DEVELOPMENT OF DNA-LATERAL FLOW STRIP TEST FOR DETECTION OF INFLUENZA VIRUS

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DEVELOPMENT OF DNA-LATERAL FLOW STRIP TEST FOR DETECTION OF INFLUENZA VIRUS

NATDANAI KONGWIMARN

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DEVELOPMENT OF DNA-LATERAL FLOW STRIP TEST

FOR DETECTION OF INFLUENZA VIRUS

BY

NATDANAI KONGWIMARN

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Author	NATDANAI KONGWIMARN
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Influenza is a disease caused by Influenza virus (IV). It is an infection that can spread through the air, making it a major public health problem in many countries around the world, especially during the colder times. The infection is higher every year. In children or immunocompromised patients, it can be fatal. Currently, IV detection is performed using reverse-transcriptase polymerase chain reaction (RT-PCR) methods, which are costly, time-consuming and require expertise. Therefore, in this research, two amplification methods were developed: reverse-transcriptase polymerase chain reaction (RT-PCR), reverse-transcriptase-loop-mediated isothermal amplification (RT-LAMP) combined with a lateral flow dipstick (LFD) for detection of IV. Referring to the limit of detection, RT-PCR-LFD and RT-LAMP-LFD could detect IV as like as 0.4 ng/µl ($6x10^7$ particles). Both assays showed no cross reaction to other related bacterial and viral DNA in humans, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, Corona virus, Respiratory syncytial virus, Human immunodeficiency virus, Chikungunya virus, Hepatitis B virus and Dengue virus serotype 1-4. The RT-LAMP-LFD method is an easy and rapid which would be accomplished by using a single temperature method. Hence, it is suitable for planning, surveillance and controlling of Influenza disease.

Keyword : Influenza virus, Reverse-transcriptase loop-mediated isothermal amplification, Lateral flow dipstick

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NATDANAI KONGWIMARN

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I
INTRODUCTION

Influenza disease is a disease caused by Influenza virus (IV) belonging to the *Orthomyxovirus* family. The virus affects human around the world in terms of living, physical health especially the respiratory system, which is the main organ system that suffers from the symptoms of the disease.

This infection can be very easily transmitted as it is spread through the air, so we may be infected via inhalation through the nostril or the droplets that come out from the patient. The virus, in the form of aerosols, is able to contaminate objects such as Mobile phones, toys, etc. The infection can show acute symptoms within 1-2 days after transmission. The severity of the disease depends on the type and species of the virus.

Humans are the main host of IV infection. When humans are infected with the virus, the main symptoms would be found in the respiratory system. They will begin chilling, coughing, sneezing, stuffy nose, the temperature inside the body will start to rise in the range 38-39 ° C, and aches and pains in different muscles of the body. Mortality rate of influenza in patients is usually very high because the symptoms are similar to ordinary flu, so they are usually delayed treated until the symptoms are in the extreme condition.

Influenza virus diagnosis can be performed using viral culture as a cell culture in culture media for 7-10 days or enzyme-link immunosorbent assay (ELISA), which is an IV antigen testing checking within 1-2 days and molecular methods such as Polymerase chain reaction (PCR) and real-time PCR methods that are virus nucleotide tests. However, these methods showed disadvantages in terms of long operating time, high cost and the complicated procedure. Therefore, this research has developed a molecular technique combined between Loop-mediated Isothermal Amplification (LAMP) and lateral flow dipstick (LFD) for the IV diagnosis. The assay is rapid, low cost, and convenient which is suitable for point-of-care diagnosis. In addition, the assay assists the prevention and control of virus.

CHAPTER II
LITERATURE REVIEW

2.1 Influenza disease (Biology)

Influenza virus (IV) is a virus that causes human disease and affects public health in many countries around the world (Figure 1).

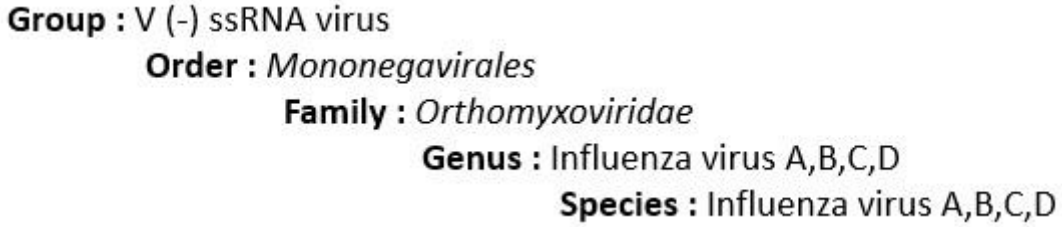

Figure 1 Taxonomy of Influenza virus.

IV is a virus that causes disease through the glycoproteins, called virulent factors, located in the envelope including Hemagglutinin (HA) and Neuraminidase (NA)[1]. There are 3 human transmitting IV serotypes, A[2], B[3] and C[4], While serotype D is usually found in cattle and pigs[5]. In addition, IV subtypes can also be classified according to the virulent factor. Although the World Health Organization (WHO) announced in 2010 that IV spreading can be controlled, but many countries still report influenza virus transmission[6].

2.2 The feature of IV virus

In humans, IV have 4 pathogenic strains of A, B, C and D.

2.2.1 Influenza virus A (IVA)

IVA is a virus that contains virion size is 80-120 nm in diameter. The genetic material of IVA is a negative single-strand RNA. Genome total size is 13.5 kb containing 8 segments with various segment size range from 890 to 2,341 kb. These segments code for 11 proteins consisting of 3 RNA polymerases which had been reported their high mutations, capsid, nucleoprotein (NP), nonstructural protein 1 (NS1), nuclear export

protein (NEP/NS2), membrane protein 1 (M1), membrane protein 2 (M2), hemagglutinin (HA) and neuraminidase (NA)[7](Figure 2).

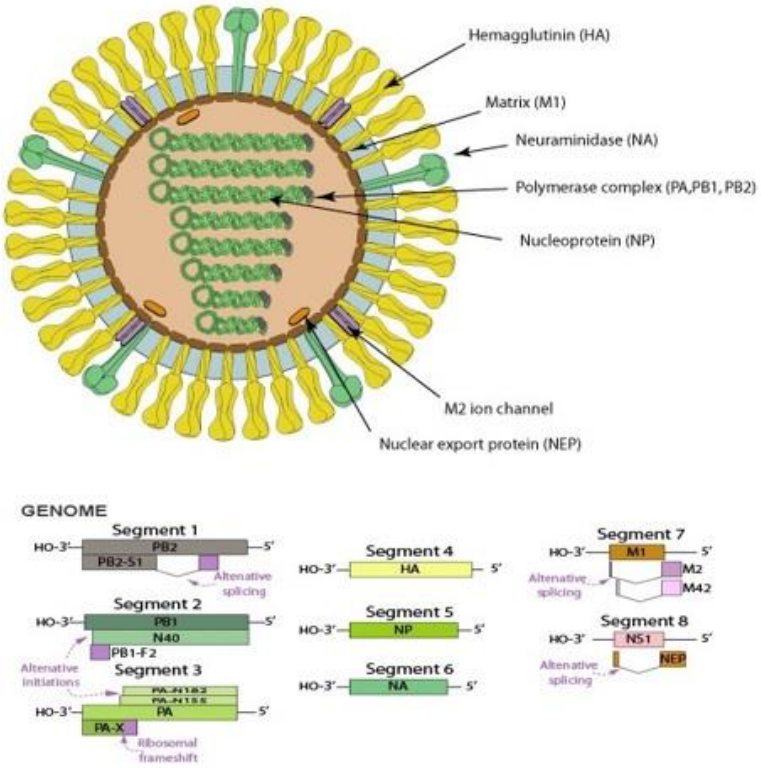

Figure 2 Virion and genome of influenza virus A.

(https://viralzone.expasy.org/223)

2.2.2 Influenza virus B (IVB)

IVB is a virus similar to strain A that contains the same virion size. The genetic material of IVB is also a negative single-strand RNA. Genome total size is 13.5 kb containing 8 segments with various segment size range from 890 to 2,341 kb. These segments code for 11 proteins consisting of 3 RNA polymerases which had been reported their high mutations, capsid, nucleoprotein (NP), nonstructural protein 1 (NS1), nuclear export protein (NEP/NS2), membrane protein 1 (M1), BM2 ion channel (BM2), hemagglutinin (HA) and neuraminidase (NA)[8](Figure 3).

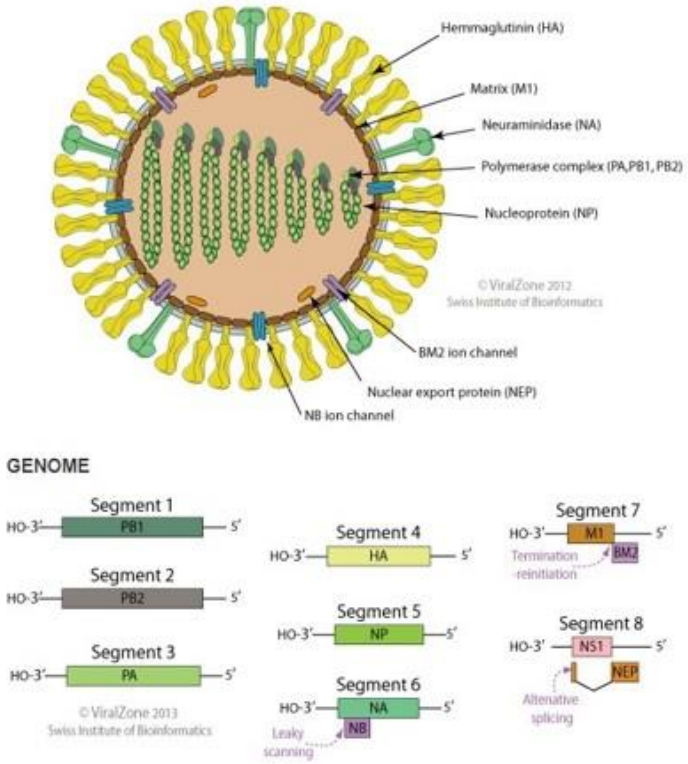

Figure 3 Virion and genome of influenza virus B.

(https://viralzone.expasy.org/80)

2.2.3 Influenza virus C (IVC)

IVC is a virus that contains virion size is 80-120 nm in diameter. The genetic material of IVC is a negative single-strand RNA. Genome total size is 10 kb containing 7 segments with various segment size range from 900 to 2,300 kb. These segments code for 9 proteins consisting of polypeptide (PB1,PB2,PA), hemagglutinin-esterase fusion (HEF), nucleoprotein (NP), matrix (M) protein, nonstructural protein (NS1,NS2) and CM2 ion channel (CM2)[9, 10](Figure 4).

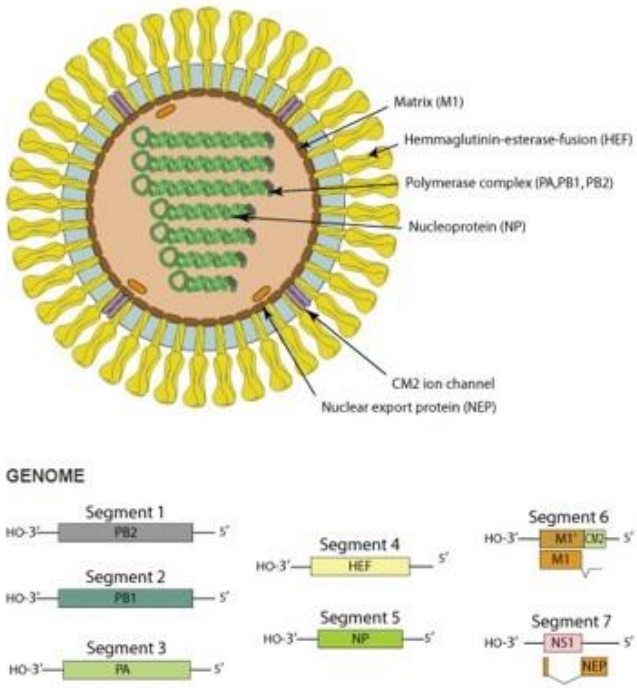

Figure 4 Virion and genome of influenza virus C.

(https://viralzone.expasy.org/81)

2.2.4 Influenza virus D (IVD)

IVC is a virus that contains virion size is 80-120 nm in diameter. The genetic material of IVC is a negative single-strand RNA. Genome total size is 10 kb containing 7 segments with various segment size range from 760 to 2,320 kb. These segments code for 7 proteins consisting of polypeptide (PB1,PB2,PA), hemagglutinin-esterase fusion (HEF), nucleoprotein (NP), matrix (M) protein and nonstructural protein (NS1)[5] (Figure5).

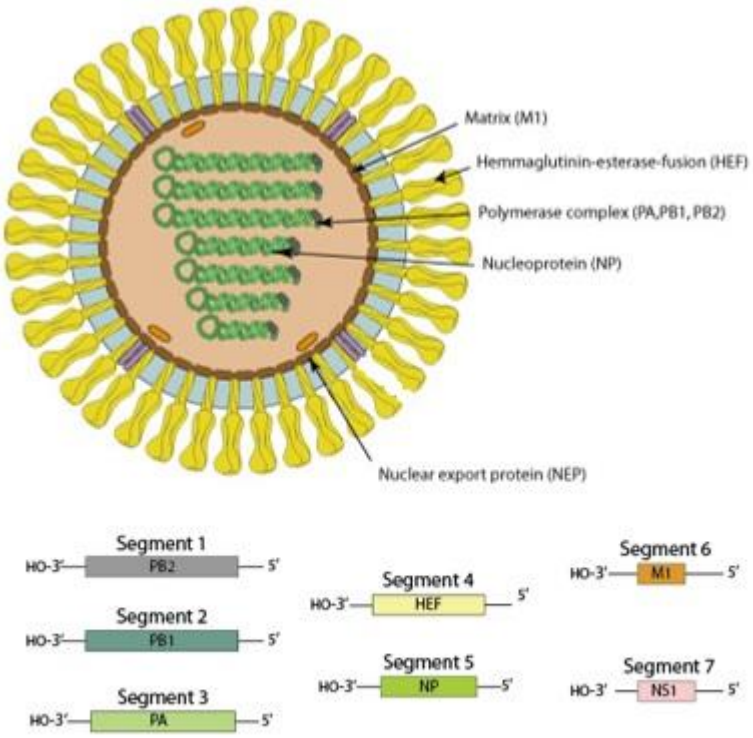

Figure 5 Virion and genome of influenza virus D.

(https://viralzone.expasy.org/6077)

2.3 Life-cycle of IV

The life-cycle of influenza virus in the lung epithelium is based on the binding with hemagglutinin (HA) on the surface of IV and sialic acid on the plasma membrane of the target cell through α-2,6 linkage, which can be found in alveolar type II cells and in the upper and lower human airway epithelium. The HA is firstly cleaved by protease and IV then enter into the cell via endocytosis and is delivered to the lysosome where acidification activates the proton selective matrix protein-2 viral channel (M2), which will lead to membrane fusion and the separation of the viral ribonucleoprotein (RNP) core. the RNP core will then be sent to the nucleus where viral RNA will be replicated. Progeny viral RNP cores are produced at the cytosol and incorporated with the surface proteins HA, neuraminidase (NA) and other viral proteins that display a similar density close to the fat at the plasma membrane. Budding out of the plasma membrane, that causes a new generation of viruses, is relied on neuraminidase activity via cleaving the linkage between HA and sialic acid. Ultimately, the release of new viruses and spread to

other cells will occur. (This step can be prevented with the use of NA inhibitors). The alveolar epithelial cell is shown as a mimic of life cycle similar to that in the airway epithelium[11](Figure 6).

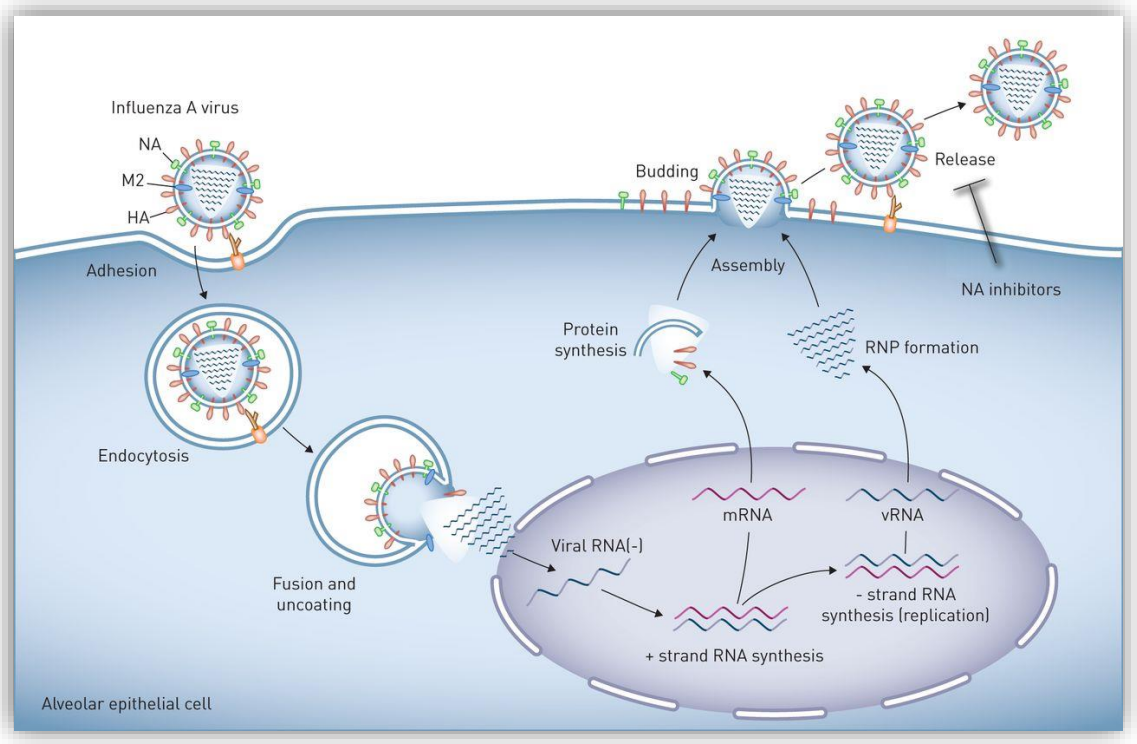

Figure 6 Schematic diagram of the life cycle of Influenza virus[11].

2.4 Nomenclature

Every strain of Influenza virus has 2 main glycoproteins located on the core-enclosing envelope: hemagglutinin (HA) and neuraminidase (NA)[12]. HA is a lectin that helps virus attachment to the target cells and the viral genome transmission into the target cells, while NA will help the new virus release by cutting off the sugar attaching on the particles of the mature virus[13], Hence, these glycoproteins are targets of antiviral drug production[14] and also an antigen that stimulates the antibody production.

Influenza virus A can be classified as a subtype based on the antibody response to HA and NA. Both glycoproteins lead to the basis of different H and N numbers. For example, H5N1[15] has H 18 and N 11 subtypes, as currently known.

2.5 Transmission and spread

The beginning of an epidemic usually occurs when a patient coughs or sneezes causing for nearly 500,000 virus particles spreading to nearby people[16]. In healthy adults, virus can spread even better, But the spread of the virus occurs 1 day before the illness[17]. In children, it is more easily transmitted than adults and spread the virus from before the symptoms until 2 weeks after infection[18] and, viral distribution can persist for more than 2 weeks in immunodeficient patient[19].

IV can transmit infection in 3 main ways[20, 21]

2.5.1 Infection through air is can cause by an infected person has a cough sneezing or spitting, and then droplets spraying to nearby people.

2.5.2 Direct contact has been found once a patient with sneezing and then nasal passage directly into the eyes, nose or mouth of a person.

2.5.3 Indirect contact means a person touches an area where the mucus of an infected person is exposed to their own eyes, nose, or mouth.

2.6 Symptoms

When the patient is infected with the influenza virus, the symptoms appear suddenly within 1-2 days after the infection. The first phase of the infection is chilling, some cases may have a fever with a body temperature at a range of 38-39 °C[22]. There is pain in the muscles at various parts of the body especially back and legs. When passing the first phase, there are other symptoms such as cough, stuffy nose, sore throat, runny nose, red face skin, rash, bleeding spots[23]. In young patients, the symptom may include vomiting, diarrhea, and abdominal pain[24, 25] (may be more severe in type B influenza patient[26]) And may cause complications of influenza, such as viral pneumonia, secondary bacterial pneumonia[27], infected ventricles and exacerbation of

existing health problems, such as asthma or a heart attack. However, approximately 33% of influenza patients often do not show symptoms[17].

2.7 Treatments

Patients should rest, drink water, avoid smoking and drinking alcohol. Acetaminophen (paracetamol) may use to reduce fever and relieve pain after the infection. Patients also should avoid contraction with others to reduce the spread of infection and should avoid aspirin during an influenza virus A infection (Especially type B) because it may lead to Reye syndrome, a fatal rare liver disease[28]. Influenza is a viral disease, so antibiotics do not affect the infection, unless the patient Receive medication that is prescribed according to symptoms. Anti-retroviral drugs are effective if received within 48 hours after initial symptoms, but some strains of influenza are resistant to standard antiretroviral drugs.

2.8 Prevention

Prevention of influenza virus infection can be managed in 2 main ways.

2.8.1 Vaccination: WHO and CDC recommend to receive influenza vaccines for high-risk groups such as children, the elderly, public health officers and chronic disease patients such as asthma, diabetes, heart disease and those with immunodeficiency[29].

2.8.2 Infection control: In order to effectively reduce the spread of the flu virus, it depends on good personal hygiene, such as avoiding to touch your eyes, nose, or mouth when touching foreign objects. washing hands frequently (coupling with soap or alcohol-based hand gel)[30] using your hands to cover your mouth when coughing or sneezing.

2.9 Diagnostic

2.9.1 Conventional Assay

2.9.1.1 Viral culture

Viral culture is a method often used in a hospital diagnostic room according to the collecting swab samples from the nose, throat, various parts of the respiratory system[31] to then be cultured on the petri dishes. This method gives good

results, but there is a limitation in the culture period that should be last 3-10 days before reporting[32].

2.9.1.2 Viral isolation

Virus isolation is a standard method of IV testing used to confirm the results of other diagnostic methods, such as nasal swab testing from patients. The method used to confirm IV is the Hemagglutination inhibition test (HI) that uses antibody to detect IV resulting in inhibiting hemagglutination[33] (Figure 7).

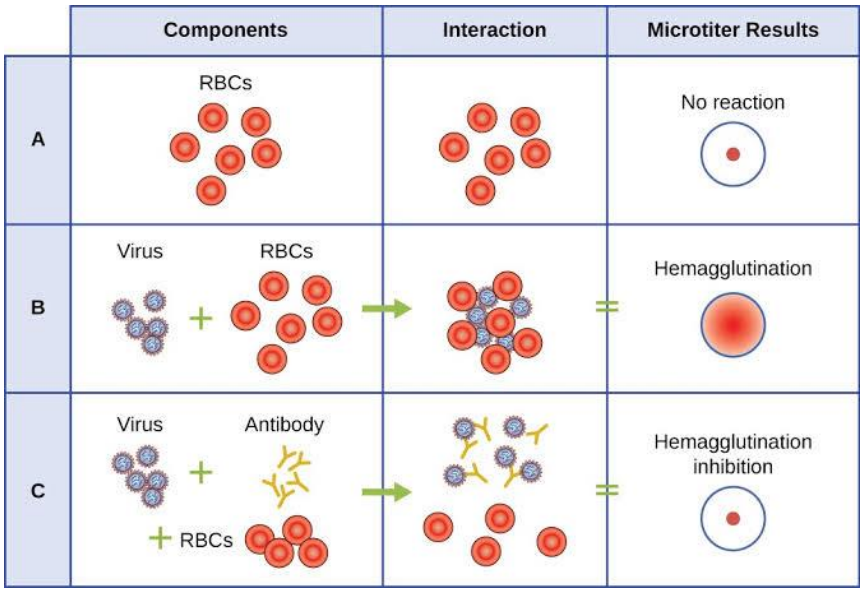

Figure 7 Principle of Hemagglutination inhibition test.

(https://courses.lumenlearning.com/microbiology/chapter/isolation-culture-and-identification-of-viruses/)

2.9.1.3 Enzyme-linked immunosorbent assay (ELISA)

ELISA is another immunological assay based on the principle of Antigen-antibody complex, which uses synthesized antibodies to coupled specifically with the antigen of IV. In this case, antibody-conjugating enzymes, such as peroxidase, help to colorize when interacting with the substrate. The color intensity depends on the amount of antigen. Sandwich ELISA had been applied to detect neuraminidase of IVA

(H7 N9) in China[34], nonetheless, this method has a high cost of monoclonal antibody production, requires a lot of expertise to operate and low sensitivity (Figure 8).

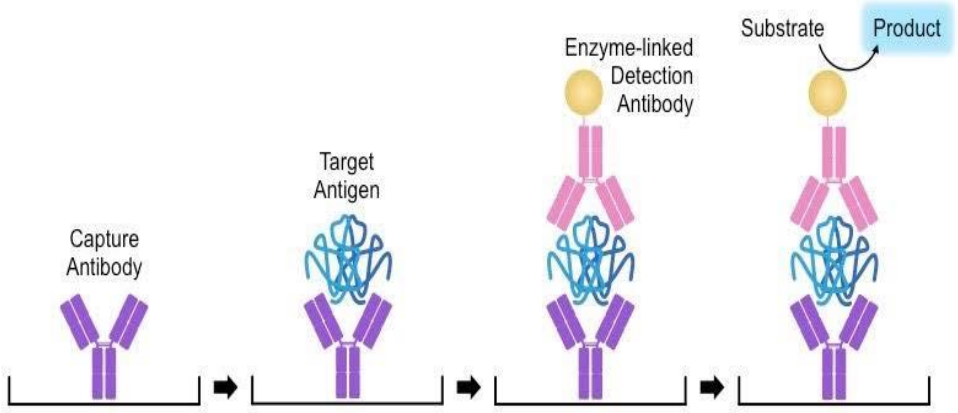

Figure 8 Principle of sandwich ELISA.

(https://ib.bioninja.com.au/options/untitled/b4-medicine/elisa.html)

2.9.2 Molecular Based Assay

2.9.2.1 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The RT-PCR is considered as a gold standard assay[31] for the diagnosis of IV due to the specificity of the genome of IV and their high sensitivity to the appropriate samples. Nevertheless, the disadvantage of this method is the need to renew the primer used in the test annually, because IV can easily evolve resulting in making it difficult to detect with an old primer.

2.9.2.2 Recombinase polymerase amplification (RPA)

RPA is one of the molecular biology methods similar to the polymerase chain reaction (PCR) method. RPA can detect RNA by adding a reverse transcriptase. Into the reaction without the process of changing the RNA to cDNA because this method is isothermal. The RPA can operate in the temperature range 37-42 ° C, so just by holding can cause the reaction simultaneously. Previously, the RPA technique was used combined to the Lateral flow dipstick for detecting the RPA product of IVA detection[35].

The principle of RPA is based on three main enzymes: a recombinase, a single strand DNA binding site (SSB) and strand-displacing polymerase. A recombinase

has the ability to capture oligonucleotide primer with homologous sequence in duplex DNA. SSB bind to displaced strands of DNA and prevent the primers from being displaced. Finally, strand-displacing polymerase begins DNA synthesis where the primer has bound to the target DNA (Figure 9).

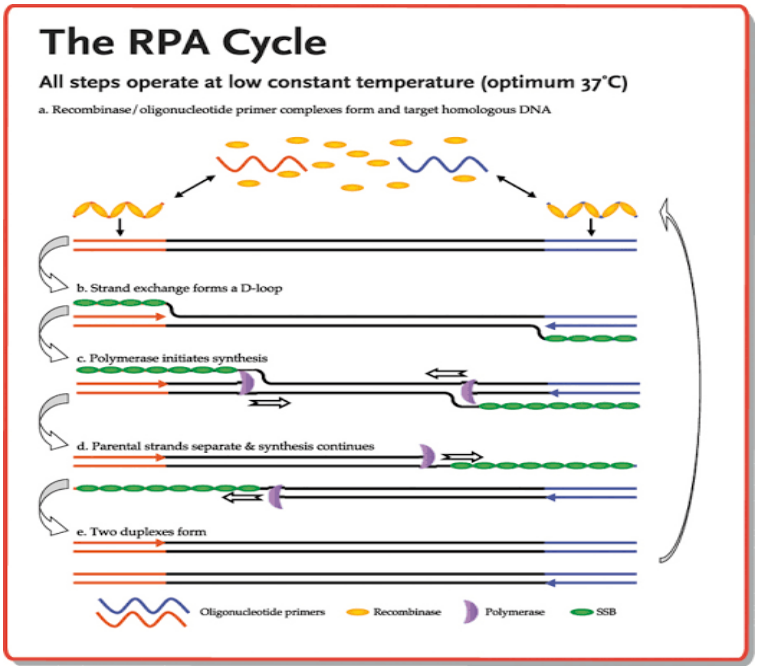

Figure 9 Principle of recombinase polymerase amplification (RPA).

(https://www.ibric.org/myboard/read.php?Board=new_protech&id=187917)

2.9.2.3 Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) is an isothermal method for amplifying target DNA. This method displays a high specificity due to the use of 2 sets of primers from 6 different DNA sequences.

Recently, the LAMP method has been used ubiquitously in laboratories around the world, such as Bacteria detection, virus detection, foodborne pathogens, etc. However, the LAMP method is limited in field work because of equipment and tools requirement.

LAMP is one of the most popular molecular diagnostic methods currently[36], which increases the amount of genetic material by starting the reaction of the LAMP method at a temperature about 60 ° C, which *Bst* DNA polymerase works well and Use 2-3 pairs of specific primer sets at different areas of the target gene. The set primer consists of 2 outer primer (forward outer primer - F3 and backward outer primer - B3), 2 inner primer (forward inner primer - FIP and backward inner primer - BIP), and sometimes There may be 2 loops primer (loop forward primer - LF and loop backward primer - LB) to reduce the time for genetic material amplification[37] (Figure 10).

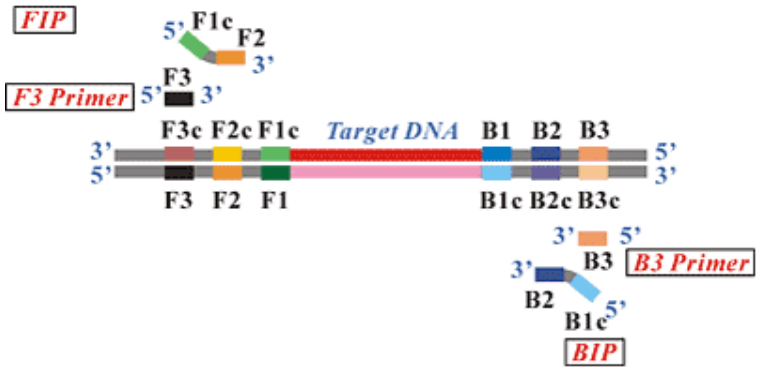

Figure 10 Primers of LAMP.

(https://malariaworld.org/blog/loop-mediated-isothermal-amplification-method-lamp-low-and-effective-cost-novel-tool-molecular)

The LAMP reaction begins with the F2 of the FIP primer binding to the DNA target in the F2 area of the DNA template, resulting in the synthesis of the DNA strand. After that, F3 will bind to the F3c of the DNA template to synthesize the DNA strand resulting in dropping out of the previously synthesized DNA strand. It then captures within the DNA line at the position of F1c and F1. On the side of the BIP reacted same as the FIP, causing the structure of the DNA called a dumbbell-like structure, Next, the amplification process continues by using a dumbbell-like structure as a template, resulting in creating different structures LAMP reactions (Figure 11).

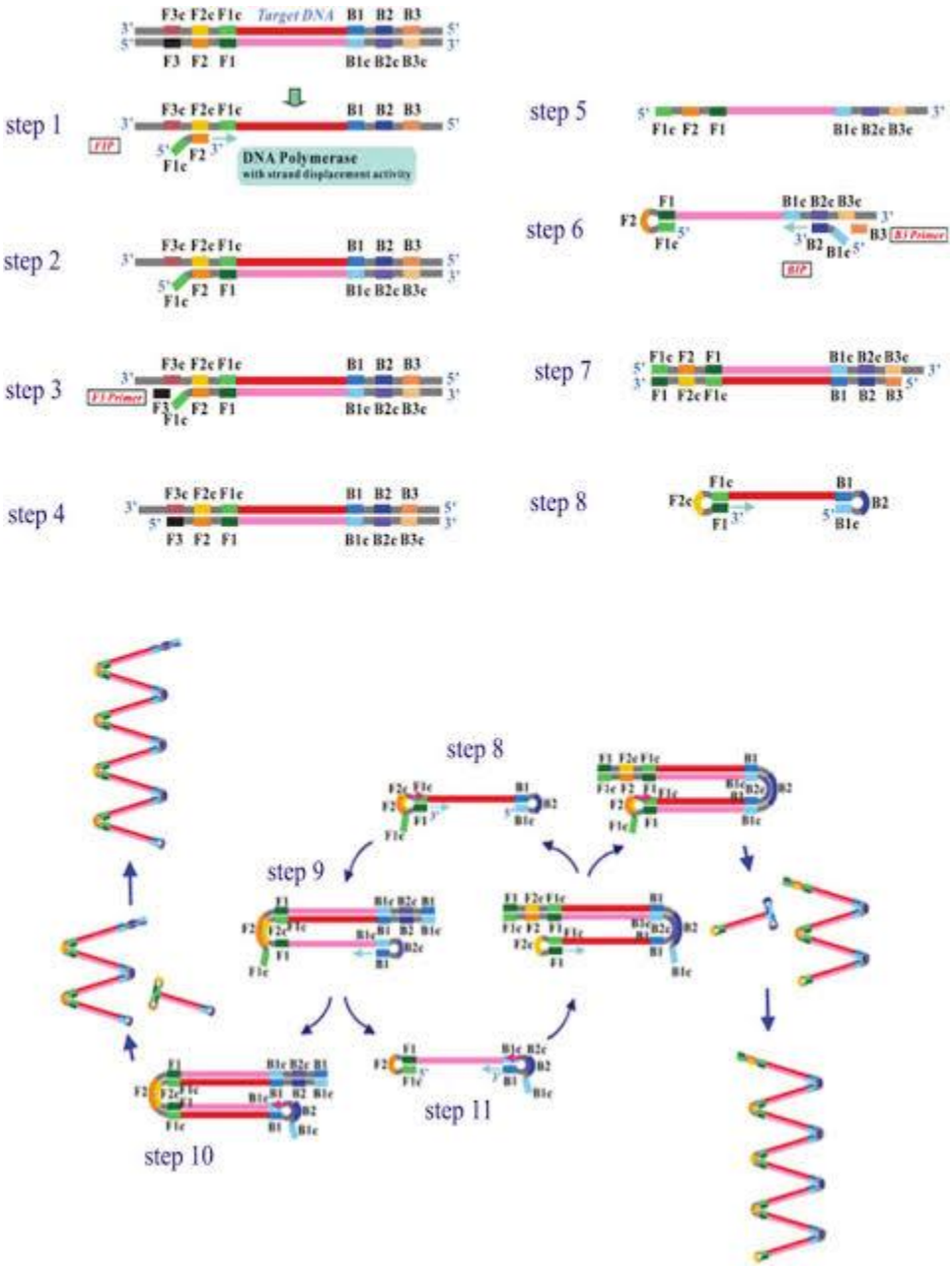

Figure 11 Principle of loop-mediated isothermal amplification (LAMP) method.

(https://www.researchgate.net/figure/Diagram-representing-the-mechanism-of-the-loop-mediated-isothermal-amplification-A-LAMP_fig5_290907479)

2.9.2.3.1 Detection of LAMP Product

In the LAMP reaction, the specific product size is 180-200 bp in length, which can check the positive result by 2 methods: 1. To see the turbidity caused

by magnesium pyrophosphate as a product of reaction, this way can be observed by looking with the naked eye. 2. Agarose gel electrophoresis[38] is the use of fluorescence dye, which helps to see the ban of the product on the agarose sheet gel.

2.9.2.4 Biosensor

Biosensor or biological analyzers Is a method used to analyze specific samples. It has been developed based on the principle of collaboration between biological components and transducers[39] to translate the result in the form of colors or electrical signals etc. (Figure 12).

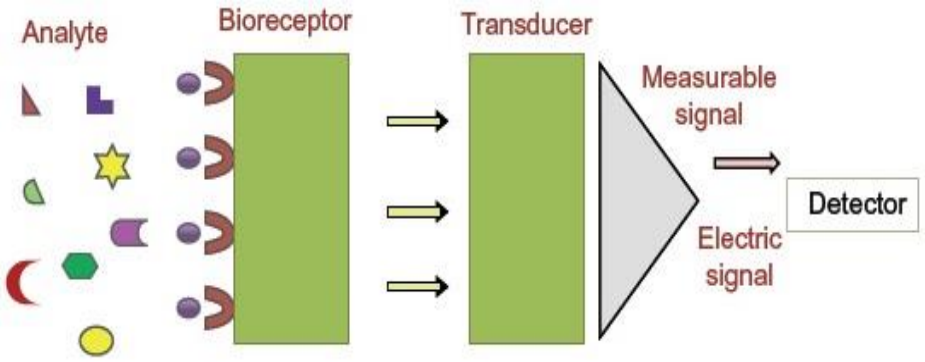

Figure 12 The component of biosensor.

(https://www.dovepress.com/enzyme-based-electrochemical-biosensors-for-food-safety-a-review-peer-reviewed-fulltext-article-NDD)

The principle of Biosensor is based on the specific binding between Ligand (Analyze substrate) such as biomolecules, proteins, antigen or drugs, etc. and the biological component such as enzyme, nucleic acid, antibody, protein or tissue attached to the signal transducer. These combination cause unique changes such as ionization, electrons, humidity, oxygen, heat, color. The transducer then converts those characteristics into electrical signals[40] or appropriate signal transmitted to the detector for further analysis and display (Figure 13).

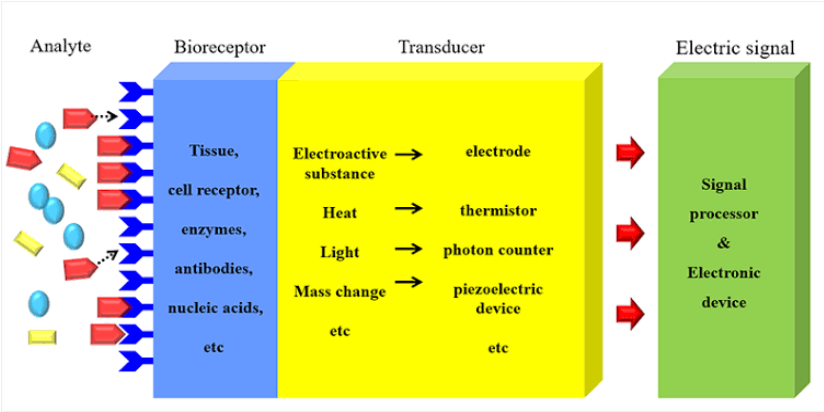

Figure 13 Principle of Biosensor.

(http://www.proteometech.com/en/rd/)

The principle of DNA biosensor is the complementary coupling between the specific DNA sequences within target analytes and the specific nucleotide sequence immobilized onto the transducer. The advantages of biosensor using nucleic acid are fast, high sensitivity and specificity.

2.9.2.5 Lateral Flow Dipstick (LFD)

LFD is a diagnostic method based on chromatographic principle. This method is widespread due to its easy of use and speed. The specificity and sensitivity of the test results are also reliable[41]. The main components of the LFD strip are 4 components on the nitrocellulose membrane consisting of a sample pad, conjugate pad, reaction membrane and Absorbent pad. Each component can transport solution of the The capacity of LFD strip can transport solution along the surface membrane. Nowadays it is often used to examine products obtained from PCR, RPA, LAMP etc. (Figure 14).

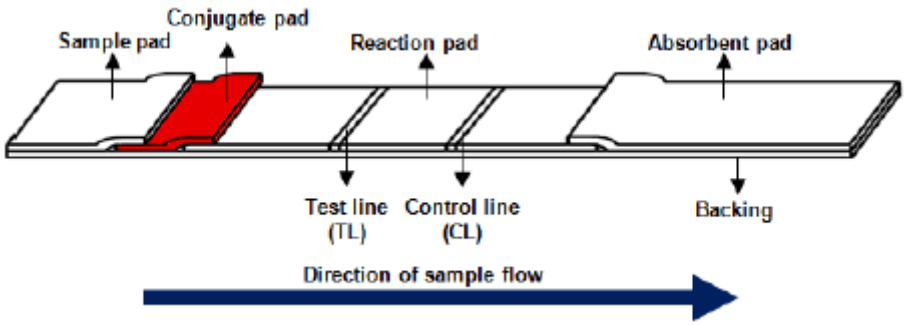

Figure 14 Component of Lateral Flow Dipstick (LFD).

(http://www.kck.usm.my/diagnosticbook/?q=content/chapter-32-hurdles-making-diagnostics-accessible-bottom-billions-can-lateral-flow-immunoassa)

2.9.2.5.1 Sample pad

Sample pad is the first area receiving the sample solution that needs to be inspected and put into this area before passing through the next part.

2.9.2.5.2 Conjugate pad

The sample solution from the sample pad will shift to the conjugate pad, which consists of antibody specific to target sample (Anti-Fluorescein isothiocyanate or FITC antibody) conjugated to gold nanoparticle. Once the solution moves to the conjugate pad, it will be able to bind to the specific antibody and deliver it to the next area.

2.9.2.5.3 Reaction membrane

The reaction membrane is hydrophobic nitrocellulose membrane onto which marker molecules for an example antibodies and biotin ligand are immobilized in test line and control line. If the sample solution contains the substance to be examined, the formation of particles on the test line will occur and appear as a band which can be seen by the naked eye.

2.9.2.5.4 Absorbent pad

The absorbent pad is the final area of the LFD which prevents the solution from flowing backwards.

The components of the LFD strip are usually assembled together within the plastic cassette with the sample pad and when the reaction is displayed on the control line and test line.

The principle of LFD is to start by dropping the sample solution onto the sample pad and it then moves to the conjugate pad. The sample solution will hybridize with the FITC DNA probe allowing it to bind to the Anti-fitc attached to the gold nano particle. After that, the complex will move to the test line and control. If the sample solution contains a sample that we are interested in, the biotin attached to the product of previous technique will bind to the biotin ligand code that is on the test line tab. The remaining solution will move to control line and will handle with Anti-anti-fitc That is coated on the control. The positive result is identified by the appearance of the red-purple color at both C and T lines while the negative result shows only the control line (Figure 15).

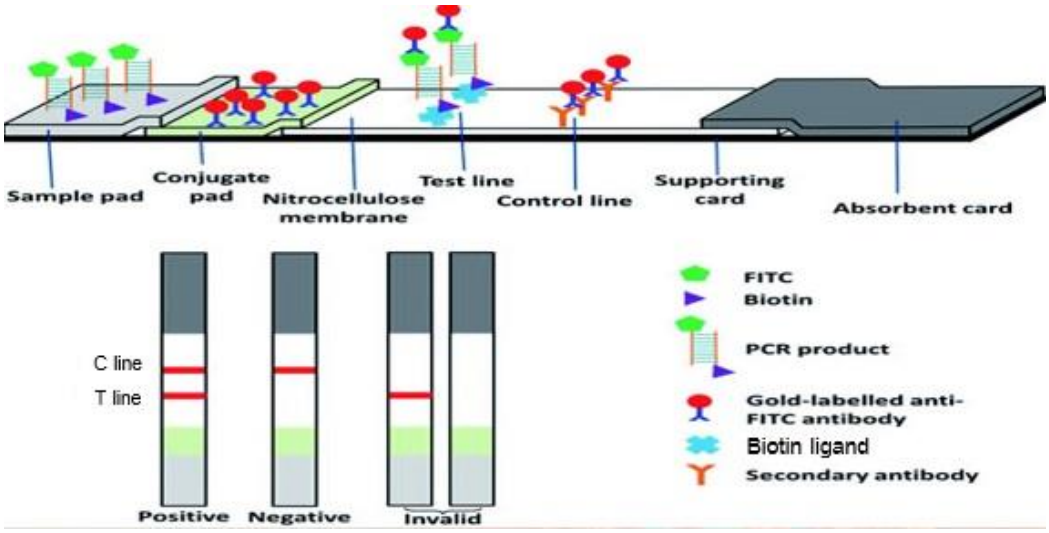

Figure 15 Principle and component of Lateral Flow Dipstick (LFD).

(https://pubs.rsc.org/en/content/articlehtml/2019/ra/c9ra05060d)

In this research, LAMP-LFD biosensor test kits will be developed for the diagnosis of IV as a point-of-care testin.

CHAPTER III
MATERIALS AND METHODS

3.1 Sample collection

The specimens were RNA Influenza virus in sputum which were provided from Panyananthaphikkhu Chonprathan Medical Center, Srinakharinwirot university.

3.2 Gene RNA Extraction and Validation

The sputum was extracted by using viral nucleic acid extraction kit II (Geneaid). The genomic RNA was stored in distilled water at 4 °C until use. RNA concentration was determined by Nano Drop™ 2000 Spectrophotometer (Thermo Scientific; Wilmington, DE, USA). The sample was confirmed via standard primer set[12, 42] from WHO.

3.3 The proposed detection process for Influenza virus

The proposed schematic diagram for Influenza virus detection in this study was illustrated in Figure 16.

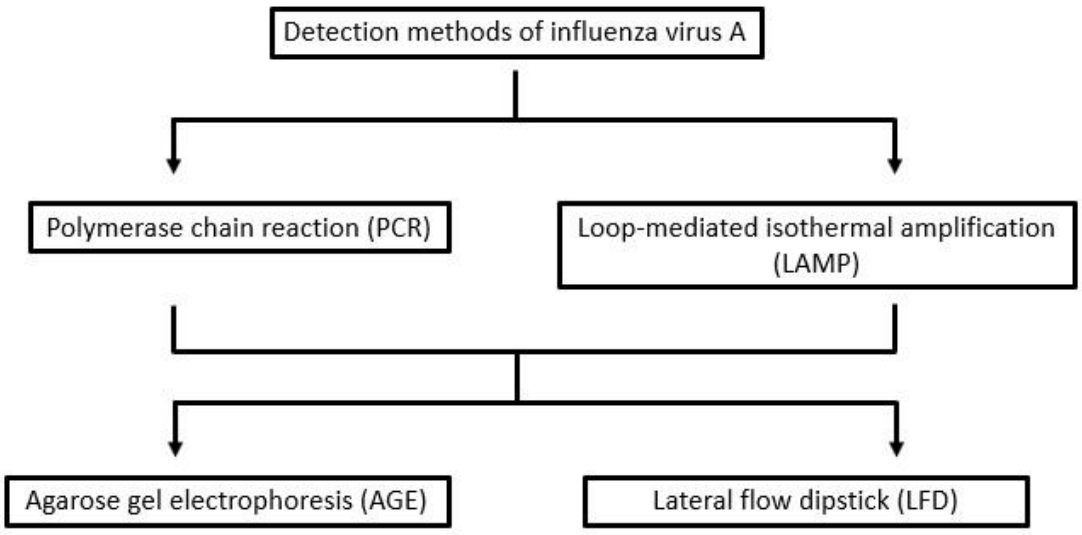

Figure 16 The proposed schematic diagram for Influenza virus detection.

3.4 PCR amplification of Influenza virus matrix gene

PCR amplification was completed by using primers designed based on the Influenza virus matrix gene from Influenza virus A (GenBank accession on.;

AAD51928.1). The forward primer; Influenza virus-F3 and the reverse primer; Influenza virus-B3 were tested by using software Primer explorer V5 programed (http://primerexplorer.jp/lampv5e/index.html). Influenza virus-probe was designed in the region between Influenza virus-F3 and Influenza virus-B3.

3.4.1 The condition of PCR amplification of Influenza virus matrix gene

The condition of PCR amplification for Influenza virus matrix protein gene was prepared in 25 µL reaction composing 1 µL of genomic DNA, 10X PCR buffer, 10 mM dNTPs, $MgCl_2$, *Taq* polymerase (Invitrogen, USA) and 10 µM of each primers. The reaction mixture will be performed by using C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Ltd.; California, USA). The steps of PCR amplification was contained pre-denaturation step at 95 °C for 3 minutes followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing 59 °C for 30 seconds and extension 72 °C for 1 minute, and finally post-extension 72 °C for 5 minutes. The PCR products were analyzed by using 2% agarose gel electrophoresis in 0.5X Tris/Acetate/EDTA (TAE) buffer at 100 volts. The DNA pattern was visualized under UV light by using gel-doc (UVITEC Cambridge).

3.4.2 Optimization of PCR amplification

3.4.2.1 Annealing temperature

The annealing temperature of PCR amplification was accomplished from 55 °C to 65 °C and analyzed by using 2% agarose gel electrophoresis in 0.5X Tris/Acetate/EDTA (TAE) buffer at 100 volts.

3.4.2.2 Concentration of $MgCl_2$

The appropriate $MgCl_2$ of PCR amplification was achieved 0.5 mM to 4.0 mM and evaluated by using 2% agarose gel electrophoresis in 0.5X Tris/Acetate/EDTA (TAE) buffer at 100 volts.

3.5 LAMP amplification of Influenza virus matrix gene

LAMP amplification was completed by using primers were designed based on the Influenza virus matrix gene from Influenza virus A (GenBank accession on.; AAD51928.1). The forward primer; Influenza virus-F3, the reverse primer; Influenza virus-B3, the forward inner primer; Influenza virus-FIP and the reverse inner primer; Influenza

virus-BIP were tested by using software Primer explorer V5 programed (http://primerexplorer.jp/lampv5e/index.html).

3.5.1 The condition of LAMP amplification of Influenza virus matrix protein

The condition of LAMP amplification for Influenza virus matrix protein gene was prepared in 25 µL reaction composing 1 µL of genomic DNA, 10x Isothermal buffer, Betaine, 25 mM dNTP, $MgSO_4$, *Bst* 2.0 DNA polymerase (New England Biolabs, Ipswich, MA,USA), 10 µM of outer primer (Influenza virus-FIP and Influenza virus-BIP) and 100 µM of inner primer (Influenza virus-F3 and Influenza virus-B3). The reaction mixture was incubated at 61 °C for 60 minutes by using C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories Ltd.; California, USA) and tested by using 2% agarose gel electrophoresis in 0.5X Tris/Acetate/EDTA (TAE) buffer at 100 volts. The DNA pattern was visualized under UV light by using gel-doc (UVITEC Cambridge).

3.5.2 Optimization of LAMP amplification

3.5.2.1 Temperature

The temperature of LAMP amplification was accomplished from 60 °C to 65 °C and analyzed by using 2% agarose gel electrophoresis in 0.5X Tris/Acetate/EDTA (TAE) buffer at 100 volts.

3.5.2.2 Concentration of Betaine

The appropriate Betaine of LAMP amplification was achieved 0.1 mM to 0.8 mM and evaluated by using 2% agarose gel electrophoresis in 0.5X Tris/Acetate/EDTA (TAE) buffer at 100 volts.

3.5.2.2 Concentration of $MgSO_4$

The appropriate $MgSO_4$ of LAMP amplification was achieved 2.0 mM to 10.0 mM and evaluated by using 2% agarose gel electrophoresis in 0.5X Tris/Acetate/EDTA (TAE) buffer at 100 volts.

3.6 Lateral Flow Dipstick assay

3.6.1 Fluorescein isothiocyanate (FITC)-labeled DNA probes for LFD

The specific oligonucleotide probe was designed based on the Influenza virus matrix protein gene from Influenza virus A (GenBank accession on. ;

AAD51928.1).The specific oligonucleotide probe was located between Influenza virus-F and Influenza virus-R in order to hybridization with amplified products. The FITC-labelling probes were synthesized by Bio Basic, Canada.

3.6.2 LAMP-LFD

After LAMP amplification, 9 µL of LAMP product was conveyed to a new microcentrifuge tube and 1 µL of FITC-DNA probe was added. Then, the reaction was incubated at 61 °C for 10 minutes before the addition of 100 µL of assay buffer. Finally, the LFD strips (Milenia HybriDtect, Germany) were placed into the hybridization mixture and leave 10 minutes prior to dipping into the water for another 10 minutes to stop reaction.

3.6.3 Optimization of DNA probe

The concentration of FITC-DNA probe for hybridization, was carried out at 10, 1 and 0.1 µM.

3.7 The limit of detection and Specificity tests

To investigate the detection limit of both techniques, it was able to be optimized using 10 times the diluted influenza virus A cDNA, starting from 40 ng / µL to 400 fg / µL. To test the specificity of this study, other microorganisms are *Staphylococcus aureus*, *Streptococcus pneumoniae*, Corona virus, Respiratory syncytial virus, Human immunodeficiency virus, Chikungunya virus, Hepatitis B virus, Dengue virus serotype 1-4, respectively.

CHAPTER IV
RESULTS

4.1 Optimization of PCR and LAMP of Matrix protein gene

4.1.1 PCR

Optimization of PCR of matrix protein gene found that the appropriate temperature and concentration of MgCl2 were 59 °C and 2 mM, respectively (Figures 17and18).

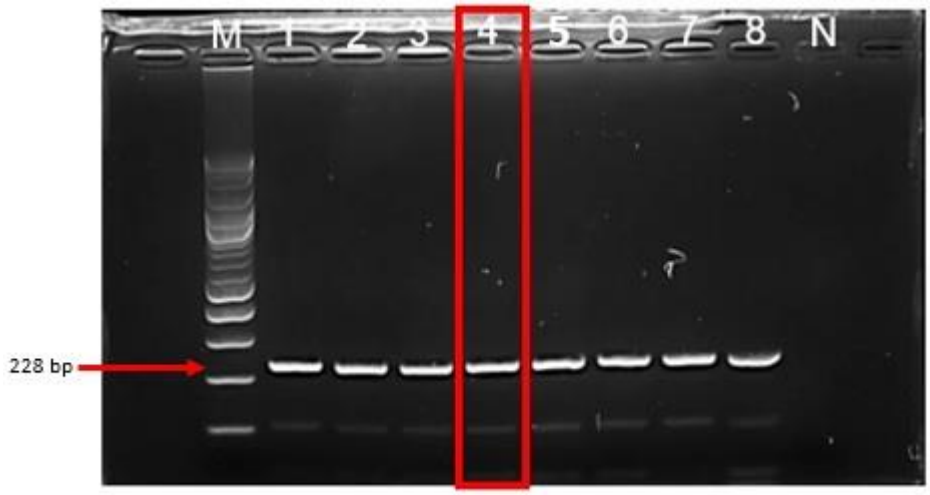

Figure 17 The optimized temperature tested by using 2% agarose gel electrophoresis. Lane M represents 100 bp DNA marker plus. Lane 1-8 represent optimized temperature were 55 °C, 55.7 °C, 57 °C, 59 °C, 61.4 °C, 63.3 °C, 64.5 °C and 65 °C, respectively. Lane N represents negative control.

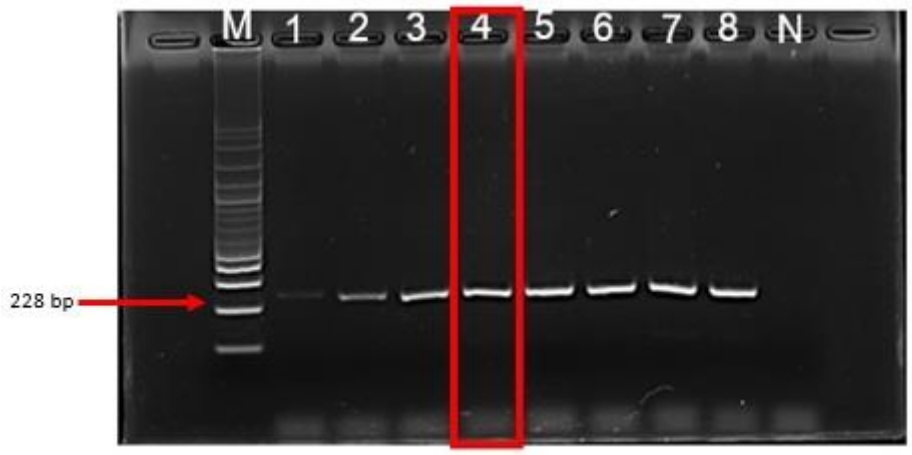

Figure 18 The optimized concentration of $MgCl_2$ tested by using 2% agarose gel electrophoresis. Lane M represents 100 bp DNA marker plus. Lane 1-8 represent optimized concentration of $MgCl_2$ at 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM, 3.0 mM, 3.5 mM and 4.0 mM, respectively. Lane N represents negative control.

4.1.2 LAMP

Optimization of PCR of matrix protein gene found that the appropriate temperature and concentration of betaine including $MgSO_4$ were 61 °C, 0.5 M and 4 mM, respectively (Figures19-21).

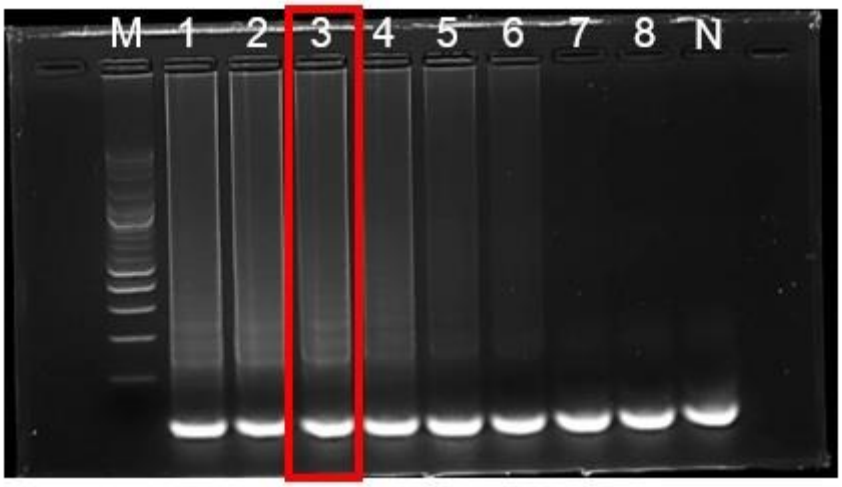

Figure 19 The optimized temperature tested by using 2% agarose gel electrophoresis. Lane M represents 100 bp DNA marker plus. Lane 1-8 represent optimized temperature at 60 °C, 60.4 °C, 61 °C, 61.9 °C, 63.1 °C, 64 °C, 64.6 °C and 65 °C, respectively. Lane N represents negative control.

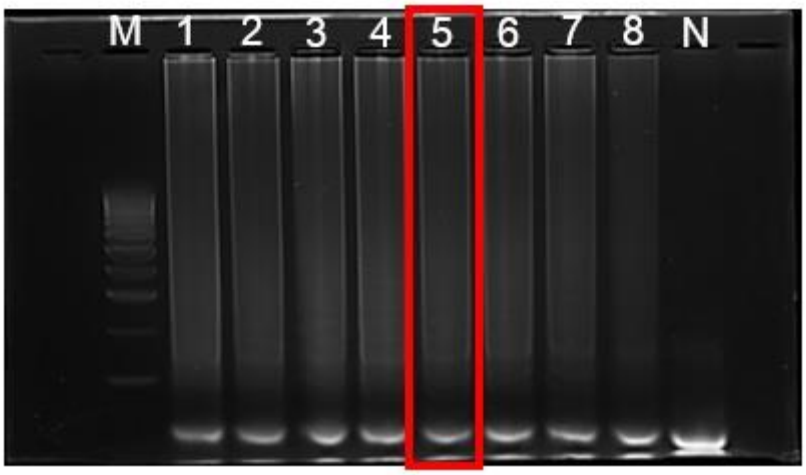

Figure 20 The optimized concentration of betaine tested by using 2% agarose gel electrophoresis. Lane M represents 100 bp DNA marker. Lane 1-8 represent optimized concentration of betaine at 0.1 M, 0.2 M, 0.3 M, 0.4, 0.5 M, 0.6 M, 0.7 M and 0.8 M, respectively. Lane N represents negative control.

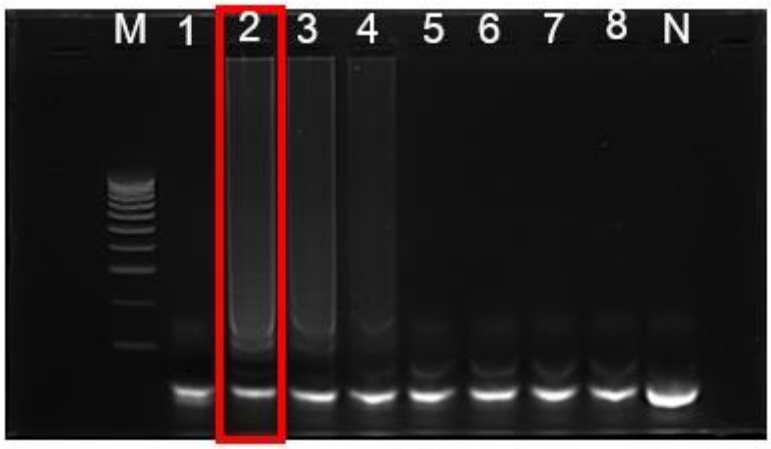

Figure 21 The optimized concentration of $MgSO_4$ tested by using 2% agarose gel electrophoresis. Lane M represents 100 bp DNA marker. Lane 1-8 represent optimized concentration of $MgSO_4$ at 2 mM, 4mM, 6 mM, 8 mM, 10 mM, 12 mM, 14 mM and 18 mM, respectively. Lane N represents negative control.

4.1.3 The limit of detection and specificity test of PCR and LAMP assays of IV

The optimized conditions detailed above was used to investigate the LOD and specificity of IV. The LOD of PCR and LAMP assays were 0.4 ng/µL. The both assays showed no cross reaction to other microorganism DNA such as *S.aureus*, *S.pneumoniae*, Corona virus, Respiratory syncytial virus, Human immunodeficiency virus, Chikungunya virus, Hepatitis B virus, Dengue virus serotype 1-4, respectively (Figures 22 and 23).

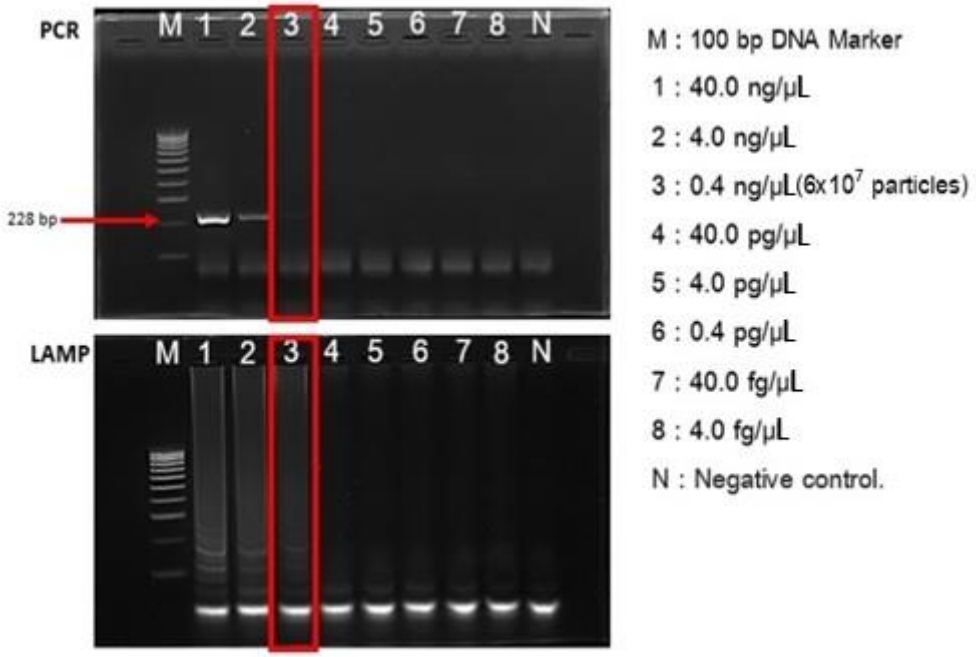

Figure 22 The limit of detection of PCR and LAMP assays. Lane M represents 100 bp DNA marker. Lane 1-8 represents amplification product of DNA concentration at 40.0 ng/µL, 4.0 ng/µL, 0.4 ng/µL, 40.0 pg/µL, 4.0 pg/µL, 0.4 pg/µL, 40.0 pg/µL and 4.0 fg/µL, respectively. Lane N represents negative control.

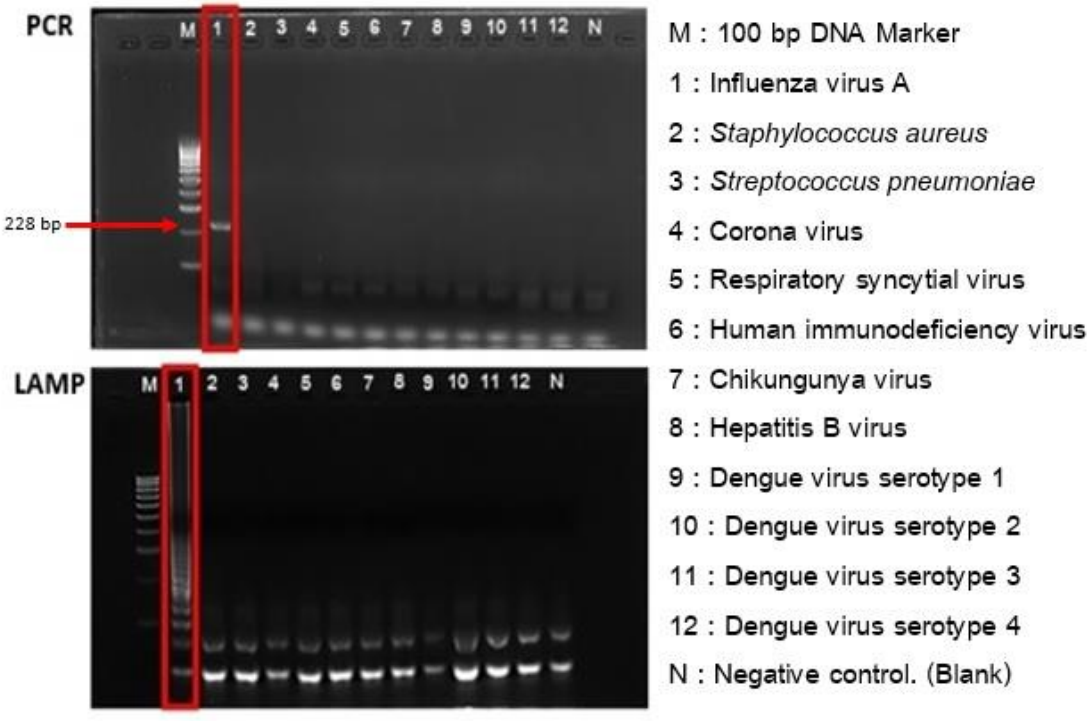

Figure 23 Specificity test of PCR and LAMP assays. Lane M represents 100 bp DNA marker. Lane 1-8 represents *S.aureus*, *S.pneumoniae*, Corona virus, Respiratory syncytial virus, Human immunodeficiency virus, Chikungunya virus, Hepatitis B virus, Dengue virus serotype 1-4, respectively. Lane N represents negative control.

4.2 Lateral Flow Dipstick (LFD) biosensor detection

4.2.1 Optimization of DNA probe concentration

The optimization of DNA probe concentration of PCR and LAMP assays was 10 μM (Figure 24).

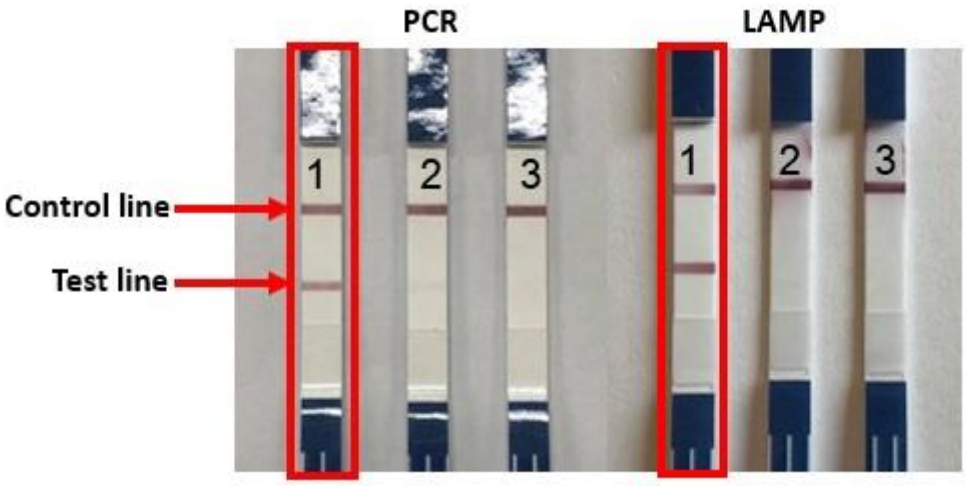

Figure 24 The optimization of DNA probe concentration for PCR and LAMP assays. Strips 1,2 and 3 represents DNA probe concentration at 10 μM, 1 μM and 0.1 μM, respectively.

4.2.2 The limit of detection and specificity of PCR-LFD and LAMP-LFD

The LOD of PCR-LFD and LAMP-LFD were 0.4 ng/μL. The both assays showed no cross reaction to other microorganism DNA such as *S.aureus*, *S.pneumoniae*, Corona virus, Respiratory syncytial virus, Human immunodeficiency virus, Chikungunya virus, Hepatitis B virus, Dengue virus serotype 1-4, respectively (Figures 25 and 26).

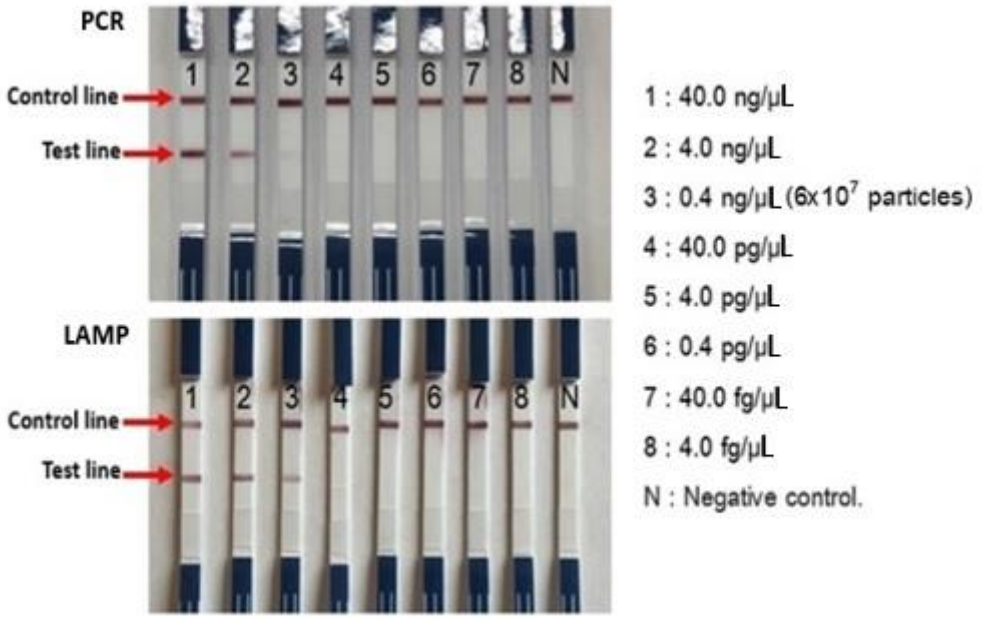

Figure 25 The limit of detection of PCR-LFD and LAMP-LFD assays. Strips 1-8 represents amplification product of DNA concentration at 40.0 ng/µL, 4.0 ng/µL, 0.4 ng/µL, 40.0 pg/µL, 4.0 pg/µL, 0.4 pg/µL, 40.0 fg/µL and 4.0 fg/µL, respectively. Strip N represents negative control.

Figure 26 Specificity test of PCR-LFD and LAMP-LFD assays. Strips 1-8 represents *S.aureus*, *S.pneumoniae*, Corona virus, Respiratory syncytial virus, Human immunodeficiency virus, Chikungunya virus, Hepatitis B virus, Dengue virus serotype 1-4, respectively. Strip N represent negative control.

CHAPTER V
DISCUSSION

Influenza spreads globally including Thailand especially during the low-temperature periods of the year[43]. The etiology of influenza has found from the infection of Influenza virus A (IVA) with a high infection rate and mortality each year.

At present, the IVA detection can be performed by both conventional and molecular based assays, but the recent approved gold-standard method is RT-PCR. Even though the high sensitivity and specificity, the RT-PCR has the limitation due to its costly, time-consuming, requirement of the operating room and depending on complicated expensive equipment. Herein, the RT-LAMP-LFD was developed for detection of IVA by using specific primers and DNA probes designed based on matrix protein gene[44, 45]. The matrix protein gene is considered as a conserved region widely used in the design of the primers due to a lower mutation rate. Therefore, this gene is suitable for designing primers and DNA probes to enhance the specificity and efficiency of IVA testing. The RT-LAMP including DNA hybridization was easily performed at the isothermal temperature of 61°C by using a simple portable heating box prior to detection on LFD. The overall process was rapid since it can be achieved within approximately 60-70 mins (Table 1). The sensitivity and specificity of RT-LAMP-LFD were comparable to those of RT-PCR. Therefore, the technique was suitable for on-site diagnosis and early detection of the IVA as well as for further planning and control of the outbreak.

Referring to the analytical sensitivity test, the limit of detection of IVA by using RT-PCR-AGE, RT-LAMP-AGE, RT-PCR-LFD, and RT-LAMP-LFD were 0.4 ng/μL ($6x10^7$ particles) (Table 1). These limits of detections were similar to those of PCR-LFD and LAMP-LFD for detection of *Cyprinid herpesvirus* 2 at the virus concentration around 0.18 pg/μL[46].

According to analytical specificity test, no cross-reactions were observed against other strains including *S.aureus*, *S.pneumoniae*, Coronavirus, Respiratory syncytial virus, human immunodeficiency virus, Chikungunya virus, Hepatitis B virus and Dengue virus serotype 1-4, respectively (Table 1). In previous studies, Respiratory

syncytial virus has been used to specificity test[47]. This indicated that primers and DNA probes using for RT-PCR- and RT-LAMP-based methods were specific to IVA.

In conclusion, both RT-PCR-LFD and RT-LAMP-LFD based techniques suggested a good promising sensitivity and specificity. However, RT-LAMP-LFD was promising for further routine usage as on-site simple and rapid test by the reason of its stronger benefits on the utilization of a single temperature, less time-consuming, compromising cost, less equipment for operation, and the proper on-site inspection. As stated by all the above reasons, the RT-LAMP-LFD is very useful for the improvement of infection-controlling plan to reduce the spread of the disease.

Table 1 Comparison of methods.

Method	Limit of detection (ng/μL)	Specificity	Time (Hr.)	Equipment
PCR-AGE	0.4	No cross-hybridization	3-4	Thermocycler
PCR-LFD	0.4	No cross-hybridization	2	Thermocycler
LAMP-AGE	0.4	No cross-hybridization	1.5-2	Heating block
LAMP-LFD	0.4	No cross-hybridization	1-1.5	Heating block

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APPENDIX

APPENDIX

Material

100 bp DNA marker	Biolabs
100 bp DNA marker plus	Biolabs
Agarose	Vivantis
Betaine	Sigma
Bst DNA polymerase	Biolabs
Acetic acid	Bio Basic Canada Inc.
Deoxynucleotide triphosphate (dNTP)	Biolabs
Ethylenediarrinetetraacetic acid (EDTA)	Bio Basic Canada Inc.
GeneHlow ^{TM} Gel/PCR Kit	Geneaid
Hydrochloric (HCl)	Mark
LFD strip kit	Krestel biosci so.,ltd
Magnesium sulfate ($MgSO_4$)	Biolabs
Magnesium chloride ($MgCl_2$)	Vivantis
Nucleic acid extraction kit II	Geneaid
Sodium chloride (NaCl)	Vivantis
Taq DNA polymerase	Invitrogen®
Tris-HCl	Amaesco®
TwistAmp® Basic kit Quick Guild	Intabas

1. Buffer preparation

1.1 10X Tris Acetate EDTA (TAE) buffer (pH 8.5)

Tris	242 g
Glacial acetic acid	57.1 mL
0.5 M EDTA pH 8	100 mL

Dissolve the following reagents in 750 ml distilled water. Then the solution was adjusted to pH 8.5 with NaCl. Bring volume to 1 liter, autoclave or sterilize by filtration.

1.2 0.5X Tris Acetate EDTA (TAE) buffer (pH 8.5)

10X TAE	50 mL
dH_2O	950 mL

The 0.5X TAE buffer was prepared by adding 0.5 volume of 10X TAE buffer to 9.5 volume of sterilized distilled water.

Table 2 Proceeding of IV DNAsensor kit

No.	Date	Invention
1	January 21-22, 2021 (Online : January 17, 2021)	991 st International Conference on Medical and Health Science (ICMHS)

Table 3 Patent of primer and DNA probe for detection of IVA

No.	Patent	Status
1	Primers and DNA probe for detection of Influenza virus A using Loop-mediated isothermal amplification combined with lateral flow dipstick	Patent submission number : 2103001165 Since April 29, 2021

MF-04-version-2.0
วันที่ 18 ก.ค. 61

หนังสือยืนยันการยกเว้นการรับรอง
คณะกรรมการจริยธรรมสำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์
มหาวิทยาลัยศรีนครินทรวิโรฒ

(เอกสารนี้เพื่อแสดงว่าคณะกรรมการจริยธรรมสำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์ ได้พิจารณาโครงการวิจัยนี้)

ชื่อโครงการวิจัย : การพัฒนาชุดทดสอบตรวจเชื้อไวรัสไข้หวัดใหญ่
ชื่อหัวหน้าโครงการวิจัย : ศาสตราจารย์ ดร.ไกสูน ชินหรัศมี
หน่วยงานต้นสังกัด : คณะแพทยศาสตร์
รหัสโครงการวิจัย : SWUEC-083/2563X

โครงการวิจัยนี้เป็นโครงการวิจัยที่เข้าข่ายยกเว้น (Research with Exemption from SWUEC)

วันที่ยินยัน : 3 มีนาคม 2563
ยินยันโดย : คณะกรรมการจริยธรรมสำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์
มหาวิทยาลัยศรีนครินทรวิโรฒ

คณะกรรมการจริยธรรมสำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์ มหาวิทยาลัยศรีนครินทรวิโรฒ ดำเนินการรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นสากล ได้แก่ Declaration of Helsinki, the Belmont Report, CIOMS Guidelines และ the International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

ออกให้ ณ วันที่ 14 เมษายน 2563

ลงชื่อ.....
(ผู้ช่วยศาสตราจารย์ ดร. ทันตแพทย์หญิงณปภา เอี่ยมจิรกุล)
กรรมการและเลขานุการคณะกรรมการจริยธรรม
สำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์

(ลงชื่อ).....
(แพทย์หญิงสุรีพร ภัทรสุวรรณ)
ประธานคณะกรรมการจริยธรรม
สำหรับพิจารณาโครงการวิจัยที่ทำในมนุษย์

หมายเลขรับรอง : SWUEC/X-083/2563

สำเนา

แบบ สป/สผ/อสป/001-ก
หน้า 1 ของจำนวน 2 หน้า

คำขอรับสิทธิบัตร/อนุสิทธิบัตร	สำหรับเจ้าหน้าที่	
☐ การประดิษฐ์	วันรับคำขอ 29 มิ.ย. 2564	เลขที่คำขอ 2103001165
☐ การออกแบบผลิตภัณฑ์	วันยื่นคำขอ 29 มิ.ย. 2564	
☒ อนุสิทธิบัตร	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	
ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ. 2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ. 2535 และ พระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ. 2542	ใช้กับแบบผลิตภัณฑ์ ประเภทผลิตภัณฑ์	
	วันประกาศโฆษณา	เลขที่ประกาศโฆษณา
	วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร
	ลายมือชื่อเจ้าหน้าที่	

1. ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์ ชุดไพรเมอร์ (Primer) และตัวตรวจจับ (DNA Probe) สำหรับตรวจหายีนเมทริกซ์โปรตีน (Matrix protein gene) ในเชื้อไวรัสไข้หวัดใหญ่สายพันธุ์ เอ (Influenza virus A) ด้วยปฏิกิริยา[illegible] (Loop-mediated isothermal amplification) หรือ แลมป์ (LAMP) ร่วมกับแผ่นตรวจวัดแบบแถบสี (Dipstick)

2. คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่ ในจำนวน คำขอ ที่ยื่นในคราวเดียวกัน

3. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ☐ บุคคลธรรมดา ☑ นิติบุคคล ☐ หน่วยงานรัฐ ☐ มูลนิธิ ☐ อื่นๆ
ชื่อ มหาวิทยาลัยศรีนครินทรวิโรฒ
ที่อยู่ 114 ซอยสุขุมวิท 23
ตำบล/แขวง คลองเตยเหนือ อำเภอ/เขต วัฒนา จังหวัด กรุงเทพมหานคร รหัสไปรษณีย์ 10110 ประเทศ ไทย
อีเมล
☐ เลขประจำตัวประชาชน ☑ เลขทะเบียนนิติบุคคล ☐ เลขประจำตัวผู้เสียภาษีอากร 0 9 9 4 0 0 0 1 5 8 1 8 1 ☐ เพิ่มเติม (ตัวแทน)
ในกรณีที่กรมฯ ติดต่อกับท่าน ท่านสะดวกใช้การ ☐ อีเมลผู้ขอ ☑ อีเมลตัวแทน

3.1 สัญชาติ ไทย
3.2 โทรศัพท์ 0 26495000 ต่อ 11015,11081
3.3 โทรสาร 0-2259-1822

ยกเว้นค่าธรรมเนียม

4. สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5. ตัวแทน (ถ้ามี)
ชื่อ นางสาวนิยดา รุ่งเรืองผล
ที่อยู่ 114 มหาวิทยาลัยศรีนครินทรวิโรฒ ซอยสุขุมวิท 23
ตำบล/แขวง คลองเตยเหนือ อำเภอ/เขต วัฒนา จังหวัด กรุงเทพมหานคร รหัสไปรษณีย์ 10110 ประเทศ ไทย
อีเมล [illegible]@gmail.com
เลขประจำตัวประชาชน 3 2 2 0 1 0 0 2 9 3 9 1 7 ☐ เพิ่มเติม (ตัวแทน)

5.1 ตัวแทนเลขที่ 2454
5.2 โทรศัพท์ 0 26495000 ต่อ 11015,11081
5.3 โทรสาร 0-2259-1822

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ ☐ ชื่อและที่อยู่เดียวกับผู้ขอ
ชื่อ รองศาสตราจารย์[illegible] จันทร์ศิริ
ที่อยู่ 114 ถนนสุขุมวิท 23
ตำบล/แขวง คลองเตยเหนือ อำเภอ/เขต วัฒนา จังหวัด กรุงเทพมหานคร รหัสไปรษณีย์ 10110 ประเทศไทย
อีเมล
เลขประจำตัวประชาชน 5 1 0 0 6 0 2 2 2 6 7 6 1 ☑ เพิ่มเติม (ตัวแทน)

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม
ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ในวันเดียวกับคำขอรับสิทธิบัตร
เลขที่ วันยื่น เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ
☐ คำขอเดิมมีการประดิษฐ์หลายอย่าง ☐ ถูกคัดค้านเนื่องจากผู้ขอไม่มีสิทธิ ☐ ขอเปลี่ยนแปลงประเภทของสิทธิ

หมายเหตุ ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียดเพิ่มเติมดังกล่าวด้วย

สำหรับเจ้าหน้าที่

ข้อมูลสิทธิบัตร/อนุสิทธิบัตร			
☐ กลุ่มวิศวกรรม	☐ กลุ่มเคมี	สิทธิบัตรการออกแบบ	อนุสิทธิบัตร
สิทธิบัตรการประดิษฐ์ (วิศวกรรม)	สิทธิบัตรการประดิษฐ์ (เคมีเทคนิค)	☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 1)	☐ อนุสิทธิบัตร (วิศวกรรม)
สิทธิบัตรการประดิษฐ์ (ไฟฟ้า)	สิทธิบัตรการประดิษฐ์ (ชีวเคมี)	☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 2)	☐ อนุสิทธิบัตร (เคมี)
สิทธิบัตรการประดิษฐ์ (ฟิสิกส์)	สิทธิบัตรการประดิษฐ์ (เภสัชภัณฑ์และเครื่องสำอาง)	☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 3)	
	สิทธิบัตรการประดิษฐ์ (ปิโตรเคมี)		

VITA

NAME	NATDANAI KONGWIMARN
DATE OF BIRTH	18 SEPTEMBER 1995
PLACE OF BIRTH	YASOTHORN
INSTITUTIONS ATTENDED	2014-2018 : Bachelor's Degree in Medical science from Huachiew Chalermprakiet University 2018-2020 : Master's Degree in Molecular biology program, Faculty of Medicine, Srinakharinwirot University.
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