การพัฒนาวิธี Reverse Transcription Loop-Mediated Isothermal Amplification เพื่อตรวจหา
ไวรัสไข้หวัดใหญ่ ชนิดเอ อย่างรวดเร็ว

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THE DEVELOPMENT OF REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR RAPID DETECTION OF INFLUENZA A VIRUSES

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A Thesis Submitted in Partial Fulfillment of the Requirements
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โรคไข้หวัดใหญ่เป็นโรคที่มีความสำคัญทางสาธารณสุข ในรอบศตวรรษที่ผ่านมามีการระบาดใหญ่ของโรคไข้หวัดใหญ่ 4 ครั้ง รวมถึงการระบาดของไวรัสไข้หวัดใหญ่สายพันธุ์ใหม่ 2009 วิธีการตรวจพิสูจน์เชื้อไวรัสไข้หวัดใหญ่มีหลากหลายวิธี เช่น วิธีการเพาะแยกเชื้อไวรัส, วิธีอิมมูโนฟลูออเรสเซนซ์, วิธีปฏิกิริยาลูกโซ่โพลิเมอเรส และ วิธีปฏิกิริยาลูกโซ่โพลิเมอเรสในสภาพจริง อย่างไรก็ตามบางวิธีไม่เหมาะสมและนำไปปรับใช้ในประเทศกำลังพัฒนา เนื่องจากมีข้อจำกัดในเรื่องห้องปฏิบัติการและไม่สะดวกในการนำไปใช้ในการออกพื้นที่ การศึกษาครั้งนี้ได้พัฒนาวิธี one step reverse transcription loop-mediated isothermal amplification (one step RTLAMP) ซึ่งเป็นวิธีที่มีความไวสูง และใช้เวลาน้อย การศึกษาครั้งนี้ได้ออกแบบชุดของไพรเมอร์ที่มีความไวและความจำเพาะสูง และหาอุณหภูมิที่เหมาะสมของปฏิกริยาคือ 63°C นาน 1 ชั่วโมง ตามด้วย 80°C นาน 10 นาที วิธี one step RT-LAMP ที่ถูกพัฒนาขึ้นมานี้มีความไวในการตรวจหาเชื้อมากกว่าวิธีปฏิกิริยาลูกโซ่โพลิเมอเรส 10 เท่า และมีความไวเท่ากับวิธีปฏิกิริยาลูกโซ่โพลิเมอเรสในสภาพจริง วิธีนี้ยังมีความจำเพาะต่อเชื้อไวรัสไข้หวัดใหญ่จากโฮสต์หลายๆ ชนิด และมีความไวในการตรวจหาเชื้อจากการป้ายเชื้อทางหลอดลมและทางทวารร่วม โดยสรุปวิธีที่พัฒนานี้ เป็นวิธีที่มีความง่าย และสะดวก เหมาะสมในการนำไปใช้ในพื้นที่ที่มีการระบาด

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Influenza A infection is a major public health problem world wide. Four major pandemics in the past century including pandemic H1N1 2009 were caused by influenza A viruses. Different approaches of diagnosis assays have been developed to detect influenza A viruses. However, some techniques such as viral isolation, immunofluorescence assay (IFA) and other molecular assays including RT-PCR and real time RT-PCT have limitation to apply in mobile surveillance and unequipped laboratories in developing countries. In this study, one step reverse transcription loop-mediated isothermal amplification was developed as a rapid, sensitive to detect influenza A viruses. To reach the overall goal, a lamp primer set was designed by PrimerExpoler V4 and developed for a sensitive and specific amplification. The optimal amplification reaction is 63°C for 60 minutes then followed by 80°C for ten minutes. The developed assay is ten times more sensitive than conventional RT-PCR and comparable as real time RT-PCR. It is also highly specific for influenza viruses of different hosts. The colorimetric assay of LAMP products is also sensitive as gel electrophoresis. This developed one step RT-LAMP reveals comparable sensitive to detect the Influenza A viruses in both cloacal and tracheal samples. This method is also an easy to use technique and suitable for field surveillance and screening.

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| Department : Veterinary Public Health | Student's Signature ............ |
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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AMV RT | Avian Myeloblastosis Virus Reverse Transcriptase |
| B3 | Backward Outer Primer consists of the B3 region |
| B3c | Complementary region to B3 Primer |
| BIP | Backward inner primer |
| bp | Base pairs |
| Bst | *Bacillus stearothermophilus* |
| C | Cysteine |
| CDC | Center for Disease Control and Prevention |
| cDNA | complementary DNA |
| CF | Complement fixation test |
| CPE | Cytopathic effect |
| CSFV | Classical swine fever virus |
| DNA | Deoxyribonucleic acid |
| dNTPs | Deoxyribonucleotide triphosphates |
| e.g. | For example |
| EIA | Enzyme Immnunoassay |
| et al. | et alii |
| F3 | Forward Outer Primer consists of the F3 region |
| F3c | Complementary region to F3 primer |
| FIP | Forward inner primer |
| G | Glycine |
| HCl | Hydrogen chloride |
| HI | Hemagglutination inhibition test |
| HPAI | High pathogenic avian influenza |
| IBV | Infectious bronchitis virus |
| IFA | Immunofluorescence assay |
| IgA | Immunoglobulin A |
| IgG | Immunoglobulin G |

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| | |
|---|---|
| IgM | Immunoglobulin M |
| kcal | Kilocalorie |
| KCl | Potassium chloride |
| LAMP | Loop mediated isothermal amplification |
| LPAI | Low pathogenic avian influenza |
| MDCK | Madin-Darby Canine Kidney Cells |
| MEGA | Molecular evolutionary genetics analysis |
| $MgSO_4$ | Magnesium sulfate |
| mM | Mili molar |
| mol | Mole |
| NDV | Newcastle disease virus |
| NEB | New England Biolabs |
| $(NH_4)_2SO_4$ | Ammonium sulphate |
| No. | Number |
| ng | nanogram |
| PCR | Polymerase chain reaction |
| pg | pictogram |
| PPHSN | Pacific Public Health Surveillance Network |
| PRRSV | Porcine reproductive and respiratory syndrome virus |
| RNA | Ribonucleic acid |
| RNAse | Ribonuclease |
| RNP | Ribonucleoprotein |
| RT | Reverse transcription |
| WHO | World Health Organization |
| U | Unit |
| USA | united States of America |
| VDL | Veterinary Diagnosis Laboratory |
| VTM | Viral transport media |
| VIT | Viral isolation technique |
| µg | Micro gram |

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| μl | Micro liter |
| μM | Micro molar |

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## Influenza Genes and Their Function

| Gene | Segment | Size (nt) | Function |
|---|---|---|---|
| PB 2 | 1 | 2341 | Transcriptase: cap binding |
| PB1 | 2 | 2341 | Transcriptase: elongation |
| PA | 3 | 2233 | Transcriptase: vRNA replication |
| HA | 4 | 1778 | Haemagglutinin: Host cell attachment |
| NP | 5 | 1565 | Nucleoprotein: RNA binding, part of transcriptase complex, nuclear cytoplasmic transport of vRNA |
| NA | 6 | 1413 | Neuraminidase: viral release |
| M | 7 | 1027 | Matrix: M1 major component of virion |
| M2 | | | Integral membrane protein-ion channel |
| NS | 8 | 890 | Nonstructural: NS1 nucleus; effects on cellular RNA transport, splicing, translation. |
| NS2 | | | Anti-interferon protein also known as NEP (nuclear export protein) |

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# CHAPTER I
# INTRODUCTION

Influenza is caused by a zoonotic virus which occurs in animals and birds as well as in humans. Influenza viruses are segmented negative-sense RNA viruses that belong to the Orthomyxoviridae family. The influenza viruses are classified into 3 types, including type A, B and C on the basis of the nucleocapsid (NP) or matrix (M) antigens. Type A influenza virus can be further divided into different subtypes (H1-H16 and N1- N9) according to its surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). Among these 3 types of Influenza viruses, Influenza A virus inflicts disease in humans, pigs, horses, seals, whales and a variety of domestic and wild birds (Webster et al., 1992). Influenza B viruses cause the same spectrum of disease as influenza A. However, influenza B viruses do not cause pandemics. This property may be a consequence of the limited host range of the virus. Influenza B virus can infect to humans and seals, so it may difficult to generate of new strains by reassortment. The influenza type C viruses are serologically different from influenza type A and B. Influenza C virus infection usually causes only a mild or unapparent illness, and rarely to isolate (Kilbourne, 1975).

Nowadays influenza virus infection is a major public health problem worldwide, causing millions cases of illness associated with various respiratory syndromes and approximately 500,000 human deaths annually (Nicholson et al., 2003). In the last century, influenza A viruses caused three major pandemics, i.e. Spanish flu in 1918, Asian flu in 1957 and Hong Kong flu in 1968, with a high mortality rate (World Health Organization, 2005). Recently a new strain of Influenza A virus has emerged and caused the pandemic flu so called “Pandemic H1N1 2009” (World Health Organization, 2009). Most of the influenza A outbreaks of humans arose from the viruses with novel hemagglutinin (HA) and/or neuraminidase (NA) proteins to which humans have no immunity (Matrosovich et al., 1999). Since the pandemic H1N1 2009, many surveillance networks had been established, of which the goals are to prevent and control the pandemic virus and to prepare for the next influenza pandemic.

Currently, the viral isolation technique (VIT) using egg innouculation has been used as a standard method for diagnosis of influenza A. However, an expert and certified laboratories are required to perform the test. It also takes 3 to 10 days for the availability of the results. Some rapid and easy detection methods have been developed, e.g. immunofluorescence assays (IFA) (Waner et al., 1991) and reverse transcription-PCR (RT-PCR) (Yamada et al., 1991). However, IFA have sometimes limited sensitivity and also need the sophisticated equipments for the application of the techniques. The sensitivity of IFA has been reported ranging from 65 to 100 % (Dominguez et al., 1993; Grandien et al., 1985; Leonardi et al., 1994; Waner et al., 1991)

A choice of diagnostic technology is based on a combination of factors that includes fitness-for-purpose, technical ease, speed, sensitivity, specificity and cost (Charlton et al., 2009). Nowadays, both conventional RT-PCR and real time RT-PCR are widely applied in Influenza A screening and diagnosis due to their high sensitivity and specificity. However, these molecular assays require the specialized equipments e.g. thermocycler and real time machine, that are not widely available in most developing countries. Therefore, there have still limitation to apply the PCR techniques in some developing countries. The reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay is a PCR- based technique that has been developed to overcome those drawbacks of the previously-existing methods. The technique has the increased sensitivity and does not require expensive equipment. It is rather easy to perform as well. The RT-LAMP has been to intend to use for both human and animal influenza diagnosis. Those specific for subtypes H1 and H3 (Ito et al., 2006), subtype H5 (Imai et al., 2006) and H9 (Chen et al., 2008) has been developed. There was a report about LAMP detection of human influenza A viruses (Poon et al., 2005). However, there is no RT-LAMP method for detection of animal influenza A virus to date and most of the two step RT-LAMP method is time-consuming because it needs to transcribe from RNA to cDNA before the LAMP reaction.

To fill the gap of previous report, we need to develop the one step RT-LAMP, which can be applied as a rapid detection of influenza A viruses from both human and animal. The newly developed technique will be useful for the surveillance of influenza A

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in both human and animal hosts. The RT-LAMP method would be suitable for the mobile surveillance and for unequipped laboratories in developing countries.

In this study, we intend to develop one step RT-LAMP technique as an applicable molecular diagnostic technique of influenza A virus, which will be a rapid screening of Influenza A by combing with visual detection of the LAMP products.

## Objective of the study

The objective of this study is to develop a rapid and sensitive RT-LAMP method for rapid detection of phenotypically and genotypically diverse influenza A viruses.

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# CHAPTER II
# REVIEW LITERATURES

The aim of this chapter is to provide through selective references to some of the literatures, a clearer understanding on the influenza viruses, their genetic nature and how important for both human and animal health, then followed by different diagnostic techniques for typing of influenza viruses. The brief interpretations on different diagnostic techniques are also ruled out. At last, the principle of LAMP technology and the detail in the steps of RT-LAMP are reviewed. Different methods of detection for LAMP products are also referred.

## 1. Background of influenza virus

Influenza virus is a member of Orthomyxoviridae family composed of 3 types A, B and C on the basic of the nucleocapsid (NP) and matrix (M) antigens. Only the type A and B cause widespread outbreaks (Nicholson et al., 2003). Type A viruses can be sub typed according to the antigenicity of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). Currently, sixteen of HA (H1-H16) and nine of NA (NA1-NA9) have been identified in mammalian and avian influenza A viruses (Fouchier et al., 2005; Stevens et al., 2006). Influenza type A viruses can infect a variety of avian and mammalian species and cause severe disease in most of animal species (Lee et al., 2004) such as swine, horses and humans, but with only selected HA and NA subtypes, including H1N1 in swine, H3N8 in horses and H1N1 and H3N2 in humans (Suarez et al., 2003). Almost all 16 HA and 9 NA subtypes have been isolated from the birds especially in aquatic birds that are believed to be a natural reservoir for influenza viruses (Lee et al., 2004; Nicholson et al., 2003). Avian influenza viruses can be divided into highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) based on the severity of clinical signs in susceptible species and presence of multiple basic amino acids in the cleavage site of the precursor hemagglutinin molecules (HA0) (Capua and Alexander, 2004).

The virus particle has a pleomorphic morphology, with the size of approximately 80-120 nm in diameter (Lamb and Krug, 2001). Influenza A virus genome composes of eight separated RNA segments encoding 10-11 different polypeptides (PB1, PB2, PA, HA, NP, NA, M1, M2, NS1, NS2 and sometimes PB1-F2). The virus contains glycoprotein spikes of two types, rod-shaped (hemagglutinin; HA) and mushroom-like shaped (neuraminidase; NA). The virion composes of viral matrix protein (M1) which underlines beneath the lipid bilayer of viral envelope and viral matrix protein (M2) forms the viral ion channel. Inside the virion are the ribonucleoprotein (RNP) structures which form twin-stranded helix RNA segments and consist of four proteins NP, PA, PB1 and PB2 (Lamb and Krug, 2001). The membrane protein, M gene of influenza A viruses encode two proteins, M1 and M2. M1 is an essential role in virus assembly and budding (Ito et al., 1991)but M2 is undefined role in virus replication (Lamb et al., 1985). The available evidence indicates that M genes are highly conserved (Lamb and Lai, 1981; Schild et al., 1972) and that the same M gene has been conserved throughout the antigenic shift of HA and NA in human pandemics (Hall and Air, 1981)

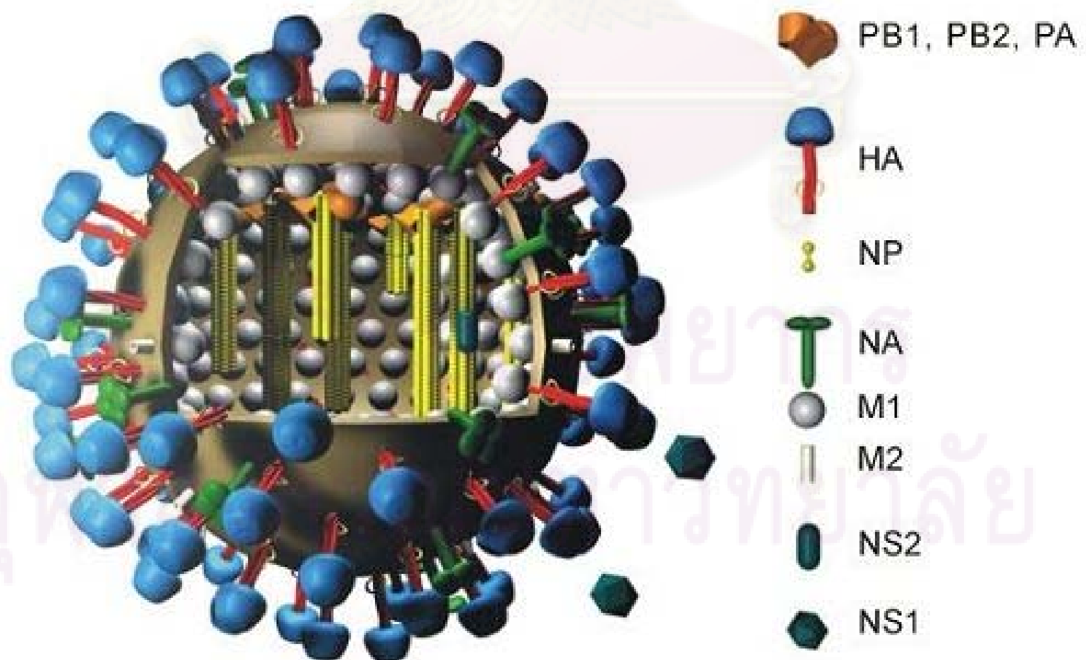

Figure 1. Structure of an influenza A virus. Image copyright by Dr. Markus Eickmann, Institute for Virology, Marburg, Germany. (Kamps et al., 2006)

## 2. Global Impact of Influenza epidemics and pandemics

Influenza causes a serious respiratory disease in both human and animals. It can be debilitating and cause complications that lead to hospitalization and death, especially in elderly and younger ages. Every year, global burden of influenza epidemics is believed to cause in both human and animal population. The risk of serious illness and death is highest among person aged >65 years, children aged < 2 years and the person who have medical conditions and placed at a high risk of developing complication from influenza (Center for Disease Control, 2005).

New epidemics Influenza A strains emerge every 1 to 2 years by the appearance of selected point mutation on two surface glycoproteins: HA and NA. These permanent and usually minor changes in the antigenicity of influenza A viruses are called “Antigenic drift” and regularly occur in influenza epidemics. In addition, multiple lineage of the same subtype can co-circulate, persist and reassort in epidemiologically significant ways.

In contrast to epidemics, pandemics are rare which occur every 10 to 50 years. In the last 400 years, at least 31 pandemics have been recorded (Lazzari and Stohr, 2004). During the twentieth century, three major influenza pandemics were occurred and their mortality ranged from devastating to moderate or mild (Simonson, 2004). The 1918 pandemic was caused by a H1N1 virus of apparently avian origin (Reid et al., 1999), whereas the subsequent pandemic strains- H2N2 in 1957 and H3N2 in 1968 were reassortant viruses containing genes from avian viruses: three in 1957 (hemagglutinin, neuraminidase, and the RNA polymerase PB1) and two (hemagglutinin and PB1) in 1968 (Kawaoka et al., 1989). These major changes in the antigenicity of an influenza virus are called .antigenic shift (Figure 2). Recently the new pandemic was occurred which is known as pandemic H1N1 2009.

Table 1: Antigenic shifts and pandemics (Kamps et al., 2006)

| | Designation | Resulting Pandemic | Death Toll |
|---|---|---|---|
| 1889 | H3N2 | Moderate | ? |
| 1918 | H1N1 (Spanish) | Devastating | 50-100 million |
| 1957 | H2N2 (Asian) | Moderate | 1 million |
| 1968 | H3N2 (Hong Kong) | Mild | 1 million |
| 2009 | H1N1 (Pandemic 2009) | Mild | ------- |

* H = hemagglutinin; N = neuraminidase

## 3. Diagnosis of influenza viruses

Although virus isolation remains the gold standard of diagnosis and is necessary for virus characterization, rapid laboratory confirmation of suspected influenza disease in routine diagnostic laboratories is usually performed by immunochromatographic or immunofluorescence detection of influenza virus antigens, or reverse transcriptase (RT). Regarding laboratory tests of avian influenza disease; many factors should be considered in deciding which tests to use. Sensitivity, specificity, turn-around-time, repeatability, ease of performance governmental regulation and costs should all be taken into account.

### 4.1 Isolation method

Virus isolation or culture is a technique whereby a specimen is inoculated in a live culture system and the presence of live virus infection is then detected in this culture system. Since the virus replicate in the culture system, it is more sensitive than direct methods, with the exception of RT-PCR. Virus isolation is only of use if the live system or cells are sensitive for the virus that one intends to isolate. Isolation requires the rapid transport of specimens to the laboratory, since delays may lead to inactivation of virus (Allwinn et al., 2002).

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a. Embryonated egg culture

Specimens are inoculated into the amniotic cavity of 9 -11 day embryonated chicken eggs. High yields of virus can be harvested after 3 days of incubation (World Health Organization, 2005). Since this technique requires a supply of fertilized specific pathogen free chicken eggs and special incubators, it is no longer used for the routine diagnosis of influenza infection. However egg isolation provides high quantities of virus and is a very sensitive culture system. Reference laboratories therefore utilize this culture system to ensure high sensitivity and to enable the production of virus stocks for epidemiological monitoring.

b. Cell culture

Conventional culture: Various cell-lines are used to isolate influenza viruses, most commonly primary monkey kidney cells and Madin-Darby canine kidney (MDCK) cells. Some authors recommend the use of trypsin to aid virus entry into the cell lines (World Health Organization, 2005). Conventional cell culture takes up to two weeks but has a very high sensitivity. Cytopathic effects (CPE) such as syncytia and intracytoplasmic basophilic inclusion bodies are observed. The presence of influenza virus can be ascertained using hemadsorption using guinea pig red blood cells (Weinberg et al., 2005), or immunofluorescence on cultured cells. The latter can also be used to type the isolated virus. Immunofluorescence has a higher sensitivity in detection of positive cultures than hemadsorption.

c. Laboratory animals

Ferrets are commonly used in research as a human model but not common in routine diagnosis.

4.2 Serology

Serology refers to the detection of influenza virus-specific antibodies in serum (or other body fluids). Serology can either detect total antibodies or be class-specific (IgG, IgA, or IgM). Different serological techniques are available for influenza diagnosis:

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hemagglutination inhibition (HI), enzyme immunoassays (EIA) and indirect immunofluorescence. Serological diagnosis has little value in diagnosing acute influenza. In order to diagnose acute infection, an at least four-fold rise in titer needs to be demonstrate, which necessitates both an acute and a convalescent specimen. However it may have value in diagnosing recently infected patients. In animals, serological tests are used for sero-surveillance and subtyping purpose and Serology is also used to determine the response to influenza vaccination (Prince and Leber, 2003).

a. Hemagglutination inhibition (HI)

Various red blood cells such as guinea pig, fowl and human blood group "O" erythrocytes are used. A 0.4- 0.5% red blood cell dilution is generally used. Serum is pre-treated to remove non-specific hemagglutinin and inhibitors. A viral hemagglutinin preparation that produces visible hemagglutination (usually 4 hemagglutination units) is then pre-incubated with two-fold dilutions of the serum specimen. The lowest dilution of serum that inhibits hemagglutination is the HI titer. HI is has advantage that it is more specific in differentiating between HA subtypes (Julkunen et al., 1985). HI assays are labor intensive and time- consuming assays that require several controls for standardization. However the assay reagents are cheap and widely available.

b. Enzyme immunoassay (EIA)

Enzyme immunoassay is antibody detecting assay by using specific antigen. EIAs are more sensitive than HI or complement fixation (CF) assays (Bishai and Galli, 1978; Julkunen et al., 1985). Various commercial EIAs are available but are not indicative of acute infection. EIA is also a rapid and easy to use serological test to detect specific antibody against influenza A viruses. The specificity of the assay depends on the purity of the antigen but usually even rather crude viral antigen preparations have been sufficiently specific for diagnostic purpose (Turner et al., 1982; Ukkonen et al., 1980).

c. Indirect immunofluorescence

Indirect immunofluorescence is not commonly used as a method to detect influenza virus antibodies. However, it also has the advantages that pooled antisera can be used to screen for viral infection using a single anti-antibody conjugated to a fluorescence dye (Stevens and Watkins, 1969)

4.3 PCR detection

RT-PCR is generally more sensitive than serology and culture and the combination of RT-PCR with serology more sensitive than the combination of any other two methods (Zambon et al., 2001). RT-PCR can only be performed in well-equipped laboratory facilities by trained personnel. These methods can either detect both influenza A and B or differentiate between types (influenza A or B). The only direct technique that has the potential to differentiate between subtypes (i.e. on the basis of hemagglutinin and neuraminidase) is RT-PCR.

Reverse transcription polymerase chain reaction (RT-PCR) is a process whereby RNA is first converted to complementary DNA (cDNA) and a section of the genome is then amplified through the use of primers that bind specifically to this target area. This allows for exponential amplification of small amounts of nucleic acid, through the action of a thermo stable DNA polymerase enzyme, which enables highly sensitive detection of minute amounts of viral genome. Not only does RT-PCR have superior sensitivity (Steininger et al., 2002) but it can also be used to differentiate between subtypes (Allwinn et al., 2002). RNA degradation of archival samples can decrease the sensitivity of RT-PCR (Frisbie et al., 2004). Therefore specimens should be processed as fast as possible after collection.

4.4 Real-time PCR

The principle of real-time PCR is based on monitoring of a fluorescent signal, which arises during the amplification process. Real-time PCR eliminates post-PCR processing of PCR products. This helps to increase throughput and reduces the chances of carryover contamination. The real-time PCR system is based on the

detection and quantification of a fluorescent reporter (Heid et al., 1996). Taq Man probes are one of the main fluorescence monitoring systems for DNA amplification. Real-time reverse-transcription PCR (rRT-PCR) was initially introduced for AI virus detection in clinical samples in 2001, when it was used for testing poultry in live bird markets (Spackman et al., 2002; Spackman and Suarez, 2005). It was then used to help control an outbreak of low pathogenic avian influenza (LPAI) virus in commercial poultry in 2002 (Akey, 2003), where it was field-validated for use in chickens and turkeys. Since then, a standard protocol has been developed in the United States, where numerous veterinary diagnostic labs have implemented the test. Subsequently, rRT-PCR has been employed as a diagnostic test worldwide during numerous outbreaks for the detection of AI virus infection in numerous species (Cattoli et al., 2004; Spackman et al., 2002; Spackman and Suarez, 2005). Real-time RT-PCR has also been used extensively for wild bird monitoring and in research as an alternative to virus isolation, particularly for quantification (Lee and Suarez, 2004).The primary and most reliable application of rRT-PCR is the detection of any type A influenza virus from any species. Tests for pan-type A influenza detection are often targeted to the conserved matrix (M) or nucleoprotein genes (Spackman et al., 2002; Spackman and Suarez, 2005).

4.5 Other direct antigen detection methods

Different methods exist for direct detection of influenza viruses. Some methods such as enzyme immunoassays (EIAs) can be suitable for bedside testing, others such as direct immunofluorescence allow for the preparation of slides onsite in clinics and the posting of fixed slides to a central laboratory (Allwinn et al., 2002).

In this direct antigen detection, EIAs utilize antibodies directed against viral antigen thatare conjugated to an enzyme. An incubation step with a chromogenic subtrate follows and a colour change is indicative of the presence of viral antigen. Certain enzyme immunoassays as well as similar assays using immunochromatography allow for bedside testing (Allwinn et al., 2002) taking 10-30 minutes. These rapid assays are generally more expensive than direct immunofluorescence or virus culture. Sensitivities of EIAs vary between 64% and 78% (Allwinn et al., 2002). Different rapid

tests can detect either influenza A or B virus without distinguishing the type, influenza A virus only or detect both influenza A and B and identify the type (Herrmann, 1978; Rahman et al., 2007).

For direct immunofluorescence, potentially infected respiratory epithelial cells are fixed to a slide and viral antigens contained in the cells is detected by specific antibodies which are either directly conjugated to a fluorescent dye (direct immunofluorescence) or detected by anti-antibodies linked to a fluorescent dye (indirect immunofluorescence). In both cases reactions are visualised under the fluorescence microscope and positive cells are distinguished on colour intensity and morphology of fluorescent areas.Direct immunofluorescence allows getting faster results but less sensitive than indirect immunofluorescence. Although, some limitations and variation exist in reporting of immunofluorescence tests since interpretation is subjective and accuracy depends on competence and experience of the operator.

4.6 Rapid Tests

The clinical value of a diagnostic test for influenza is to a large extent dependent on the particular test's turnaround time. The first diagnostic tests that were developed for influenza diagnosis were virus isolation and serological assays. At that stage it took more than two weeks to exclude influenza infection. Although shell vial tests have reduced the turn-around time of isolation, they are not generally regarded as rapid tests. The development of direct tests such as immunofluorescence enabled the diagnosis within a few hours (1 to 2 incubation and wash steps). Immunofluorescence tests however necessitate skilled laboratory workers and the availability of immunofluorescence microscopes. Enzyme immunoassay for serology is also a rapid and easy to use technique but the limitation on its specificity and the availability of antigens (Rahman et al., 2007). The later developed antigen detection molecular assays especially real time PCR and RT-PCR are very sensitive and specific technologies. However, the applicable of those techniques still limit in some developing countries and field diagnostic laboratories because sophisticated equipments are needed. A variety of commercial rapid test kits for Influenza detection and typing can be available (Table 2).

Table 2: Rapid Influenza Diagnosis Tests (Kamps et al., 2006)

| Name of test | Influenza types detected | Time for results |
|---|---|---|
| 3M™ Rapid Detection Flu A+B Test | A and B | 15 minutes |
| Direct EZ Flu A+B (Becton-Dickinson) | A and B | Less than 15 minutes |
| BinaxNow Influenza A&B (Inverness) | A and B | Less than 15 minutes |
| Osmo® Influenza A&B (Genzyme) | A and B | Less than 15 minutes |
| QuickVue Influenza Test (Quidel) | A and B | Less than 15 minutes |
| SAS FluAlert (SAS Scientific) | A and B | Less than 15 minutes |
| TRU FLU (Meridian Bioscience) | A and B | Less than 15 minutes |
| XPECT Flu A&B (Remel) | A and B | Less than 15 minutes |

The characteristics of the different test methods available for influenza diagnosis can be compared as shown in Table 3.

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Table 3: Comparison of test characteristics* (Kamps et al., 2006)

| Test | Sensitivity | Turnaround time | Ease of performance | Affordability |
|---|---|---|---|---|
| Direct Detection | | | | |
| Rapid tests (EIA / Chromatography) | -2 | +2 | +2 | 0 |
| Immunofluorescence | 0 | +1 | +1 | +1 |
| Gel electrophoresis RT-PCR | +2 | 0 | -1 | -2 |
| Real-time RT-PCR | +2 | +1 | -1 | -2 |
| Viral Culture | | | | |
| Routine viral culture | +2 | -2 | -1 | +2 |
| Shell viral culture | +1 | 0 | -1 | +1 |
| Serology | | | | |
| EIA | +2 | -2 | +1 | +1 |
| Hemagglutination inhibition | +1 | -2 | -1 | +2 |
| Complement fixation | 0 | -2 | -2 | +2 |

*Relative criteria for favorability of tests (5 point ordinal scale)

-2: very unfavorable characteristic
-1: unfavorable characteristic
0: average characteristic
+1: favorable characteristic
+2: very favorable characteristic

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### 5. Loop mediated isothermal amplification (LAMP) method

LAMP is a sensitive method used to amplify both RNA and DNA under isothermal conditions. The result can be visualized directly by the naked eye upon the addition of an intercalating dye or by gel-electrophoresis. This method is developed based on auto-cycling strand displacement DNA synthesis which is achieved by a DNA polymerase with high strand displacement activity and by using a set of two specially designed inner and outer primers. Initially in the LAMP reaction, all four primers are used but later during the cycling reaction only the inner primers are used for strand displacement DNA synthesis. The inner primers include forward inner primer (FIP) and the backward inner primer (BIP) respectively and each compose of two distinct sequences matching to the sense and antisense sequences of the target DNA, one for priming in the first stage and the other for self-priming in the later stages. The two outer primers consist of namely B3 and F3 complementary to B3c and F3c, respectively. The LAMP reaction is then initiated by addition of the *Bst* DNA polymerase large fragment and carried out at 65$^{\circ}$C for one hour (Notomi et al., 2000). LAMP has been shown to be as sensitive and specific as real-time PCR, and several LAMP assays have been developed to detect different viruses (Boldbaatar et al., 2009; Chen et al., 2010a; Chen et al., 2009; Chen et al., 2010b; Qin et al., 2009). The effects of different concentration of regents on LAMP reactions were examined in a previous report (Blomstrom et al., 2008).

### 6. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) method

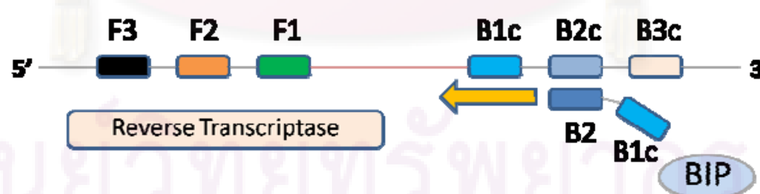
The principle of reverse transcription loop-mediated Isothermal Amplification (RT-LAMP) method was described in the Eiken Genome site. Use the RNA extraction kit to extract the RNA and then transcribe from RNA to cDNA. RT-LAMP method can synthesize cDNA from RNA template by the activity of AMV reverse transcriptase and can apply to amplify and detect the RNA present in the sample. RNA template sample in addition with the reagents of DNA amplification (primers, DNA polymerase with strand displacement activity, substrates, etc.), AMV reverse transcriptase is added to the reaction mixture. After that mixing and incubating at a constant temperature between

60-65°C by using only water bath, both amplification and detection can be carried out in a single step.

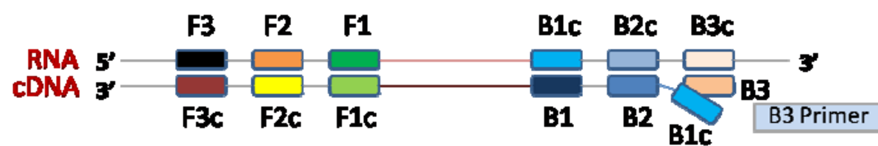
The principle of RT-LAMP reaction includes the reverse transcription steps, which backward inner primer (BIP) anneals to the template RNA and transcribe into cDNA by the activity of reverse transcriptase. Then B3 primer anneals to the region outside the BIP and synthesizes the new cDNA by reverse transcriptase. The single strand cDNA synthesized from BIP is released and FIP primer anneals to the single strand cDNA. In the step of structural formation, FIP linked DNA strand contains complementary sequences at both end which self-anneals and forms a dumbbell-liked structure. The dumbbell-liked structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis. And also BIP plays the same like FIP. During the reaction time, it can produce different size of DNA products because of its self-priming activities. So, we can see ladder liked pattern after gel electrophoresis of LAMP products. The detail of the RT-LAMP reaction processes are as follows.

A. Reverse Transcription Processes

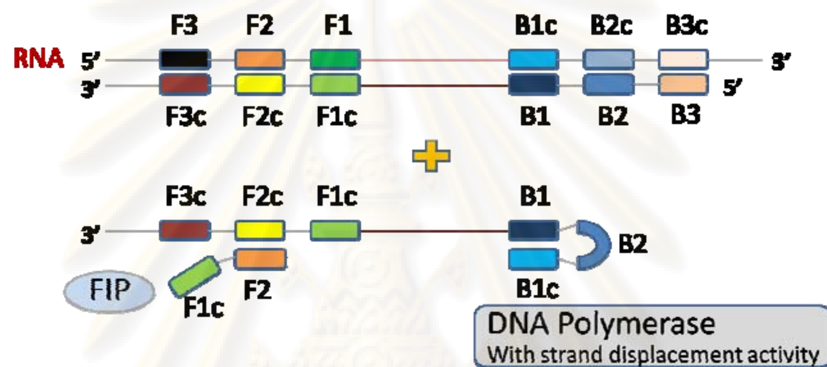
STEP 1: RNA was extract from the samples by using RNA extraction and then prepares the sample solution. The RNA sample solution was mixed with the reaction solution and incubate at a constant temperature between 60-65°C. BIP anneals to the RNA template, and with the activity of reverse transcriptase, cDNA is synthesized.




STEP 2: B3 primer anneals to the region outside of the BIP, with the activity of reverse transcriptase, a new cDNA is being synthesized, while concurrently releasing the cDNA strand previously formed by the BIP.

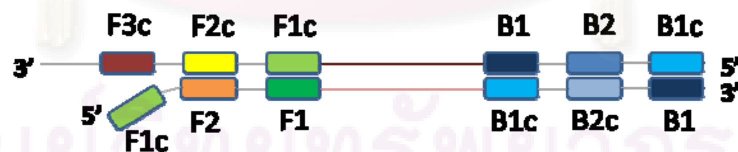



STEP 3: From step (2), the single stranded cDNA synthesized from BIP is released. The FIP then mean to this single strand cDNA.

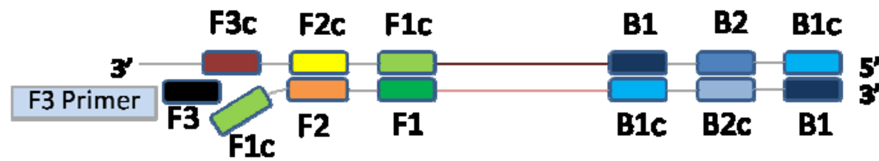



B. Structure Formation Processes

STEP 4: From step (3), through the activity of the DNA polymerase with strand displacement activity, the 3' end of F2 region in FIP becomes the starting point to synthesize complementary DNA strand.

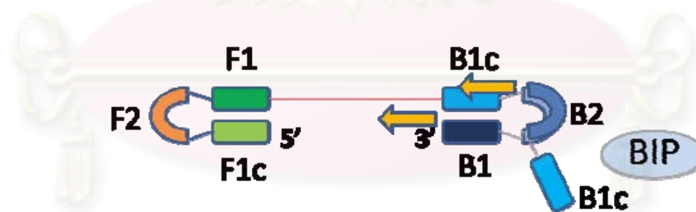



STEP 5: F3 primer anneals to the region outside of FIP and its 3' end becomes the starting point to synthesize while concurrently releasing the DNA strand previously formed by FIP.



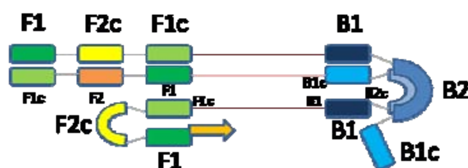

STEP 6: The DNA strand synthesized by F3 primer together with the template DNA strand forms a double stranded DNA.

STEP 7: Since the FIP linked DNA strand, which was released in step (5), contains complementary sequences at both ends, it self-anneals and forms a dumbbell-like structure. This structure (7) becomes the starting structure of the LAMP cycling amplification.

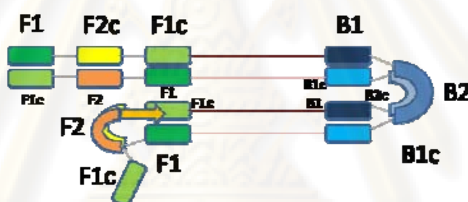



C. LAMP cycling amplification steps

STEP 8: The dumbbell-like DNA structure (7) is quickly converted into a stem-loop DNA by self-primed DNA synthesis, which unfolds the loop at 5' end to extend DNA synthesis. The BIP anneals to the single stranded region in the stem-loop DNA to start DNA synthesis in step (8) while releasing the previously synthesized strand.




STEP 9: This released single strand forms a stem-loop structure at the 3’ end because of complementary F1c and F1 regions. Then, starting from the 3’ end of the F1 region, DNA synthesis starts using self-structure as a template, and releases BIP-linked complementary strand. Structure 9 is formed.




STEP 10. The released BIP-linked single strand then forms a dumbbell-like structure as both ends have complementary F1-F1c and B1c-B1 regions, respectively. This structure is the turn-over structure of structure (7).

STEP 11. Similar to step (7), structure (10) proceeds self-primed DNA synthesis starting from the 3’ end of the F1 region. Furthermore, the FIP anneals to the F2c region and starts synthesizing DNA strand. This FIP-linked DNA strand is released by the strand displacement of self-primed DNA synthesis. Accordingly, similar to step (7), (8) and (10), step (10) and (11) proceeds, and structure (7) is once again being formed.

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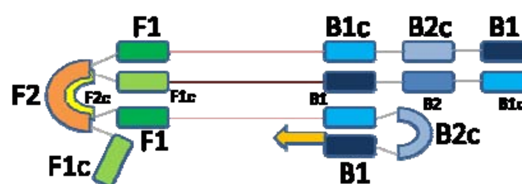
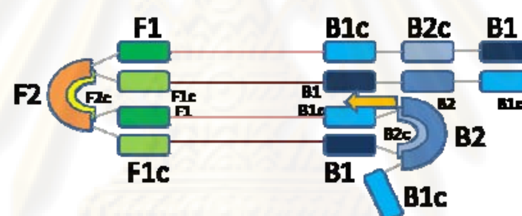



STEP 12. With the structure produced in step (9) (or step (12)), the FIP (or BIP) anneals to the single stranded F2c region (or B2c region), and DNA synthesis continues by releasing double stranded DNA. As a result of this process, various sized structures consisting alternately inverted repeats of the target sequence on the same strand are formed.




7. Detection of LAMP products

There are three methods used to detect amplified DNA products from the RT-LAMP products; (1) agarose gel electrophoresis: 5 µl aliquot of the RT-LAMP reaction mixture was separated on a 2% agarose gel stained with ethidium bromide. (2) Visual observation of white precipitates formed by magnesium pyrophosphate in the positive RT-LAMP reaction mixture. This method is less sensitive but volatilization of RT-LAMP product can be avoided. (3) Colorimetric assay: this method is performed by using intercalating dyes for double stranded DNA (dsDNA) and can detect the DNA products by visual inspection. The most popular intercalating dye is SYBR Green I (Invitrogen) which is added to the RT-LAMP reaction mixture after the reaction and reaction mixture will change color from yellow to fluorescence green when it is positive (Newhouse et al., 2009; Njiru et al., 2008).

CHAPTER III

# MATERIALS AND METHODS

In this study, the experiment was conducted with 3 steps (Figure 2): including Step 1: Designing the LAMP primers set and optimization of the amplification conditions Step 2: Tests for detection limit and specificity of the assay, Step 3: Visual detection of LAMP products and testing on spiked samples and blind samples.

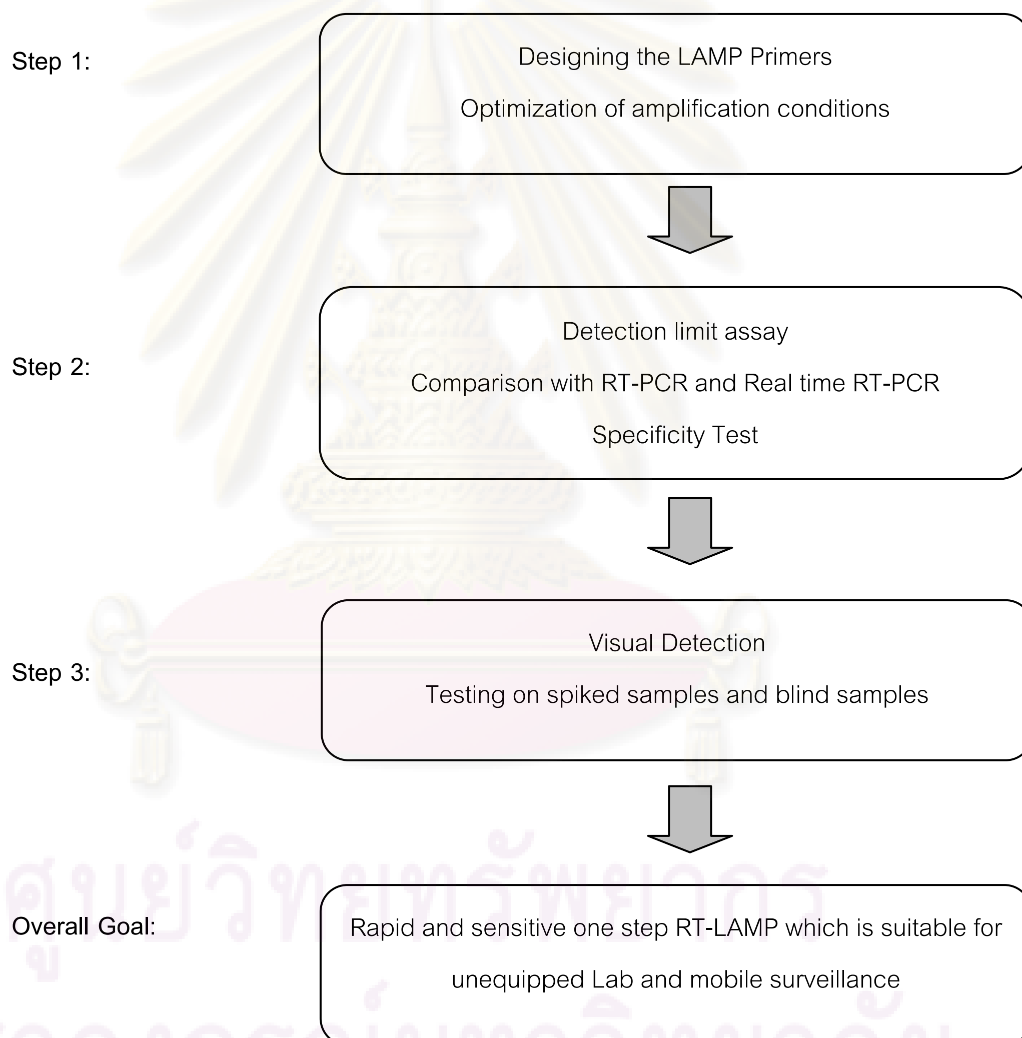



Figure 2: The conceptual framework in this study

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**Step 1: Designing the LAMP primers set and optimization of the reaction time and temperature**

1.1 Designing the LAMP primers set

M genes sequences of Thailand influenza isolates including both human and animal viruses were retrieved from Gene Bank database and aligned by using MEGA 4. The conserve region (approximately 200 base pairs) was identified for the LAMP primer designing. The RT-LAMP primer set including F3, B3, FIP and BIP were designed based on this 200 base pairs conserved region by using PrimerExpoler v4 software.

In detail, the LAMP primers were designed based on the six distinct regions (F3, F2, F1, B3, B2 and B1) on the target sequence, A/chicken/Thailand/CUK2/04 (H5N1). Forward inner primer (FIP) was designed by including F2 sequence of it 3' end which complementary to F2c region and the same sequence as F1c region at 5' end. Furthermore, backward inner primer (BIP) was designed including both B2 and B1c. To command the PrimerExpoler v4, easy mode was used. The Tm for each region is designed to be about 63°C (60 - 65°C) for F1c and B1c and about 55°C (53 - 58°C) for F2, B2, F3, and B3. The 3' ends of F2/B2, F3/B3, and the 5' end of F1c/B1c are designed by the free energy ($\Delta G$) of 4 kcal/ mol or less. Primers were designed by GC content of 40 to 60 %.

After primer designs for the entire target sequences were obtained, the best primer set was selected. Follow by changing the nucleotides of each primer to multi-nucleotides on mutation sites. But we paid attention to avoid too much multi-nucleotides in each primer. We followed the instruction of manual book to design the best LAMP primer set.

1.2 Optimization of the amplification conditions

To amplify the target sequence of a gene by RT-LAMP method, there are some factors affecting to the amplification condition. Bst DNA polymerase plays a crucial role during the LAMP assay. *Bst* DNA polymerase is activated in the temperature range of 60 to 65°C and around an hour to finish the amplification. In addition, concentration of MgSO4 also effects on amplification condition. Therefore, an evaluation of the effects

on the one step RT-LAMP assay e.g. different concentrations of MgSO4 and Bst DNA Polymerase, the different amplification temperature and reaction time were carried out.

The reaction of the LAMP assay was performed in a 25μl reaction mixture including 1X ThermoPol buffer (NEB, USA) which contain 20mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1 % Triton X-100 and pH 8.8 @ 25°C, 0.4mM dNTPs, 0.2μM each F3 and B3 primers, 1.6 μM of FIP and BIP primers and 0.04 M betaine (Sigma). For reverse transcription in a single step, 5U cloned AMV reverse transcriptase was added. 2μl of RNAs were added into the reaction mixture.

The effect of different MgSO4 concentration on amplification condition was evaluated by using 2, 4, 6 and 8mM of MgSO4 Different concentrations of *Bst* DNA polymerase (0.5, 1 and 1.5 Unit) were added to the reaction mixture and evaluated their effects on amplification reaction. Different time (30, 40, 50, 60 and 70 minutes) and temperature (60, 63, 65$^{o}$ C) were applied to evaluate their effects. When evaluation on MgSO4 and *Bst* DNA polymerase concentration, recommended temperature and time (63C for 1 hour) were applied. To terminate the amplification reaction, temperature was raised to 80$^{o}$ C as recommended. The procedure of the LAMP assay is shown in figure 5 (Figure 5).

**Step 2: Detection limit and specificity tests of the LAMP assay**

2.1 Selections of standard influenza isolates

Influenza viruses from both human and animal hosts were selected to evaluate the sensitivity of this method. At least 10 isolates from each of human and animal hosts were selected to detect the sensitivity of this RT-LAMP method. Standard avian influenza isolate (A/chicken/Thailand/CUK2/04 (H5N1)) from Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University was used as a positive control to optimize the LAMP reaction and to detect the sensitivity. Avian influenza isolates were received from Veterinary Diagnosis Laboratory and the Department of Pathology, Faculty of Veterinary Science. Human influenza isolates were received from Faculty of Medicine, Chulalongkorn University. The following table showns the list of isolates which were used in this study.

Table 4: List of Influenza isolates used in this study

| Sample ID | Subtype | Host | HA test |
|---|---|---|---|
| CUK2 (H5N1) | H5N1 | Chicken | Positive |
| H7N4 (duck) | H7N4 | Duck | Positive |
| H3N2 Duck | H3N2 | Duck | Positive |
| 31 | H5N1 | Chicken | Positive |
| 212 | H5N1 | Chicken | Positive |
| BB205 | H5N1 | Chicken | Positive |
| BB177 | H5N1 | Duck | Positive |
| BB219 | H5N1 | Chicken | Positive |
| 13/6 | H5N1 | Chicken | Positive |
| 15/6 | H5N1 | Chicken | Positive |
| 23/6 | H5N1 | Chicken | Positive |
| 27/6 | H5N1 | Chicken | Positive |
| 45/6 | H5N1 | Chicken | Positive |
| 48/6 | H5N1 | Chicken | Positive |
| 47/6 | H5N1 | Chicken | Positive |
| 100/6 | H5N1 | Chicken | Positive |
| 101/6 | H5N1 | Chicken | Positive |
| 06cb1 | H1N1 | Swine | Positive |
| 05cb2 | H3N2 | Swine | Positive |
| H1N2 Swine | H1N2 | Swine | Positive |
| k1 | H1N2 | Swine | Positive |
| k2 | Not finished sub-typing | Swine | Positive |
| k3 | Not finished sub-typing | Swine | Positive |
| Ms | Not finished sub-typing | Swine | Positive |
| k4 | H1N2 | Swine | Positive |
| 17 | H1N2 | Swine | Positive |
| 412 | H1N1 | Swine | Positive |
| 6.3 | H3N2 | Swine | Positive |

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| H286 | H1N1 (seasonal) | Human | Positive |
|---|---|---|---|
| B1257 | H1N1 (seasonal) | Human | Positive |
| B1463 | H3N2 (seasonal) | Human | Positive |
| B1425 | H3N2 (seasonal) | Human | Positive |
| B1456 | H1N1 2009 | Human | Positive |
| B1447 | H1N1 2009 | Human | Positive |
| B42 | H3N2 (seasonal) | Human | Positive |
| H16 | H1N1 (seasonal) | Human | Positive |
| H565 | H1N1 2009 | Human | Positive |
| H611 | H1N1 2009 | Human | Positive |
| H612 | H1N1 2009 | Human | Positive |
| H613 | H1N1 2009 | Human | Positive |
| H615 | H1N1 2009 | Human | Positive |
| H647 | H1N1 2009 | Human | Positive |
| H653 | H1N1 2009 | Human | Positive |
| H657 | H1N1 2009 | Human | Positive |
| H671 | H1N1 2009 | Human | Positive |
| H688 | H1N1 2009 | Human | Positive |
| H705 | H1N1 2009 | Human | Positive |
| B5E1A | H1N1 2009 | Human | Positive |
| B938 | H1N1 2009 | Human | Positive |
| H340 | H1N1 2009 | Human | Positive |

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2.2 Detection limit and comparison of detection limit with RT-PCR and Real-time PCR

The amount of viral RNA was measured by the NanoDrop 1000 Spectrophotometer (Thermo Scientific) according to the recommendation of manufacturer and was recorded as the concentration (µg/µl). Serial dilution of viral RNA in RNAse free distilled water was performed.

The sensitivity of optimized RT-LAMP method was evaluated by using those serially diluted viral RNA. The serially diluted RNA samples used in RT-LAMP assay

were tested by both conventional RT-PCR and real time RT-PCR using the primers for conserved region on M gene. The comparison of the detection limits to those of three assays was conducted. Figure 3 shows the work flow about comparison of detection limit among different assays.

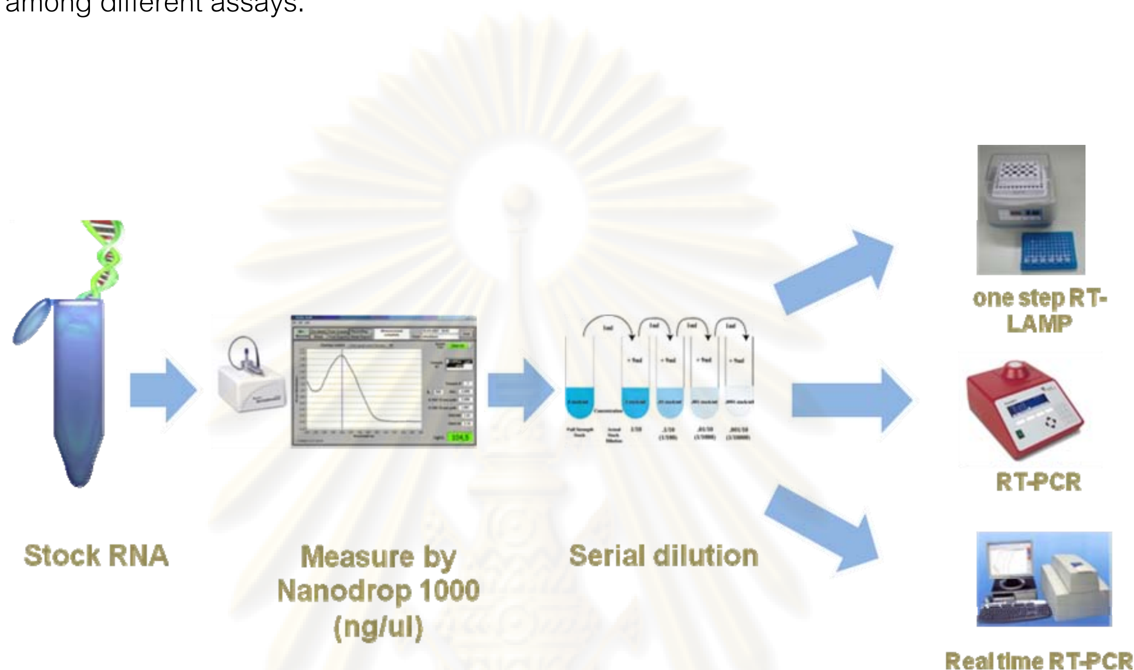


Figure 3: Comparison of detection limit among three assays

2.3 Specificity Test

The specificity of RT-LAMP was tested against other RNA viruses of poultry and swine diseases i.e. porcine reproductive and respiratory syndrome virus (PRRSV), classical swine fever virus (CSFV), Infectious bronchitis virus (IBV) and Newcastle disease virus (NDV) and also human influenza B virus. The criteria to choose the other RNA viruses for specificity test are RNA viruses which cause major problem and epidemic outbreaks in animal industry and also depend on the availability of the isolates. The specificity test was compared with conventional RT-PCR by using the specific primers on the conserved region of Matrix gene.

2.4 Test on influenza viruses from both human and animal hosts

At least 10 influenza isolates (known influenza subtypes) from both human and animal hosts were used to test the specificity of the assay.

Step 3: Visual detection of LAMP products and testing on spiked and blind samples

3.1 Visual detection of LAMP products by SYBR Green

Visual analysis by colorimetric method was developed in this study. The aim of using colorimetric assay is to replace the gel electrophoresis, SYBR Green I (Invitrogen) was used as a colorimetric assay. 1µl to 1/10 dilution of SYBR Green I was added to the LAMP products and direct inspection to the color changes. The positive reaction was changed to green and negative can be shown as brown color (Figure 4).

Figure 4: Visual detection of LAMP products

3.2 Testing on spiked samples and blind samples

The viral solution was spiked into both pure viral transport media (VTM) and VTM with cloacal and tracheal swabs. The positive viral solution was spiked into both VTM alone and VTM with cloacal and tracheal swabs from pathogens free chickens. Negative control (nuclease free water) was also placed into both VTM and VTM with cloacal and tracheal swabs. After that RNA was extracted from every spiked sample and the samples were then tested by the developed one step RT-LAMP. Each sample was duplicated to confirm the results. In addition, the samples were randomly assigned with blind numbers by one graduate student. The samples were then tested by developed one step RT-LAMP method. The blind numbers were then decoded. The results were then evaluated for the sensitivity and specificity of the assay.

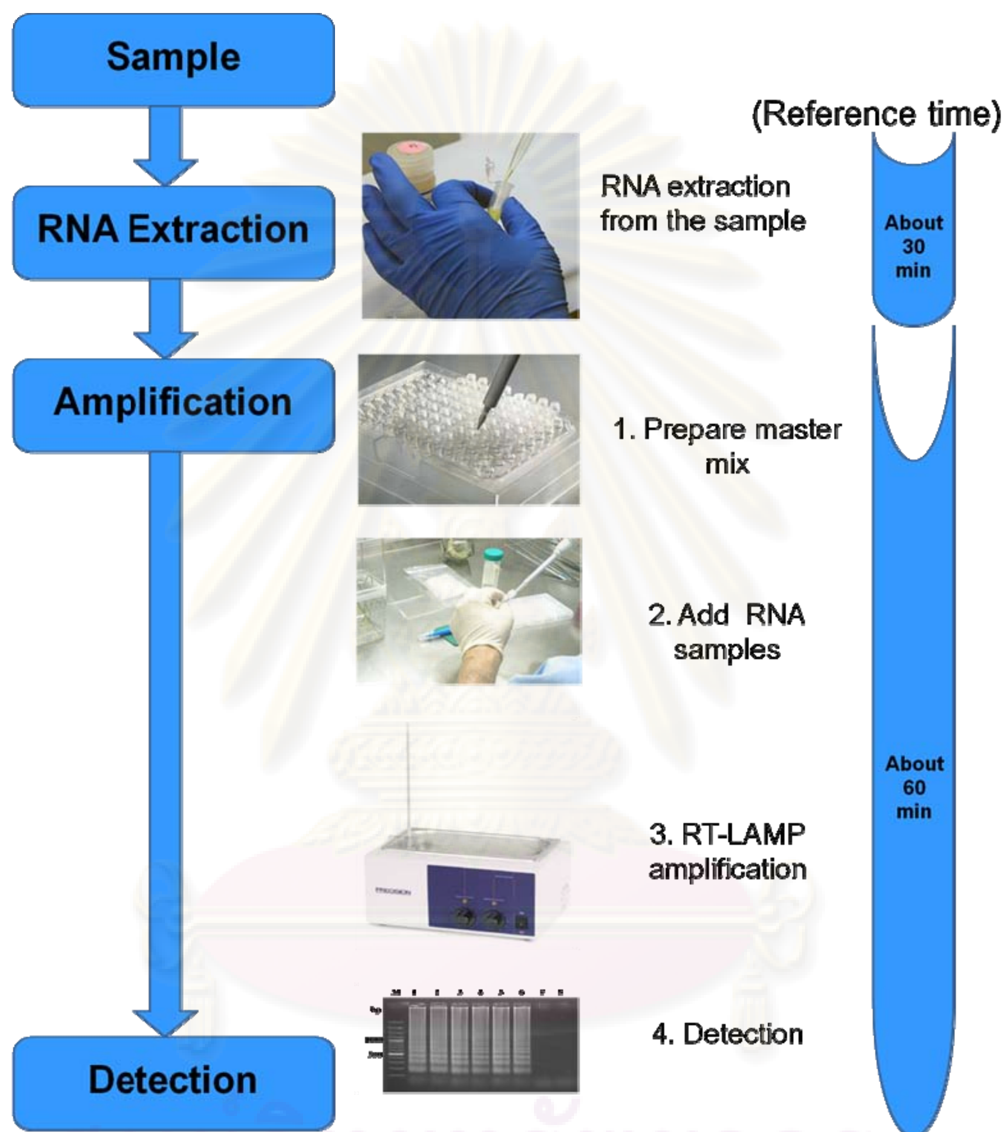


Figure 5: Flow chart for RT-LAMP Method

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# CHAPTER IV

# RESULTS

## 1. Designing the LAMP primer set

By using the PrimerExpoler 4 program, one primer set was designed according to the program's recommendation. The details of designed LAMP primer set are shown in the Table 6 and the location of each primer on target sequence is also shown in Fig 6. The designed primer set has the Tm for each region about 63°C (60 - 65°C) for F1c and B1c and about 55°C (53 - 58°C) for F2, B2, F3, and B3. The 3' ends of F2/B2, F3/B3, and the 5' end of F1c/B1c were designed by the free energy ($\Delta$G) of 4 kcal/ mol or less. Primers contain GC content of 40 to 60 %. The total size of the product quoted by the designed primer set is 192 base pairs. Length of each primer segment is not over 25 nucleotides.

Table 6: Details of the LAMP primers

| label | 5'pos | 3'pos | length | Tm | 5'dG | 3'dG | GC rate | Sequence |
|---|---|---|---|---|---|---|---|---|
| F3 | 199 | 217 | 19 | 55.64 | -4.45 | -6.19 | 0.47 | TCTCTCTATCATCCCGTCA |
| B3 | 410 | 429 | 20 | 55.58 | -4.16 | -5.40 | 0.40 | CCATTTCCATTTAGGGCATT |
| FIP | | | 38 | | | | | TGAGAGCCTCGAGATCGGTG-AGATCGCGCAGAAACTTG |
| BIP | | | 42 | | | | | CCTCTGACTAAAGGGATTTTGGGA-CTGGACAAAGCGTCTACG |
| F2 | 234 | 251 | 18 | 57.13 | -4.92 | -4.16 | 0.50 | AGATCGCGCAGAAACTTG |
| F1c | 274 | 293 | 20 | 62.74 | -4.60 | -6.19 | 0.60 | TGAGAGCCTCGAGATCGGTG |
| B2 | 392 | 409 | 18 | 56.77 | -5.35 | -4.76 | 0.56 | CTGGACAAAGCGTCTACG |
| B1c | 326 | 349 | 24 | 61.97 | -5.19 | -5.37 | 0.46 | CCTCTGACTAAAGGGATTTTGGGA |

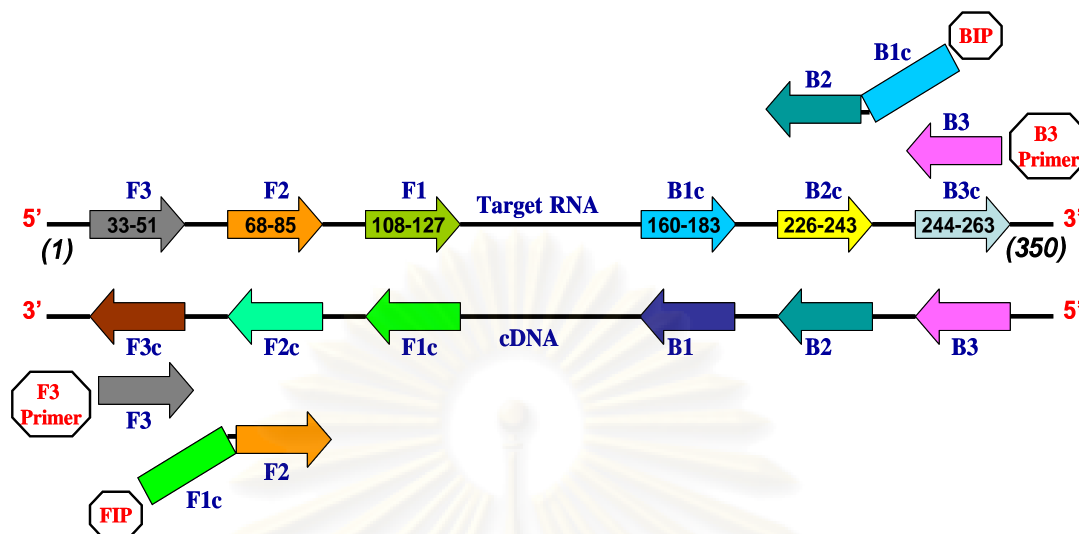


Figure 6: Location of LAMP primers on the target sequence

The locations of each primer in target sequence are shown in Figure 6. After designed by PrimerExpoler 4, the primers were confirmed by using alignments data, then high probable mutation sites were modified by degenerated nucleotides. The following shown the finalized primer sequences which were used in the study.

F3-AA2 5' TCTCTCTATCATCCCRTCA 3'

B3-AA2 5' CCATTTCCATTTAGGGCRTT 3'

FIP-AA2 5' TGAGAGCCTCRAGATCGGTG – AGATCGCGCAGARACTTG 3'

BIP-AA2 5' CCTCTGACTAAAGGGATTTTGGGA – CTGGACAAAGCGTCTACG 3'

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## 2. Optimum amplification conditions for LAMP assay

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Effect of Bst DNA polymerase

Figure 7 shows that the LAMP amplification can be activated at the present of *Bst* DNA polymerase at the minimum concentration of 0.75 Units.

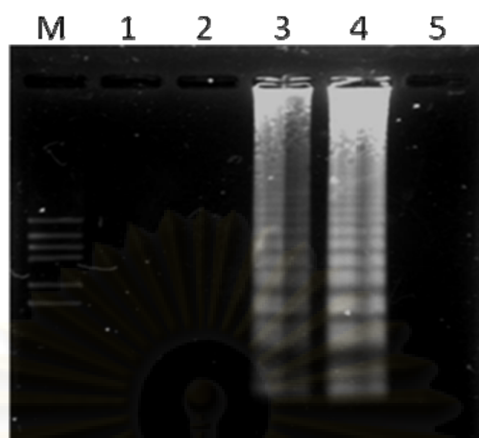


Figure 7: Effect of Bst DNA polymerase on LAMP amplification. Lane M: Marker, Lane 1-4: 0.25, 0.5, 0.75 and 1 Unit of Bst DNA Polymerase, Lane 5: negative control.

<u>Effect of MgSO4</u>

Figure 8: Effect of MgSO4 on LAMP amplification. Lane M: Marker, Lane 1-4: 2, 4, 6 and 8 mM of MgSO4 and Lane 5: negative control

The effects of MgSO4 concentration on LAMP amplification condition were evaluated by different concentration of MgSO4 (2, 4, 6 and 8 milimolars). Figure 8 shows that the LAMP amplification can actively provide the result in gel electrophoresis when minimum 4 mM of MgSO4 was added. The better result can be shown in higher concentration of 6 milimolars.

<u>Effect of temperature and amplification time</u>

LAMP assay can be active on different temperature and shows ladder shape bands in a range of temperature 60 to 65$^{O}$C. And also the amplification can be started if the reaction takes at least 50 minutes and onwards (Figure 9).

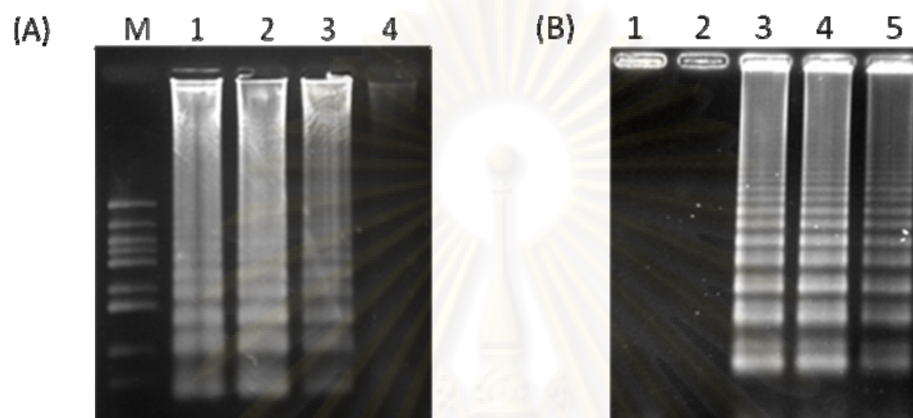


Figure 9: Effect of temperature and amplification time on LAMP amplification. (A) The effect of temperature: lane 1-3 (60, 63 and 65$^{o}$C), lane 4: negative control. (B) The effect of reaction time: lane 1-5 (30, 40, 50, 60 and 70 minutes).

**3. Detection limit and comparison of detection limit with RT-PCR and Real-time PCR**

Table 7: Comparison of detection limits between RT-LAMP, RT-PCR and Real time RT-PCR

| RNA serial dilution | RT-LAMP | RT-PCR | RRT-PCR (Ct) | RNA concentration per 1X reaction volume | |
|---|---|---|---|---|---|
| | | | | ug | pg |
| 10E7 Log EID 50 | Positive | Positive | 20.9 | 170 | 170000 |
| 10E6 Log EID 50 | Positive | Positive | 24.54 | 17 | 17000 |
| 10E5 Log EID 50 | Positive | Positive | 27.51 | 1.7 | 1700 |
| 10E4 Log EID 50 | Positive | Positive | 30.36 | 0.17 | 170 |
| 10E3 Log EID 50 | Positive | Positive | 33.65 | 0.017 | 17 |
| 10E2 Log EID 50 | Positive | Negative | 36.68 | 0.0017 | 1.7 |
| 10E1 Log EID 50 | Negative | Negative | 39.19 | 0.00017 | 0.17 |

*Ct 36 and above is positive, between 37 to 40 is suspected and over 40 is negative in term of interpretation on Ct value from real time PCR

Figure 10 shows the detection limit of different molecular assays in detection of influenza A virus by using the primers which is specific for conserved region on M gene. In term of comparison among three kinds of assay, our developed one step RT-LAMP showed ten times more sensitive than conventional RT-PCR. The developed assay can be worked out when 1.7 Pico grams (pg) of target RNAs were included in the samples (Table 7). And also the detection limit of developed assay can be compared with the sensitive molecular assay, real time RT-PCR which is very useful in most of the laboratories for screening influenza A viruses.

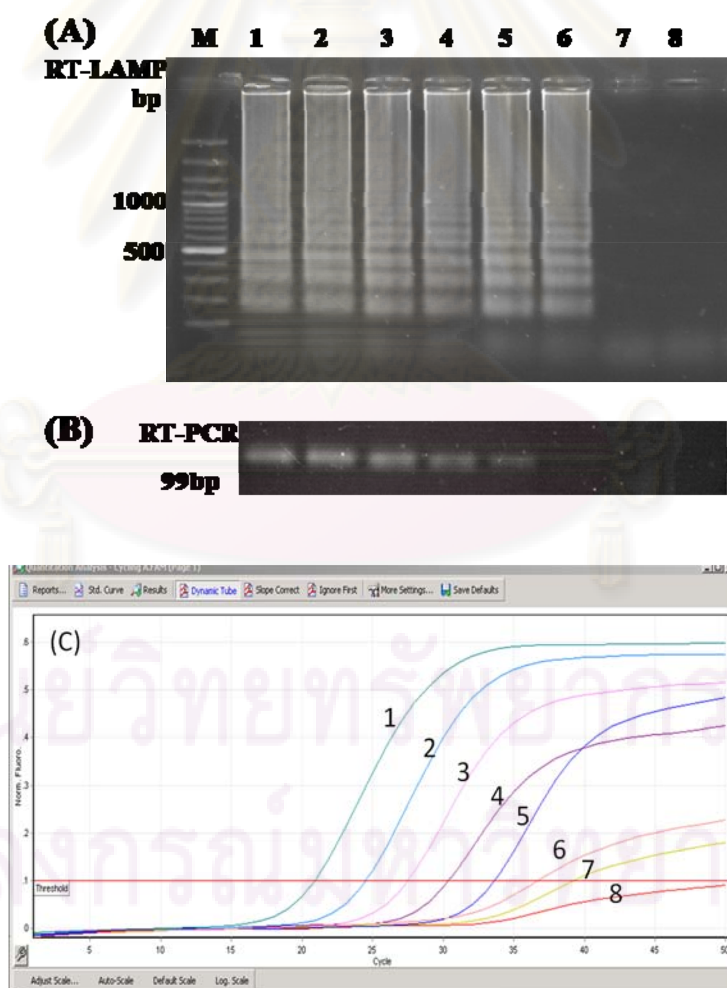


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Figure 10: (A) The sensitivity of RT-LAMP, (B) Conventional one step RT-PCR and (C) Real-time RT-PCR. (A, B) Lane M: DNA markers, lane 1-7: 170,000pg, 17,000pg,

1,700pg, 170pg, 17pg, 1.7pg and 0.17pg of RNAs respectively and lane 8: negative control. (C) Line 1-7: 170,000pg (Ct 21), 17,000pg (Ct 24), 1,700pg (Ct 27), 170pg (Ct 30), 17pg (Ct 33), 1.7pg (Ct 36) and 0.17pg (Ct 39) of RNAs and line 8: negative control.

### 4. Visual detection of LAMP products by SYBR Green

The result of colorimetric assay by using SYBR green shows no different sensitivity with the conventional method, gel electrophoresis in 2% agarose gel (Figure 11).

Figure 11: Comparison between colorimetric detection and gel electrophoresis of LAMP products. N: negative control, 1: negative and 2-7: positive.

### 5. Specificity of the LAMP assay

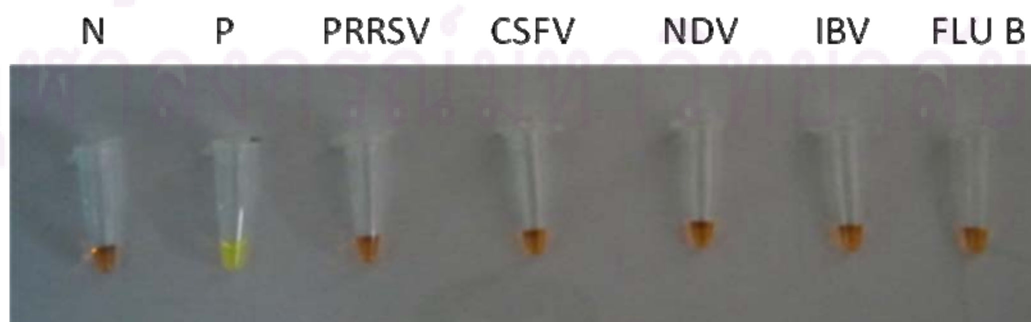

Figure 12: Specificity test by RT-LAMP assay. N: negative control, P: positive control, PRRSV: porcine reproductive and respiratory syndrome virus, CSFV: classical swine fever virus, NDV: newcastle disease virus, IBV: infectious bronchitis virus, FLU B: influenza B virus.

Our finding shows that there are no cross reaction with other important veterinary RNA viruses and human influenza B (Figure 12). This result indicated that the developed RT-LAMP assay is very specific for influenza A viruses.

**6. Test results on influenza viruses of both human and animal**

Table 8 shows the sensitivity of LAMP assay for the detection of both human and animal influenza viruses.

Table 8: Results of RT-LAMP detection on influenza A isolates from various sources

| Sample ID | Subtype | Host | RT-LAMP test |
|---|---|---|---|
| CUK2 (H5N1) | H5N1 | Chicken | Positive |
| H7N4 (duck) | H7N4 | Duck | Positive |
| H3N2 Duck | H3N2 | Duck | Positive |
| 31 | H5N1 | Chicken | Positive |
| 212 | H5N1 | Chicken | Positive |
| BB205 | H5N1 | Chicken | Positive |
| BB177 | H5N1 | Duck | Positive |
| BB219 | H5N1 | Chicken | Positive |
| 13/6 | H5N1 | Chicken | Positive |
| 15/6 | H5N1 | Chicken | Positive |
| 23/6 | H5N1 | Chicken | Positive |
| 27/6 | H5N1 | Chicken | Positive |
| 45/6 | H5N1 | Chicken | Positive |
| 48/6 | H5N1 | Chicken | Positive |
| 47/6 | H5N1 | Chicken | Positive |

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| 100/6 | H5N1 | Chicken | Positive |
|---|---|---|---|
| 101/6 | H5N1 | Chicken | Positive |
| 06cb1 | H1N1 | Swine | Positive |
| 05cb2 | H3N2 | Swine | Positive |
| H1N2 Swine | H1N2 | Swine | Positive |
| k1 | H1N2 | Swine | Positive |
| k2 | Not finished sub-typing | Swine | Positive |
| k3 | Not finished sub-typing | Swine | Positive |
| Ms | Not finished sub-typing | Swine | Positive |
| k4 | H1N2 | Swine | Positive |
| 17 | H1N2 | Swine | Positive |
| 412 | H1N1 | Swine | Positive |
| 6.3 | H3N2 | Swine | Positive |
| H286 | H1N1 (seasonal) | Human | Positive |
| B1257 | H1N1 (seasonal) | Human | Positive |
| B1463 | H3N2 (seasonal) | Human | Positive |
| B1425 | H3N2 (seasonal) | Human | Positive |
| B1456 | H1N1 2009 | Human | Positive |
| B1447 | H1N1 2009 | Human | Positive |
| B42 | H3N2 (seasonal) | Human | Positive |
| H16 | H1N1 (seasonal) | Human | Positive |
| H565 | H1N1 2009 | Human | Positive |
| H611 | H1N1 2009 | Human | Positive |
| H612 | H1N1 2009 | Human | Positive |
| H613 | H1N1 2009 | Human | Positive |
| H615 | H1N1 2009 | Human | Positive |
| H647 | H1N1 2009 | Human | Positive |
| H653 | H1N1 2009 | Human | Positive |
| H657 | H1N1 2009 | Human | Positive |
| H671 | H1N1 2009 | Human | Positive |

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| H688 | H1N1 2009 | Human | Positive |
|---|---|---|---|
| H705 | H1N1 2009 | Human | Positive |
| B5E1A | H1N1 2009 | Human | Positive |
| B938 | H1N1 2009 | Human | Positive |
| H340 | H1N1 2009 | Human | Positive |

7. Accuracy of test

| | | Reference Test (Viral isolation & RT-PCR) | |
|---|---|---|---|
| | | Positive | Negative |
| Blind Test (RT-LAMP) | Positive | 12 | 0 |
| | Negative | 0 | 6 |

From the findings from blind samples test, the test results show 100 % concordant with blind data (Appendix B). This result shows that there is no bias during the study and the obtained result is reliable.

8. Test on viral spiked samples

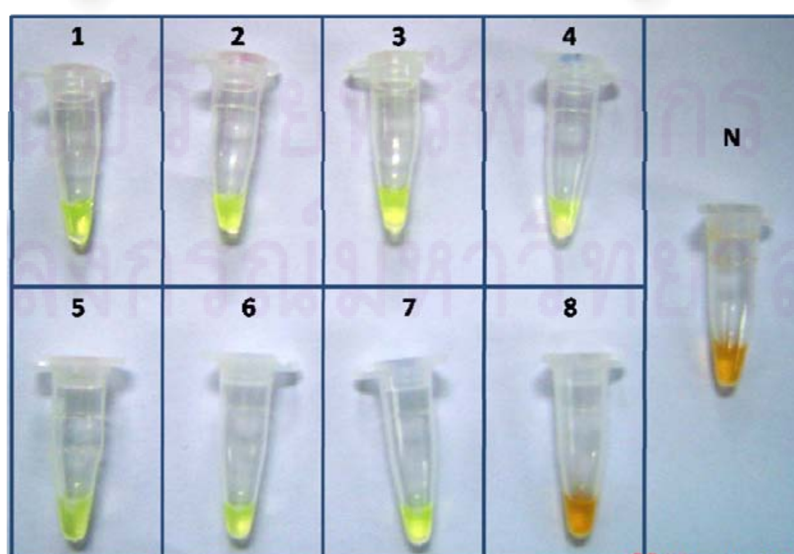


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Figure 13: Test on viral spiked samples. 1,5: Viral spiked in normal saline, 2,6: viral spiked in cloacal swab with VTM, 3,7: viral spiked in tracheal swab with VTM, 4,8: viral spiked in excess fecal samples with VTM, N: negative control.

Figure 13 shows that different kinds of samples can be detected by our developed assay but one of the viral spiked in excess fecal sample can not be detected out of duplications. It may be because the excess fecal sample interferes in RNA extraction process. So the better choice of sample for our developed assay is cloacal and tracheal swab samples.

Furthermore, the serial dilution of viral solution were spiked and tested by developed assay. Figure 14 reveals that the developed assay can detect at the minimum of $10^2$ Log EID 50 of viruses titer containing in cloacal swab samples. This finding also consent the detection limit assay of our developed assay in this study.

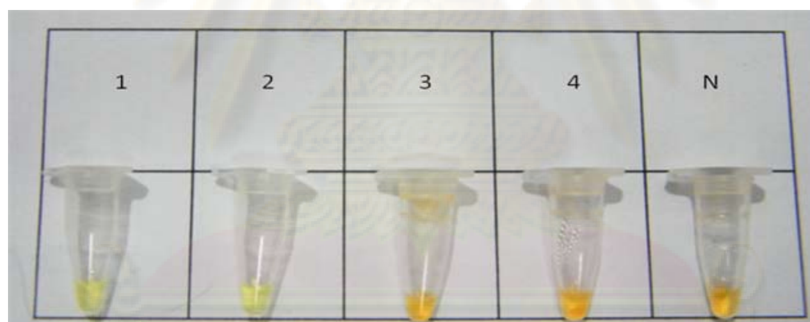


Figure 14: Detection limit by using cloacal swab samples. 1: $10^3$, 2: $10^2$, 3: $10^1$, 4: $10^0$ Log EID 50 respectively, N: negative control.

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# CHAPTER V
# DISCUSSION

Influenza A virus is concerning with one of the major crisis in the world so called Flu War. Most of the public health authorities are worrying about the emerging pandemic of influenza and high-throughput rapid diagnosis assays may help to control and response to Flu attacks. In most of developing countries, the influenza outbreaks in both human and animals are endemic and outbreak reports were uploading to World Health Organization (WHO) and World Animal Organization (OIE). However, there are still limitations in applying the recommended molecular assays in most of the developing countries because its requirements for a high precision thermocycler and elaborate methods for detecting the amplified products (Alexander, 2008; Lu, 2006). Therefore, this study intended to develop the one step RT-LAMP assay to overcome this gap in most developing countries.

LAMP is a very sensitive PCR based technique and can apply without any special equipment. The primer in the previous study can detect only human influenza and can not detect the animal influenza. And also it takes longer duration because of needing two steps to perform RT-LAMP (Poon et al., 2005). But the designed primer set in this study can detect most of the influenza A viruses from both human and animals including the recent pandemic influenza (2009 H1N1). The primer designed in the previous study is not laid on the conserved region of M genes from both human and animal influenza viruses. In this study, we identified the conserved region on different M genes sequences of influenza viruses and modified the mononucleotide into degenerated nucleotides in particular single nucleotide polymorphism (SNP). The G/C content and Tm of the designed primer set are also within the standard ranges. The location of designed primer set is also somewhat overlapping with WHO recommended primer using in influenza A detection by RT-PCR and real time RT-PCR.

LAMP assay has been developed for the detection of other gene of influenza A. For examples, Ito et al., 2006 developed a LAMP detection of influenza A typing based on the HA gene. But HA gene is a very diverse gene among influenza A subtypes so it

may not the right choice to use HA gene for detection of influenza A. Some researches reported the application of LAMP technique and identified the factor influencing on LAMP reaction such as MgS04. From our experiment, the amplicons can be seen by gel electrophoresis with minimum 4mM of MgSO4 but the best result shows at 6-8mM of MgSO4.

*Bst* DNA polymerase plays a crucial role in LAMP technique. *Bst* DNA polymerase is derived from *Bacillus stearothermophilus* and the recommended concentration for most of the LAMP assays is 1 Unit per reaction (Notomi et al., 2000). However, most researches did not rule out the minimum concentration of *Bst*. In this study, the effect of *Bst* DNA polymerase concentration on our developed assay and its compatibility with designed primer were tested. The findings show that our developed assay can worked with the presence of minimal 0.75 Units of *Bst* DNA polymerase. But for the better result and safety margin should be 1 units and it agree with the standard recommendations.

*Bst* DNA polymerase can be activated at the temperature within 60$^{o}$C and 65$^{o}$C (Notomi et al., 2000). From the findings, the optimum temperature for amplification is 63$^{o}$C but all three temperatures (60, 63 and 65$^{o}$C) tested provided positive amplicons. This finding is also identical with the former research done for shrimp viruses (Puthawibool et al., 2009). When we choose the optimum amplification temperature (63$^{o}$C), we selected the temperature for the clearest of the result and the temperature can allow lower and upper boundaries. Finally the 63$^{o}$C is an optimal temperature applying for this developed assay.

In this study, we used only 2 sets of primer, both inner and outer but excluded the loop primer set. If the loop primer set is applied into the amplification the time to produce stratified amounts of amplicons can be reduced (Notomi et al., 2000). In our findings, the amplicons were started to produce at 50 minutes after amplification started. However, we choose 60 minutes for amplification time because it will provide enough quantity of amplicons for gel electrophoresis and visual detection. This interpretations also identical to the former findings (Boldbaatar et al., 2009; Chen et al., 2009; Newhouse et al., 2009; Qin et al., 2009; Xu et al., 2009). Base on the previous data, if the

loop primer was added, the amplification time can be reduced to 30 minutes (Blomstrom et al., 2008).

LAMP is a very sensitive technique in which high amounts of amplicons can be obtained when the present of ten copies of pathogen's genome (Notomi et al., 2000). The detection limit of this assay is ten times more sensitive than conventional RT-PCR. However, some LAMP assay can be sensitive 100 to 1000 times compare with RT-PCR (Imai et al., 2006). The lower detection limit of developed assay can compare with the real time RT-PCR threshold cycle 36.68. Our results indicated the lower detection limit of developed LAMP assay is 1.7 Pico grams RNA concentration and 10 times more sensitive than conventional RT-PCR.

To replace the using of gel electrophoresis for detecting amplicons, the colorimetric assay was used. The intercalating dye used in this study is SYBR Green I which is save to handle when other are carcinogen e.g. ethidium bromide. SYBR Green can help to detect the amplicons by naked eyes and the sensitivity is the same with gel electrophoresis. So this will be very suitable for the amplicons detection in field situation and safe to use. The cost of using SYBR Green per sample is also cheaper than gel electrophoresis because it was diluted 1:1000 ratio before adding only 1 μl into the amplicons.

The developed assay is very specific for influenza A and no false positive with other RNA viruses such as PRRSV, CSFV, NDV, IBV and influenza B viruses. The RNA viruses used in specificity test are few because of the availability of samples. But the RNA viruses used in this study are clinically complicated with influenza A infection in both human and animals. If more RNA viruses include, the result will support stronger specificity of the test. The developed assay is also sensitive with influenza A viruses from different hosts (duck, pig, chicken and human) and different subtypes such as H1N1, H3N2, H5N1, H7N4. All isolates can be detected by developed one step RT-LAMP. However, the sensitivity of the assay in other subtypes could not be rule out because of the availability of samples. In this study, many pandemic H1N1, other human seasonal influenza subtypes and different swine influenza subtypes were tested. Therefore, the developed assay is very suitable in human and swine influenza virus detection.

According to the findings from blind samples test, 100 percent reliability of test shows in the experiments without any bias. So the results from different experiments in this study are reliable and acceptable.

In the spiked sample tests, we compared the ability of test to detect the viruses from different sample sources such as cloacal and tracheal swabs in viral transport media (VTM), VTM including excess fecal samples, and virus in the normal saline. Most of the samples can be detected by developed assay but only one viral spiked sample in the VTM including excess fecal samples can not be detected among two samples. It may be because of the interfering of natural inhibitor in fecal material from RNA extraction process. So, RNA extraction should be done with caution especially in the sample with excess fecal materials. The best recommendation is proper centrifugation and by adding of RNAse inhibitor when collecting fecal samples in the field. The detection limit by using cloacal swab samples also agreed with the previous detection limit assay in this study.

Overall view on this developed assay, the assay can applied in the presence of 8mM MgSO4, 1U *Bst* DNA polymerase, specific LAMP primers and the reaction can take place on isothermal condition of temperature $63^{o}$ C for 60 minutes, followed by inactivation temperature of $80^{o}$ C about 10 minutes. This developed assay can be applied in water bath or other inexpensive heating block because the reaction only needs isothermal temperature without any amplification cycles like PCR. Therefore, this technique is suitable for most developing countries and field laboratories where minimal facilities are provided. This assay is also sensitive enough to detect the influenza A viruses from different types of samples and can be applied in case of conventional and real time PCR techniques are not available. In conclusion, the developed assay is a useful influenza A screening tool with enough sensitivity and specificity.

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Conclusion and suggestions

From the finding of this study, it can be concluded that

1. Our developed one step RT-LAMP method is a sensitive and specific method for Influenza A viruses from both human and animal.
2. The detection limit of developed assay is ten time more sensitive than conventional RT-PCR and can be comparable to real time RT-PCR.
3. The developed assay is a suitable technique for minimal equipped laboratories, developing countries and also mobile surveillance system.
4. Colorimetric assay for LAMP products also sensitive like gel electrophoresis.

Taken together, the developed assay is a sensitive assay and very useful for unequipped laboratories and mobile surveillance system. However, further studies are suggested and recommended.

a. The primers for specific subtypes of Influenza A virus should be developed.
b. The easy RNA preparation should be developed to combine with this one step RT-LAMP assay in the application for field surveillance where minimal laboratory facilities were provided.
c. Our developed assay should be used as a primary screening tool in routine surveillance and mobile surveillance because it can apply with temperature stable water bath.
d. It can also be used in clinical diagnosis as a rapid diagnostic assay.

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APPENDICES

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# APPENDIX A

## Reagents and preparations

### Reagents for RT-PCR

| | |
|---|---|
| - PCR buffer | 1X |
| - dNTPs mix | 400 μM |
| - Forward primer | 0.6 μM |
| - Backward primer | 0.6 μM |
| - One step RT-PCR Master Mix | 1X |
| - RNAse inhibitor | 8 Units |
| - Template RNA | 2 μl |

### Reagents for Real time RT-PCR

| | |
|---|---|
| - 2x QuantiFast Probe RT-PCR Master Mix | 1X |
| - Forward primer | 0.8 μM |
| - Backward primer | 0.8 μM |
| - Probe | 0.2 μM |
| - Template RNA | 2 μl |
| - RNAse-free water | up to 25 μl |

### Reagents for RT-LAMP assay

| | |
|---|---|
| - 1X ThermoPol buffer (NEB, USA) | 2.5 μl |
| - dNTPs mix | 0.4 mM |
| - MgSO4 | 6-8 mM |
| - F3 primer | 0.2 μM |
| - B3 primer | 0.2 μM |
| - FIP primer | 1.6 μM |
| - BIP primer | 1.6 μM |

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- Betaine (Sigma) 0.04 M
- AMV reverse transcriptase 5 U
- *Bst* DNA polymerase 1 U
- RNAs 2 µl
- RNAse free water up to 25 µl

Reagents for agarose gel electrophoresis

1. 10 mg/ml Ethidium bromide
    - Ethidium bromide 1 g
    - Distilled deionized water 1,000 ml
2. 50X TAE (Tris-Borate buffer) 1000 ml contains
    - Tris 53.0 g
    - Boric acid 27.5 g
    - 0.5M EDTA pH 8.0 20.0 ml

    Adjust the volume to 1,000 ml with DW

TBE can diluted to 0.5X prior using in gel electrophoresis, 1x is acceptable.

Reagents for colorimetric assays

-SYBR GREEN I nucleic acid gel stain: 10,000 X solution in dimethyl sulfoxide (DMSO)

Before opening, allow the product to warm to room temperature and then briefly centrifuge to collect at the bottom of the vial. The Staining Solution should be prepared in a plastic rather than a glass container, as the stain may adsorb to glass surfaces. Prepare the Staining Solution with a 1:10,000 dilution of the product in the following buffers:

- 1X TBE buffer (89 mM Tris base, 89 mM boric acid, and 1 mM EDTA, pH 8.0)

  Then, added 1 µl of diluted SYBR Green I into the amplicons and monitor the color changes by naked eyes.

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# APPENDIX B

Coding of blind samples

| Blind Sample ID | RT-LAMP result | Decoding the samples |
|---|---|---|
| 1 | Positive | H1N1 human seasonal |
| 2 | Negative | NDV |
| 3 | Negative | Negative Control |
| 4 | Positive | H5N1 chicken |
| 5 | Positive | H5N1 duck |
| 6 | Negative | PRRSV |
| 7 | Positive | H1N1 human 2009 |
| 8 | Positive | H3N2 swine |
| 9 | Positive | H7N4 duck |
| 10 | Positive | H5N1 chicken |
| 11 | Negative | Negative Control |
| 12 | Positive | H5N1 chicken |
| 13 | Negative | IBV |
| 14 | Positive | RNA spiked sample (VTM + tracheal swab) |
| 15 | Negative | CSFV |
| 16 | Positive | H1N1 swine |
| 17 | Positive | RNA spiked sample (VTM + cloacal swab) |
| 18 | Positive | RNA spiked sample (VTM alone) |

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Sources of influenza isolates

| Sample ID | Subtype | Host | Sources |
|---|---|---|---|
| Avian | | | |
| CUK2 (H5N1) | H5N1 | Chicken | VDL |
| H7N4 (duck) | H7N4 | Duck | VDL |
| H3N2 Duck | H3N2 | Duck | VDL |
| 31 | H5N1 | Chicken | VDL |
| 212 | H5N1 | Chicken | VDL |
| BB205 | H5N1 | Chicken | VDL |
| BB177 | H5N1 | Duck | VDL |
| BB219 | H5N1 | Chicken | VDL |
| 13/6 | H5N1 | Chicken | VDL |
| 15/6 | H5N1 | Chicken | VDL |
| 23/6 | H5N1 | Chicken | VDL |
| 27/6 | H5N1 | Chicken | VDL |
| 45/6 | H5N1 | Chicken | VDL |
| 48/6 | H5N1 | Chicken | VDL |
| 47/6 | H5N1 | Chicken | VDL |
| 100/6 | H5N1 | Chicken | VDL |
| 101/6 | H5N1 | Chicken | VDL |
| Swine | | | |
| 06cb1 | H1N1 | Swine | Department of Pathology |
| 05cb2 | H3N2 | Swine | Department of Pathology |
| H1N2 Swine | H1N2 | Swine | Department of Pathology |
| k1 | H1N2 | Swine | Department of Pathology |
| k2 | Not finished sub-typing | Swine | Department of Pathology |
| k3 | Not finished sub-typing | Swine | Department of Pathology |
| Ms | Not finished sub-typing | Swine | Department of Pathology |

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| k4 | H1N2 | Swine | Department of Pathology |
|---|---|---|---|
| 17 | H1N2 | Swine | Department of Pathology |
| 412 | H1N1 | Swine | Department of Pathology |
| 6.3 | H3N2 | Swine | Department of Pathology |
| Human | | | |
| H286 | H1N1 (seasonal) | Human | Faculty of Medicine |
| B1257 | H1N1 (seasonal) | Human | Faculty of Medicine |
| B1463 | H3N2 (seasonal) | Human | Faculty of Medicine |
| B1425 | H3N2 (seasonal) | Human | Faculty of Medicine |
| B1456 | H1N1 2009 | Human | Faculty of Medicine |
| B1447 | H1N1 2009 | Human | Faculty of Medicine |
| B42 | H3N2 (seasonal) | Human | Faculty of Medicine |
| H16 | H1N1 (seasonal) | Human | Faculty of Medicine |
| H565 | H1N1 2009 | Human | Faculty of Medicine |
| H611 | H1N1 2009 | Human | Faculty of Medicine |
| H612 | H1N1 2009 | Human | Faculty of Medicine |
| H613 | H1N1 2009 | Human | Faculty of Medicine |
| H615 | H1N1 2009 | Human | Faculty of Medicine |
| H647 | H1N1 2009 | Human | Faculty of Medicine |
| H653 | H1N1 2009 | Human | Faculty of Medicine |
| H657 | H1N1 2009 | Human | Faculty of Medicine |
| H671 | H1N1 2009 | Human | Faculty of Medicine |
| H688 | H1N1 2009 | Human | Faculty of Medicine |
| H705 | H1N1 2009 | Human | Faculty of Medicine |
| B5E1A | H1N1 2009 | Human | Faculty of Medicine |
| B938 | H1N1 2009 | Human | Faculty of Medicine |
| H340 | H1N1 2009 | Human | Faculty of Medicine |

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# BIOGRAPHY

Mr. Hein Min Tun was born on May 27, 1985 in Kyaukataga Township, Myanmar. He holds the degree of Bachelor from University of Veterinary Science, Naypyitaw, Myanmar. He was awarded Gold Medal by Minister of Livestock and Fisheries, Myanmar for his highest academic achievements in every academic year. He got the license of Veterinary Surgeon approved by Myanmar Veterinary Council. He had worked with International Non-Governmental Organizations as livestock specialist and technical trainer. He is also a Member of Myanmar Veterinary Medical Association. He enrolled the degree of Master of Science in the Department of Veterinary Public Health, Faculty of Veterinary Science, Chulalongkorn University, Thailand in 2008. He has been attached with National Public Health Laboratory, Ministry of Health, Singapore to study influenza sequencing and bioinformatics studies for a month in 2009. Currently, he is pursuing his doctoral degree at The University of Hong Kong, Hong Kong SAR. He presented part of this study in 4$^{th}$ Asian Society of Veterinary Pathologists Conference (November 19-20, 2009) as the topic of “One step reverse transcription loop-mediated isothermal amplification for Influenza A rapid detection.”

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