used for RT-LAMP with LFD assay. RNA of SIV subtype H1N1 is used for this step. Figure 6 showed that RT-LAMP detection by LFD visualization was assessed and working well, which positive RT-LAMP result showed 2 lines in both control and test lines, while negative RT-LAMP result showed only one line (control line).

Figure 6. RT-LAMP assay with LFD visualization. 1: positive SIV (2 lines appeared in both control and test lines), 2: negative SIV (1 line appeared in control line)



Phase 2. Evaluation of the sensitivity, specificity and performance of newly developed RT-LAMP-LFD assay

RT-LAMP-LFD assay was evaluated for the sensitivity, specificity and performance test. In this study, RT-LAMP-LFD assay has an analytical sensitivity (minimum detection limit) at $114x10^{-5}$ ng/µl (1.14 pg/µl) and high specificity which no cross reaction with other respiratory viruses in pigs (PRRSV, PCV2, PEDV and Pseudorabies virus). Performance of RT-LAMP-LFD was evaluated with 30 samples of randomly blinded RNA/DNA of respiratory viruses of pigs (SIV, PRRSV, PEDV and Pseudorabies virus).

2.1. Sensitivity of the RT-LAMP-LFD assay

The analytical sensitivity “minimum detection limit” was assessed by determining the minimum detection of SIV that LAMP could detect in this

study, serially dilution of 114 ng/µl of reference SIV subtype H1N1 was used as a template. Our results showed that RT-LAMP assay have comparable analytical sensitivity with real time RT-PCR by using agarose gel electrophoresis and 10-fold higher sensitivity by using LFD (Table 4). Figure 7 showed the comparison of analytical sensitivity (detection limit) between RT-LAMP assay, either by agarose gel electrophoresis and LFD, with real time RT-PCR.

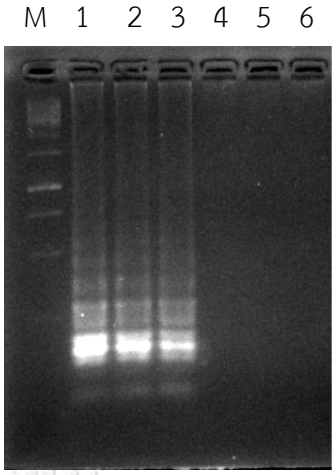
Table 4. The analytical sensitivity (detection limit) of the RT-LAMP assay compared with real time RT-PCR

Serial dilution	Assay			RNA concentration	
	RT-LAMP with agarose gel electrophoresis	RT-LAMP with LFD assay	Real time RT-PCR (Ct)*	ng/µl	pg/µl
10^{-2}	+	+	+ (21.88)	1.14	1140
10^{-3}	+	+	+ (28.87)	0.114	114
10^{-4}	+	+	+ (33.83)	0.0114	11.4
10^{-5}	-	+	- (39.45)	0.00114	1.14
10^{-6}	-	-	N/A	0.000114	0.114
Negative control (no template)	-	-	N/A	N/A	N/A

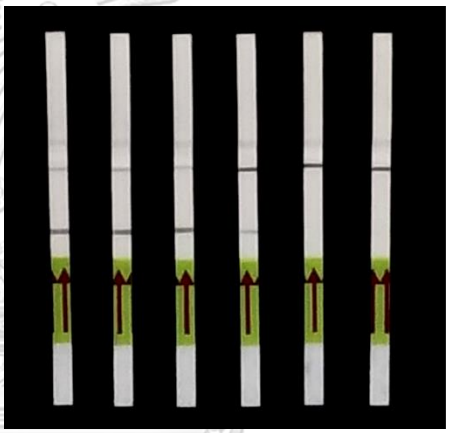
* The interpretation of real time RT-PCR result was in Ct value, where value under 36 considered as positive, from 37 to 40 as suspected and above 40 as negative.

Figure 7. The analytical sensitivity (detection limit) of the RT-LAMP assay with different visualization: agarose gel electrophoresis and LFD, compared with real time RT-PCR. M: Marker, 1: SIV dilution 10^{-2}, 2: SIV dilution 10^{-3}, 3: SIV dilution 10^{-4}, 4: SIV dilution 10^{-5}, 5: SIV dilution 10^{-6}, 6: Negative control (no template)

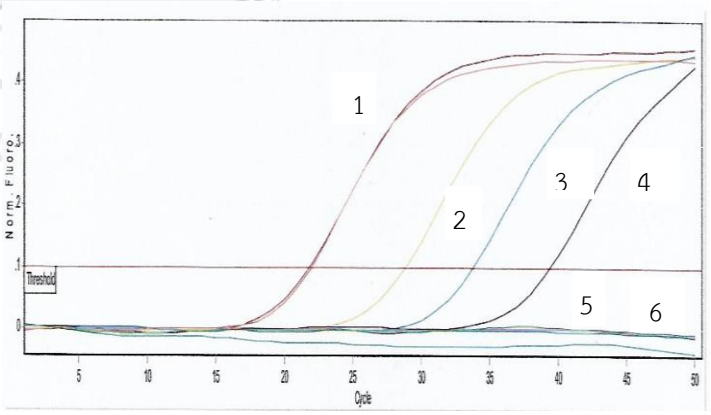
A. RT-LAMP with agarose gel electrophoresis



B. RT-LAMP with LFD assay



C. Real time RT-PCR

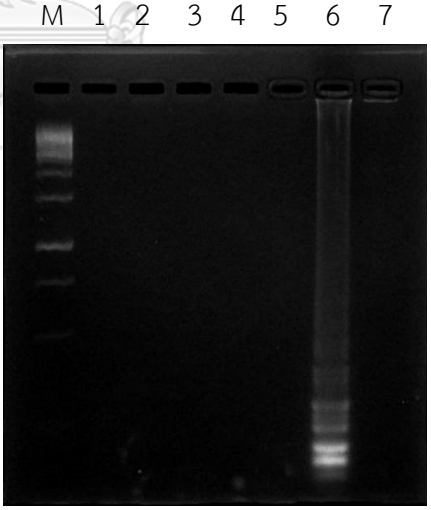


2.2. Specificity of the RT-LAMP-LFD assay

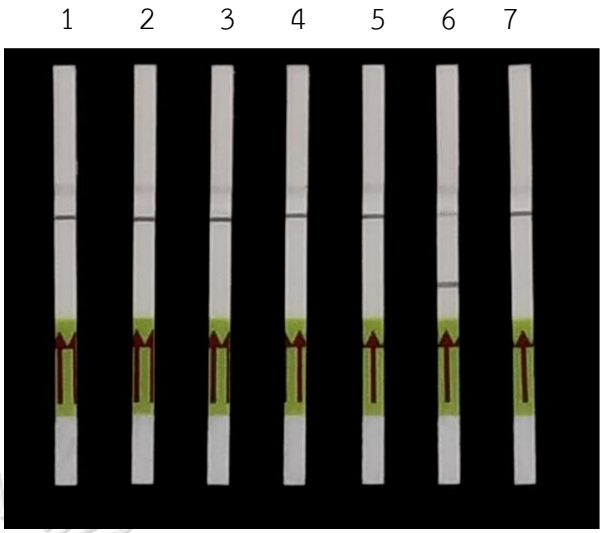
In this study, RT-LAMP assay was assessed against RNA/DNA of important respiratory viruses of pigs such as SIV, PRRSV, PCV2, PEDV and Pseudorabies viruses. Figure 8 showed that there was no cross reaction among SIV and other viruses. These results indicate that RT-LAMP assay is specific for SIV either by agarose gel electrophoresis and LFD.

Figure 8. Specificity test by RT-LAMP assay: A. visualization by agarose gel electrophoresis, B. visualization by LFD. M: Marker, 1: Pseudorabies virus, 2: PCV2, 3: PRRSV EU, 4: PRRSV US, 5: PEDV, 6: SIV, 7: Negative control (no template)

A. RT-LAMP with agarose gel electrophoresis



B. RT-LAMP with LFD assay



2.3. Performance assessment of RT-LAMP-LFD assay

Performance assessment of RT-LAMP-LFD assay was evaluated and described as diagnostic sensitivity, diagnostic specificity and the measure of agreement (Kappa value). Table 5 showed the result of RT-LAMP assay in 30 blinded samples of RNA/DNA viruses that were assessed with real time RT-PCR and RT-LAMP assay, either by agarose gel electrophoresis and LFD.

Table 5. Result of real time RT-PCR and RT-LAMP assay, either by agarose gel electrophoresis and LFD

No.	References	Virus	Methods		
			LAMP Gel elect.	LAMP LFD	Real Time RT-PCR
1	SF 21625*	SIV	+	+	+
2	SF 22300*	SIV	+	+	+
3	SF 21298 N*	SIV	+	+	+
4	SF 21299 N*	SIV	+	+	+
5	SF 21305 N*	SIV	+	+	+
6	SF 20218*	SIV	+	+	+
7	SF 20226*	SIV	+	+	+
8	SF 21302*	SIV	+	+	+
9	SF 21304*	SIV	+	+	+
10	SF 21307*	SIV	+	+	+
11	S1803 074**	PRRSV	-	-	-
12	S1803 110**	PRRSV	-	-	-
13	P97/61 1.0**	PRRSV	-	-	-
14	S 5005**	PEDV	-	-	-
15	S 5032**	PEDV	-	-	-
16	S 5039**	PEDV	-	-	-
17	S 5043**	PEDV	-	-	+
18	Neg 1	No template	-	-	-
19	Neg 2	No template	-	-	-
20	Neg 2	No template	-	-	-
21	01KB1***	PRRSV EU	-	-	-
22	01NP1***	PRRSV US	-	-	-
23	Commercial vaccine, Korea	PEDV	-	-	-
24	NIAH***	Pseudorabies virus	-	-	-
25	NRU 3488 10-2*	SIV H3N2	+	+	+
26	NRU 3336 10-1*	SIV H1N1	+	+	+

27	NRU 3336 10-2	SIV H1N1	+	+	+
28	NRU 3336 10-3	SIV H1N1	+	+	+
29	NRU 3336 10-4	SIV H1N1	+	+	+
30	NRU 3336 10-5	SIV H1N1	-	+	-

* Previously identified by viral isolation (egg inoculation) and/or real time RT-PCR

** Previously identified by PCR conventional and/or real time RT-PCR

*** Previously identified and calculated for virus titer (TCID50/ml).

The results (Table 5) then compared with reference standard by two-by-two table and then calculate for diagnostic sensitivity, diagnostic specificity and the measure of agreement (Kappa value) (Table 6). This result (Table 5) showed that RT-LAMP-LFD assay have a good performance with 100% sensitivity, 100% specificity and perfect percentage of agreement (Kappa = 1). RT-LAMP assay with agarose gel electrophoresis visualization have lower sensitivity (93.75%) than using LFD. Comparing with real time RT-PCR specificity result (92.86%), RT-LAMP assay has higher specificity.

Table 6. Result comparison and the calculation of diagnostic sensitivity, diagnostic specificity and the measure of agreement (Kappa value)

			Gold standard test result (as reference test)		
			Positive	Negative	Total
A	RT-LAMP with gel electrophoresis	Positive	*15*	*0*	*15*
		Negative	*1*	*14*	*15*
	Total		*16*	*14*	*30*
B	RT-LAMP with LFD (developed test)	Positive	*16*	*0*	*16*
		Negative	*0*	*14*	*14*
	Total		*16*	*14*	*30*
C	Real time RT-PCR	Positive	*16*	*1*	*17*
		Negative	*0*	*13*	*13*
	Total		*16*	*14*	*30*

A. RT-LAMP with gel electrophoresis

Sensitivity	93.75	%
Specificity	100	%
Kappa	0.93333333	

expected (a)	8
expected (d)	7
expected agreement	0.5
observed agreement	0.966667

B. RT-LAMP with LFD

Sensitivity	100	%
Specificity	100	%
Kappa	1	

expected (a)	8.533333
expected (d)	6.533333
expected agreement	0.502222
observed agreement	1

C. Real time RT-PCR

Sensitivity	100	%
Specificity	92.8571429	%
Kappa	-0.0762332	

expected (a)	9.066667
expected (d)	6.066667
expected agreement	0.504444
observed agreement	0.466667

CHAPTER V
DISCUSSION

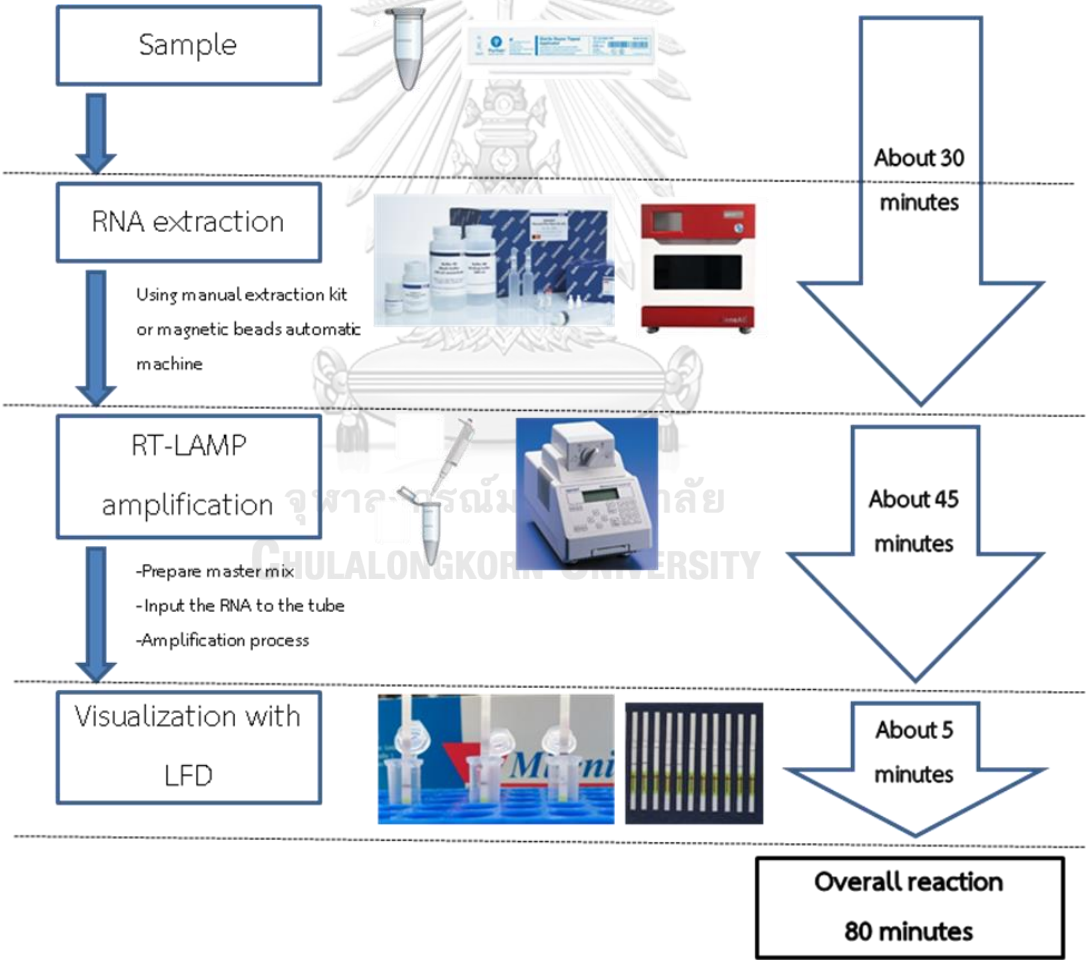
Swine influenza causes high morbidity and low mortality in pigs. The disease spreads rapidly in pig farms causing the delay of pig weight to reach market. Moreover, pigs play important role as “mixing vessel” of influenza viruses. Pigs can support infection and replication of influenza viruses from avian, human and swine origins (Brown et al., 1997; Campitelli et al., 1997; Webby et al., 2000; Qi and Lu, 2006). These lead to major concerns of the important of swine influenza which are not only the economic impacts but also the human health. In Thailand, SIV has been reported since 1981 (Kanai et al., 1981). There are three main SIV subtypes co-circulating in Thailand including H1N1, H1N2, H3N2 (Nonthabenjawan et al., 2015). With high density of pig population in Thailand, gives more opportunity of SIVs infection and subsequently genetic reassortment and the risk of inter-species transmission. Previous studies had reported the reassortment between endemic Thai SIVs with pandemic H1N1 2009 (Hiromoto et al., 2012; Nonthabenjawan et al., 2015) and an outbreak of infection by pandemic H1N1 2009 in commercial pig farm which possibility transmitted from human to pig (Sreta et al., 2010). Therefore, the need of accurate and rapid diagnosis for SIVs is critical to minimize further spreading of the viruses in pig population and to reduce the risk of multiple infections of influenza viruses.

In this study, we have developed a RT-LAMP-LFD assay which is the combination of reverse transcription loop-mediated isothermal amplification (RT-LAMP) with lateral flow device (LFD) for swine influenza virus detection. The primers for RT-LAMP assay was newly designed based on M gene of SIV The primers can be applied in RT-LAMP assay and make it possible to detect three common subtypes of SIVs (H1N1, H1N2, and H3N2). Previous studies have also been reported that RT-LAMP assay can be used for SIV detection such as: SIV-H3N2 (Gu et al., 2010), pandemic (H1N1) 2009 (Kubo et al., 2010), and swine origin influenza A H1N1 (Parida et al., 2011). In this study, six primers were used for RT-LAMP assay including two internal primers (FIP and BIP), two outer primers (F3 and B3) and two loop primers (loop F and loop B). Since the loop primers were used, the RT-LAMP reaction was enhancing for the speed and specificity (Nagamine et al., 2002). This speculation agreed with our result which the RT-LAMP reaction required only 30 minutes to process and provides suitable condition for RT-LAMP assay. It was noted that LAMP assay without loop primers (only two internal and two outer primers) requires 60 minutes (Notomi et al., 2000).

Unlike PCR, LAMP assay can be performed under isothermal temperature. This is related to the use of DNA polymerase. In this study, we used *Bst* polymerase large fragment where the optimal temperature are between 60°C and 65°C (Notomi et al., 2000). Our developed RT-LAMP assay can work under all three temperatures (60°, 63° and 65°C). The temperature 63°C was selected as our optimum condition for RT-LAMP

assay. Based on the temperature 63°C, our developed RT-LAMP assay can work under wide range of time (30 – 70 minutes). Thus, in this study, the optimum condition selected for RT-LAMP assay was 63°C 30 minutes. The time of overall process of RT-LAMP-LFD assay from RNA extraction until LFD visualization can be achieved within 80 minutes (Figure 9).

Figure 9. The overall process and time of RT-LAMP-LFD assay for SIV detection (from RNA extraction until LFD visualization)



Many studies have been reported the sensitivity of RT-LAMP assay. RT-LAMP assay has either comparable detection limit with real time RT-PCR (Kubo et al., 2010) or 10-fold higher than real time RT-PCR (Parida et al., 2011). Some studies reported that the RT-LAMP assay has detection limit 100 fold higher than conventional RT-PCR (Chen et al., 2010a). In this study, our developed RT-LAMP assay has comparable detection limit or "analytical sensitivity" with real time RT-PCR when using agarose gel electrophoresis and 10-fold higher than real time RT-PCR when using LFD. In general, visualization of RT-LAMP by using agarose gel electrophoresis contains several steps from preparing gel, loading LAMP products into the wells, and then running gel in electrophoresis chamber. These steps provide opportunities of contamination and time consuming. On the other hands, by using LFD for LAMP visualization, the steps provide limit contamination and less time which only take 5-10 minutes.

In previous reports, LAMP assay have been developed and used to detect other respiratory viruses in pigs such as PRRSV (Li et al., 2009; Chen et al., 2010a), PCV2 (Zhao et al., 2011) and Pseudorabies virus (En et al., 2008). In this study, analytical specificity was conducted with assessed RT-LAMP-LFD assay against several viruses in pigs including SIV, PRRSV, PCV2, Pseudorabies virus and PEDV. As result, RT-LAMP assay capable to detect SIV and there was no cross reaction with other viruses. This result can imply that RT-LAMP-LFD assay is a specific method. The six primers were designed to recognize eight distinct region of target gene, which expected to have high

selectivity. Moreover, the loop primers (loop F and B) that have been tagged with FAM and biotin, ensure that only targeted amplicons could show visible band in test band of LFD.

To evaluate the performance of RT-LAMP-LFD assay, we conducted SIV detection by RT-LAMP-LFD in blinded samples. After blinded samples were decoded and compared with RT-LAMP-LFD result, the diagnostic sensitivity, diagnostic specificity and percentage of agreement by Kappa value were calculated. Our result showed that RT-LAMP-LFD assay provided 100% sensitivity, 100% specificity with Kappa = 1. This result indicated that the newly developed RT-LAMP-LFD has high diagnostic sensitivity and diagnostic specificity with high perfect percentage of agreement. Meanwhile RT-LAMP test with agarose gel electrophoresis had lower sensitivity than RT-LAMP-LFD and real time RT-PCR result showed lower specificity than RT-LAMP-LFD assay. Similar to previous study, the performance of RT-LAMP-LFD assay have been examined for the detection of Influenza A (H7N9) virus with 100% sensitivity and 100% specificity (Ge et al., 2013).

With the combination of RT-LAMP assay with LFD, this assay is simple, rapid and low cost with less time than PCR based method (real time RT-PCR and conventional PCR). However, despite of the high sensitivity of RT-LAMP-LFD could offer, it gives chances of false positive result. Even with only a single trace of DNA/RNA could create contamination. RT-LAMP assay need preparation of mixing several reagents such

as thermoPol buffer, dNTPs, $MgSO_4$, betaine, *Bst* DNA polymerase, AMV buffer, AMV RT, and H_2O. Therefore, it need extra cautious in handling the preparation of master mix and should be in sterile condition. The combination of RT-LAMP assay with LFD helps to avoid contaminations in post-amplifications operations.

Overall, RT-LAMP-LFD assay was developed with newly designed primers. The RT-LAMP-LFD has high analytical sensitivity with minimum detection at 1.14 pg/µl of reference SIV. There was no cross reaction with other important viruses of pigs. The newly developed RT-LAMP-LFD has good performance with 100% sensitivity, 100% specificity and perfect percentage of agreement compared to reference assays (viral isolation and/or real time RT-PCR).

Conclusions and suggestions

The findings of this study support the conclusions that:

1. RT-LAMP-LFD assay was developed with newly designed primers (two internal primers, two outer primers and two loop primers) with optimum condition at 63°C and 30 minutes.
2. The detection limit of RT-LAMP-LFD assay is 1.14 pg/ul of RNA concentration, 10-fold higher than real time RT-PCR.

3. RT-LAMP visualization by LFD has higher analytic sensitivity (detection limit) than agarose gel electrophoresis.
4. RT-LAMP-LFD assay is specific method for the detection of SIV, there were no cross reaction with other important viruses of pigs (PRRSV, PCV2, Pseudorabies virus and PEDV).
5. The newly developed RT-LAMP-LFD has good performance with 100% sensitivity, 100% specificity and perfect percentage of agreement (Kappa = 1) compared to reference assays (viral isolation and/or real time RT-PCR).

With these conclusions, RT-LAMP-LFD can become a simple assay for the detection of SIV and suitable for the field or in-farm setting. However, to achieve better performance of this assay, recommendations and suggestions are as following:

1. Further evaluation and large-scale testing on field samples is needed to improve the validation of this RT-LAMP-LFD assay.
2. Further study on simple and rapid RNA extraction method is needed to improve the RT-LAMP-LFD assay more applicable in field or in farm setting.
3. The RT-LAMP–LFD assay has a potential for patent technology as a rapid diagnostic test and could be developed for low-cost or in-house kits.

4. The RT-LAMP-LFD assay for SIV detection could be implemented and applied at first point care of SIV outbreaks to help control and prevent the spread of disease in pig farms.

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