การพัฒนาและการประเมินวิธีการตรวจหาเชื้อไวรัสพิษสุนัขบ้าด้วยวิธี nucleic acid sequence based amplification แบบมีตัวควบคุมภายใน

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DEVELOPMENT AND EVALUATION OF METHOD TO DETECT RABIES VIRUS USING NUCLEIC ACID SEQUENCE BASED AMPLIFICATION WITH INTERNAL CONTROL RNA

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ภัททา ผู้มีศีล : การพัฒนาและการประเมินวิธีการตรวจหาเชื้อไวรัสพิษสุนัขบ้าด้วยวิธี nucleic acid sequence based amplification แบบมีตัวควบคุมภายใน (DEVELOPMENT AND EVALUATION OF METHOD TO DETECT RABIES VIRUS USING NUCLEIC ACID SEQUENCE BASED AMPLIFICATION WITH INTERNAL CONTROL RNA) อ.ที่ปรึกษา: ศ.นพ. ธีระวัฒน์ เหมะจุฑา, 99 หน้า. ISBN: 974-14-2982-7.

การศึกษาครั้งนี้เพื่อนำเทคนิค NASBA มาพัฒนาเป็น NASBA แบบมีตัวควบคุมภายใน (internal control: IC) เพื่อใช้ในการตรวจหาเชื้อไวรัสพิษสุนัขบ้า และสามารถป้องกันผลลบปลอม เพื่อเพิ่มความถูกต้องในการวินิจฉัยให้มากขึ้น โดยไม่ทำให้ sensitivity ในการตรวจลดลง การศึกษาครั้งนี้อาศัยหลักการเพิ่มปริมาณของ internal control (IC) RNA และ wild type (WT) RNA ในหลอดเดียวกัน แต่มีการตรวจวัดแยกกันด้วย probe คนละตัว โดยสร้าง IC ด้วยวิธี modified overlap extension PCR (MOE-PCR) ได้เป็น IC RNA ซึ่งมีลักษณะแตกต่างจาก WT 25 นิวคลีโอไทด์ โดยใช้สำหรับเป็นเป้าหมายของ specified probe นอกจากนั้นยังมีความยาวเพิ่มขึ้น 42 นิวคลีโอไทด์ เพื่อลดประสิทธิภาพในการแก่งแย่งกับ WT ในปฏิกิริยา amplification

จากการศึกษาพบว่า การเติม IC RNA ในช่วงก่อน amplification step ดีกว่าการเติมในช่วงก่อน isolation step เนื่องจาก detection limit ของทั้ง IC และ WT RNA ลดลงในแบบหลัง จากการตรวจเนื้อสมองจำนวน 20 ตัวอย่าง และใช้ IC RNA 25×10^{-4} fg per reaction ใน amplification step สามารถตรวจพบ IC ได้ในทุกๆ ตัวอย่าง ยกเว้นในตัวอย่างที่มีปริมาณ WT จำนวนมาก ซึ่งก็ไม่ทำให้การแปลผลผิดพลาด อีกทั้งเมื่อนำไปทดสอบกับปัสสาวะจำนวน 15 ตัวอย่างที่ได้ผ่านการตรวจสอบว่ามีหรือไม่มี inhibitor โดยวิธี nested RT-PCR แล้ว จะพบว่า IC สามารถใช้ในการป้องกันผลลบปลอมได้ กล่าวคือ ในตัวอย่างปัสสาวะที่มี inhibitor พบว่าไม่มี signal ของ IC RNAขึ้นเช่นกัน ดังนั้น การตรวจหาเชื้อไวรัสพิษสุนัขบ้าด้วยวิธี IC-NASBA แบบเติม IC RNA ใน amplification step สามารถป้องกันผลลบปลอม และเพิ่มความถูกต้องในการวินิจฉัยให้มากขึ้น โดยไม่ทำให้ sensitivity ในการตรวจลดลง

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต.....ภัททา ผู้มีศีล.....

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษา.....

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PATTA PHUMESIN: DEVELOPMENT AND EVALUATION OF METHOD TO DETECTED RABIES VIRUS USING NUCLEIC ACID SEQUENCE BASED AMPLIFICATION WITH INTERNAL CONTROL RNA. THESIS ADVISOR: PROF. THIRAVAT HEMACHUDHA, M.D., 99 pp. ISBN: 974-14-2982-7

Standard nucleic acid sequence based amplification (NASBA) method without internal control RNA (IC RNA), although uncertain, it may not avoid false negative. The objective of this study as to develop and to evaluate whether NASBA with internal control RNA (IC-NASBA) could be used for diagnosis of rabies and to avoid false negative without compromising its detection sensitivity when compare to NASBA.

In this study, the modified overlap extension by polymerase chain reaction method was used to construct rabies modified fragment containing 25 changed and 42 inserted nucleotides in N gene fragment of rabies virus as internal control in IC-NASBA. Segment of changed nucleotide was a target for specific IC probe. Its longer sequences reduced competitive capacity with WT in amplification reaction. Constructed IC RNA was added to each reaction tube in amplification step. It was amplified by the same target NASBA primers and was detected by a probe complementary to the internal nontarget sequences. IC RNA of 25×10^{-4} fg per reaction was used in the amplification step. IC-NASBA was applied in the target of brain and urine specimens. Twenty samples of rabies infected and non-infected brains were examined. The result showed that IC RNA could be detected in all except in high quantity virus samples. Fifteen urine samples of them, previously presence to contain inhibitor, were subject to IC-NASBA assay. Results were in accessed with nested RT-PCR.

In conclusion, IC-NASBA should be useful in the diagnosis of rabies and may increase accuracy in rabies detection.

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LIST OF ABBREVIATIONS

ABLV	Australian Bat Lyssavirus
AMV-RT	Avian Myeloblastosis Virus Reverse Transcriptase
bp	Base Pairs
cDNA	Complementary DNA
CNS	Central Nervous System
CSF	Cerebral Spinal Fluid
CVS	Challenge Virus Strain
DFA	Direct Fluorescent Assay
DNA	Deoxyribo Nucleic Acid
DNase	Deoxyribonuclease
DUVV	Duvenhage Virus
E. coli	*Escherichia coli*
EBL-1	European Bat Lyssavirus Type1
EBL-2	European Bat Lyssavirus Type2
ECL	Electrochemiluminescent
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscope
ER	Endoplasmic Reticulum
FAT	Fluorescent Antibody Test
fg	famtogram
FIV	Feline Immunodeficiency Virus
G	Glycoprotein
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus

IC	Internal Control
IC-NASBA	Nucleic Acid Sequence Based Amplification with Internal Control
IHC	Immunohistochemistry
L	Large Protein
LB	Luria-Bertani Media
LBV	Lagos Bat Virus
Le	Leader Sequence
M	Matrix Protein
M	Molar
µg	Microgram
µl	Microlitre
ml	Millilitre
mM	Millimolar
MOE-PCR	Modified Overlap Extension by Polymerase Chain Reaction
MOKV	Mokola Virus
MRI	Magnetic Resonance Imaging
mRNA	Massenger Ribonucleic Acid
N	Nucleoprotein
NASBA	Nucleic Acid Sequence Based Amplification
NC	Nucleocapsid
NoV GII	Noroviruses genotype II
OD	Optical Density
ORFs	Open Reading Frames
P	Phosphoprotein
PBS	Phosphate Buffer Saline
PC	Performance Control
PCR	Polymerase Chain Reaction
PEP	Post-exposure Prophylaxis

Poly A	Polyadenylation
PV	Pasture Virus
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNase H	Ribonuclease H
RNP	Ribonucleoprotein
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RV	Rabies Virus
SARS	Severe Acute Respiratory Syndrome
SC	Sample Control
SIV	Simian Immunodeficiency Virus
SLEV	Saint Louis Encephalitis Virus
SSPE	Standard Salt Buffer
Tr	Trailer Sequence
U1A mRNA	U1 Human Small Nuclear Ribonucleoprotein-Specific A Protein mRNA
WCBV	West Caucasian Bat Virus
WT-PC	Wild-Type Performance Control

CHAPTER I

INTRODUCTION

1. Background and Rationale

Rabies is a uniformly fatal disease caused by neurotropic RNA viruses in the family *Rhabdoviridae* and in the genus *Lyssavirus*. The virus is usually transmitted to other mammals including humans through broken skin or mucous membrane with rabies virus containing saliva from infected animals via bite or scratch. Spreading along peripheral nerve and within central nervous system (CNS) is possible by retrograde axonal transport. Rabies virus replicates exclusively in neurons. Recent data showed that microglia can also be infected but the replication is limited [1].

The incubation period, time between exposure and the development of clinical symptoms and signs, is variable, ranging from one week to one year. Such variability reflects the length of time rabies virus spends within muscle cells at the site of the bite, prior to gaining access to the nervous system. Once the virus has entered nerve endings, it advances relentlessly up the nerve bodies until it reaches the spinal cord and eventually the brain. After its replication in the brain, the virus is distributed centrifugally to several tissues in the respiratory, gastrointestinal and urogenital tracts including salivary glands [2-4]. The virus has been isolated and demonstrated in salivary glands, lungs, heart, tongue, gall bladder, kidneys, urinary bladder, muscles, and in myoneural junctions or motor plates [5-7]. Although tissues such as salivary

glands, cornea, skin and tonsils can be used for diagnosis, brain tissue remains the most important and reliable source for postmortem diagnosis [8-13].

Classical features in human rabies can be distinctively classified into 2 separate types, encephalitic or furious and paralytic or dumb rabies [14]. Another form "nonclassic or atypical rabies" [15,16] lacks the usual rabies characteristic symptoms and signs, thus, make diagnosis extremely difficult. Diagnosis can be aided by the use of history of patient, clinical signs, magnetic resonance imaging (MRI) and molecular techniques.

Conventional diagnostic method, the fluorescent antibody test (FAT) was introduced by Goldwasser and Kissling (1958). It is the gold standard due to its practicality and reliability when performed in brain impression smear [17]. This test requires brain tissue from humans or animals suspected of rabies. The test can be performed with post-mortem or biopsy brain tissue, although the latter may be judged too invasive. The use of this test, however, has been limited since it cannot be applied to clinical samples, such as saliva, CSF, urine during when the patient remains alive. Further, it is not reliable when performed in decomposed brain. Isolation of virus is another conventional diagnostic method that can be used when saliva is the preferred clinical sample. Cell culture isolation methods [18] are problematic due to the inability of certain rabies virus variants to propagate easily in specific cell lines [19]. The classic mouse inoculation test [20] for virus isolation can lead to a considerable delay in the estimation of an end point, requires facilities for the use of experimental animals and is labor intensive.

For ante mortem diagnosis of rabies, many tests have been advocated. Corneal smear examination [21] first developed by Schneider is too insensitive and

produced false positive and negative results [22,23]. Demonstration of rabies virus antigen in section of skin biopsy tissue is sensitive but requires cryostat section and fluorescence microscope [24]. Although immunohistochemistry technique can also be applied to formalin-fixed biopsy skin tissue, it requires processing and is time consuming. Antibody detection in CSF and serum by indirect immunofluorescence has been tried on a limited number of samples. Crepin et al. (1998) found that sensitivities of detection of rabies antibodies by ELISA and by sero-neutralization test on cell culture were very low in serum and CSF [25]. This was in accord with previous reports by Hemachudha et al [14,26,27]. Serological testing is of limited value due to the fact that seroconversion occurs late in the course of the disease [28]. Newer techniques such as the dot blot enzyme immunoassay has not been very useful in detecting rabies antigen in saliva and CSF samples [29]. Molecular technique has recently been used to confirm diagnosis. It is suitable since rabies diagnosis must be rapid and conclusive in order to prevent contamination spread to hospital personnel and design appropriate care in such patient. Moreover, it also alerts other individuals who could have been contacted with that rabid animal(s) to acquire appropriate rabies post-exposure prophylaxis (PEP). Additional advantages of molecular technique are that it can be applied with clinical samples of various sources, thus, is useful in both ante- and postmortem diagnosis. Detection of rabies RNA in decomposed brain is also possible. Result can be known within hours.

Molecular testing includes several techniques; reverse transcription polymerase chain reaction (RT-PCR), real time PCR and nucleic acid sequence based amplification (NASBA).

In RT-PCR, the technique is modified by incorporation of a preliminary step in which reverse transcriptase transforms RNA into DNA [30]. RT-PCR is used for rapid rabies diagnosis and for the detection and identification of rabies variants associated with particular hosts as well as genotypes of lyssaviruses, enabling the choice and implementation of specific control strategies. Transmission dynamic of rabies virus can also be constructed[31,32]. RT-PCR is also useful in the study of pathogenesis of rabies [25,33-46].

Real time PCR with SYBR Green I chemistry has been widely used for the diagnosis of viral infections, such as rotaviruses, HIV, Hepatitis B, rhinoviruses, noroviruses and plum pox viruses [47-54]. Probes used in Real time PCR are strain dependent and it is difficult to find a genome region that is identical in a maximum number of strains. In the detection step, fluorescence signal can be performed by integration of a specific linear dual-labelled oligoprobe in the amplification master mix and is achieved during the exponential phase of the targeted sequence amplification. This technique can also generate quantitative data by comparing the results for a given sample to those obtained from a standard curve of a serially diluted cloned amplicon [19,55-58]. Hughes et al. designed a TaqMan PCR based method for the detection of rabies virus RNA in tissue samples [19]. The assay was found to be sensitive and specific and correlated well with the concentration of infectious virus. Advantage of real-time PCR technique offers the possibility of simultaneously amplifying and detecting the targeted nucleic acids in a single step procedure, thereby eliminating the need of post-amplification processing steps and thus results in increased confidence in the results acquired [47].

An alternative isothermal RNA amplification method (NASBA) has also been tested for routine rabies detection in clinical samples. This transcription-based assay, modeled on retrovirus replication, theoretically amplifies the initial RNA target of more than 10^{12} -fold by using a combination of three enzymes: a reverse transcriptase, a T7 RNA polymerase and a RNAse H [59]. The detection of amplified RNA product is achieved by an electrochemiluminescent (ECL) probe through an ECL reader or by a dot blot specific probe hybridization procedure to prevent the misinterpretation of false positive non-specific amplification.

Comparing the sensitivity and detection limit between RT-PCR and NASBA in ante-mortem diagnosis of human rabies using clinical specimens, such as CSF, saliva and urine, NASBA is superior to RT-PCR [60-62]. There is no head-to-head comparison between NASBA and real time PCR. Nevertheless, Houde A. et al. (2006) compared RT-PCR, NASBA and TaqMan RT-PCR (Kageyama system [63] and Ando system [64]) for the detection of Noroviruses genotype II (NoV GII) in 13 clinical stool samples to evaluate their respective analytical sensitivity under controlled experimental conditions [65]. The results showed that NASBA was more sensitive, by at least 2 logs, than conventional RT-PCR in the detection of NoV GII in faecal material. Real time TaqMan RT-PCR showed similar detection limit as NASBA with the Kageyama system, but it was 1 log less sensitive with the adapted Ando system.

We have used NASBA without internal control for ante- and postmortem diagnosis of human rabies at Chulalongkorn University Hospital since 2001 [60-62]. Its sensitivity in diagnosing human rabies during life in almost 40 patients using saliva, urine and CSF as detection sources was almost 100% with 2

negative results, both of whom were the case of paralytic rabies [60,61]. NASBA is relatively easy to use and the whole process from extraction through amplification to detection takes about 4 hours.

Nevertheless, in NASBA system internal control was not included. It has been shown that clinical samples especially urine may contain enzyme inhibitors [32]. The objective of this study was to develop NASBA with internal control RNA (IC-NASBA) in order to avoid false negative results and should be useful in increasing diagnostic accuracy. Comparison of the sensitivity between conventional NASBA and IC-NASBA was also performed.

2. Research Question

Is NASBA with internal control RNA (IC-NASBA) equally sensitive as NASBA without IC and is reliable as to prevent false negative result?

3. Objective

Development and evaluation of method to detect rabies virus RNA using IC-NASBA.

4. Hypothesis

1. Sensitivity of IC-NASBA is at least equal to NASBA.
2. IC-NASBA can prevent false negative result.

5. Keywords

Rabies virus

NASBA

Diagnosis

Internal control

6. Expected Benefit & Application

1. New molecular diagnostic test.
2. A molecular test which can prevent false negative result.
3. Preliminary step for rabies NASBA quantitation.

CHAPTER II

LITERATURE REVIEWS

Rabies virus [66] is a neurotropic RNA virus that is responsible for encephalomyelitis in mammals. It is classified in the Rhabdoviridae family of the Mononegavirale order and Lyssavirus genus (from the Greek rhabdos, meaning "rod") [11,66]. The family Rhabdoviridae together with Paramyxoviridae, Filoviridae and Bornaviridae constitute the "superfamily" taxon, order Mononegaviridae, since all members are RNA viruses which contain nonsegmented, negative-sense, single-strand RNA genomes [67]. There are 7 genotypes in the genus Lyssavirus; whereas other 4 remains tentative [68-71]. Rabies virus (RV, genotype 1) is distributed worldwide among terrestrial mammals and bats. It presents the most comprehensive collection of isolates, and has been extensively studied due to its health and economic significance. The other 6 lyssaviruses reflect the genetic diversity which share among the same genus and with rabies virus, the prototype of which has a unique capability to produce fatal encephalomyelitis.

Genotypes 2-6 include Lagos bat virus (LBV; genotype 2), first isolated in Nigeria from the frugivorous bat (Eidolon helvum) in 1956 [72]. Mokola virus (MOKV; genotype 3) was first isolated from shrews in Nigeria in 1968 [73]. Duvenhage virus (DUVV; genotype 4) was isolated from a human, who died after a bat bite in 1970 in South Africa [74]. European bat lyssavirus, type 1 (EBLV-1; genotype 5) has been isolated from a number of European countries, where it was suspected since the 1950s [75]. European bat lyssavirus, type 2 (EBLV-2; genotype 6)

was isolated from a biologist in Finland, who died of rabies like illness [76]. Australian bat lyssavirus (ABLV; genotype 7) was discovered in 1996 [77-79]. Clearly, all lyssaviruses except Mokola virus are associated with bats.

Four new other tentative viruses in the genus Lyssavirus include Aravan virus which was isolated in southern Kyrgyzstan in 1991 [80], Khujand virus, isolated in Northern Tajikistan during 2001 [81], West Caucasian bat virus (WCBV) and Irkut virus have been isolated from Eurasian bats during 2002 [82]. According to preliminary identification with anti-nucleocapsid monoclonal antibodies and comparison of limited N gene sequences, they may be considered as new genotypes of the Lyssavirus genus [82-85].

The public health implications of these newly discovered lyssaviruses require introspection. Although no human infection caused by viruses other than genotypes 1, 4, 5 and 6 have been reported, such observations may be as a result of inadequate diagnostic tests or inappropriate epidemiological surveillance [84].

1. Molecular composition and morphology of rabies virus particles

All lyssaviruses share many biologic and physico-chemical features as well as amino acid sequence characteristics that classify them with other rhabdoviruses. These include the bullet-shaped morphology, helical nucleocapsid (NC) or ribonucleoprotein (RNP) core and structural proteins of the virus (**Figure 1**). The five structural proteins of the virion contain the genomic RNA which is tightly encapsidated by the viral nucleoprotein (N) and RNA polymerase complex, consisting of the large protein (L) and its co-factor, the phosphoprotein (P). Both L and P

proteins are involved in transcription and replication. One matrix protein (M) interacts with both the nucleocapsid and the plasma membrane and probably plays an important role in the virion maturation process. The fifth is a type I transmembrane glycoprotein (G). Three of the viral proteins are located in the RNP core. They are the N, the noncatalytic polymerase-associated P and the catalytic L (RNA polymerase). All three proteins are involved in the RNA polymerase activity of the virion. Both the N and P are phosphorylated in rabies virus, unlike in other rhabdoviruses, in which only the P is phosphorylated [69,86-88]. The P in rabies virus is phosphorylated by a unique cellular protein kinase and specific isomers of protein kinase C [89]. The number of molecules of each protein per virion has been estimated in different laboratories with somewhat variable results [90]. It can be argued reasonably that these differences in estimates of protein molecules per virion simply may be a reflection of variables associated with the way the studies were conducted in the different laboratories.

Nevertheless, one stoichiometric relationship that emerges from these estimates with respect to the RNP composition that appears to be valid is the 2:1 ratio of the N and P molecules per virion. During nascent protein synthesis and replication of viral progeny RNA, these two proteins interact in the same 2:1 ratio to bind to the newly synthesized progeny RNA. Also, the L is typically the protein produced in least amount. The remaining two structural proteins of the rabies virion, the G and M, are associated with the lipid-bilayer envelope that surrounds the RNP core. The M lines the viral envelope, forming an inner leaflet between the envelope and RNP core, whereas the G produces the spikelike projections or peplomers on the surface of the viral envelope [91].

At the center of the bullet-shaped virus particle is a core of the helical RNA (the viral genome) and protein, the RNP core that extends along its longitudinal axis and is surrounded by a lipid-protein envelope. The RNA is single-stranded, nonsegmented and has negative-sense or minus-strand polarity [92]. This implies that isolated (naked) minus-strand genome RNA is not infectious and it cannot be translated directly into protein [93]. The RNP that becomes the tightly coiled core of all virions is produced from a flexible right-handed helix structure that has a periodicity of approximately 7.5 nm per turn. The RNP core in standard size infectious virions measures approximately 165 x 50 nm but measures between 4.2 and 4.6 mm in length when relaxed and fully extended, like a tread, outside the virion [92,94].

During virus assembly, the RNP core is surrounded by M, one of the two membrane proteins of the virus, to form the “skeleton” structure of the virus [95]. As virus particles mature and bud through the cellular membrane, the skeleton structure acquires the lipid bilayer envelope that is 7.5 to 10 nm thick surrounding the mature virion. Located on the external surface of the viral envelope are the surface projections that measure 8.3-10 nm in length, each projection or spike containing three molecules (a trimer) of the viral G [91]. These have been described when viewed in the EM as the “short spikes extending outward with the appearance of hollow knobs at their distal ends” [94]. It is estimated that the height of the “hollow knobs” or “heads” of the spike is about 4.8 nm, the rest of the spike is made up of the thin “stalk” on which the head rests [91].

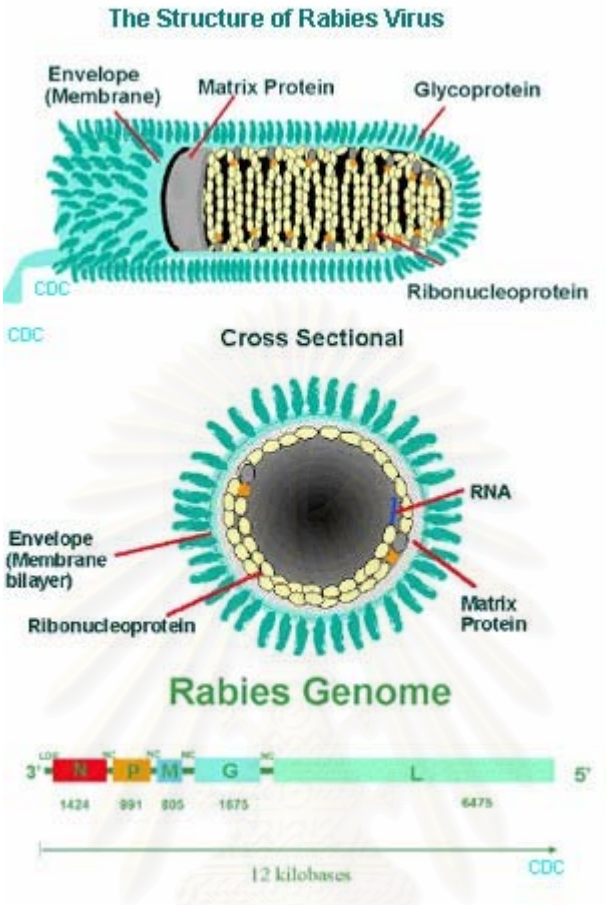


Figure 1 : Structure of rabies virus

2. <u>Genome organization</u>

The RNA is single-stranded, nonsegmented and has negative-sense or minus-strand polarity. This implies that isolated (naked) minus-strand genome RNA is not infectious and it cannot be translated directly into protein [93]. The first event in infection, therefore, is transcription of the genome RNA to produce complementary (positive strand) monocistronic messenger RNA (mRNA) molecules from each of the viral genes or cistrons in the genome. The viral proteins are synthesized from the monocistronic mRNAs. The organization and general features of the rabies virus

genome RNA are similar to other negative-strand RNA viruses within the Mononegavirales order and, in particular, to other rhabdoviruses [96]. At the 3'end (first 58 nucleotides) of the 11,932 nucleotides genome RNA of rabies virus (PV strain) is a noncoding (extragenic) leader (Le) sequence. Immediately downstream of the Le sequence, in sequential order, are the five structure genes (N, P, M, G, and L) followed by noncoding trailer (Tr) sequence (last 70 nucleotides) at the 5' end (**Figure 2**) [97,98]. The genes are separated by relatively short (dinucleotide or pentanucleotide) sequences that represent the intergenic regions (stretch of nucleotides from the 5' end of one gene to the 3' start of the next gene) of the genome. These short intergenic regions are located between the N and P genes (2 nucleotides) and between the P and M genes and the M and G genes (each 5 nucleotides long). The remaining intergenic region between the G and L genes contains a long stretch of 423 nucleotides in the rabies virus genome, 504 nucleotides in the Mokola virus genome [99], 475 nucleotides in the Australian bat lyssavirus genome [100]. The 3' half of this viral genome, which includes the first four genes of the genome and their respective open reading frames (ORFs), has been determined (Gould et al.,1998). However, the genetic diversity that occurs with in the ORFs between the different genotypes. The G-L intergenic region is sufficiently long to represent a potential gene but lacks an ORF for a detectable protein. It has been given the designation of remnent gene or pseudogene (Ψ), recognizing that it once represented an ORF of sufficient size to code for a recoverable protein [98]. Interestingly, in this long intergenic region, two sequences stand out that appear to give credence to its former function. One is a sequence motif that resembles the rabies consensus mRNA start signal (UUAU), which is located 10 nucleotides downstream (or UUGU in MOKV, 20 nucleotides downstream) from the

stop signal for the mRNA of the G gene in the rabies virus genome. The other is a stretch of 25 nucleotides located upstream from the L gene, which resembles a polyadenylation (poly A) signal located at the end of mRNA molecules. These signals suggest that the virus may have inherited and since lost a protein ORF in its evolution, analogous to the nonviral protein of the infectious hematopoietic necrosis virus, a fish rhabdovirus [98]. Strangely, the Ψ region represents the most divergent area of the genome [34].

The Le sequence at the 3'end of the genome RNA serves a multifunctional purpose in rabies viruses. Within the 3' terminal Le sequence, a specific cis-acting signal (a specific nucleotide sequence "acting within" the genome RNA) functions as a signal (or promoter) for template recognition by the viral RNA transcriptase (L-alone) or RNA polymerase complex (L and P). This particular signal initiates genome RNA transcription [101-104]. Within the first 10 to 20 nucleotides at the 3' and 5' ends of the rabies virus RNA genome there is a high level of sequence complementary, including an exact base complementarity between the first and the last 11 nucleotides at the 3' and 5' ends of the genome RNA, respectively. This is compelling evidence that the promoter sequences, which are shared in the Le and TrC (3' end of the antigenome RNA that is complementary to the 5' end of the genome) regions, provide a common function in transcription and replication [93].

During transcription, a positive stranded leader RNA and five mRNAs are synthesized. The replication process yields nucleocapsids containing full length antisense genomic RNA, which in turn serves as a template for the synthesis of positive sense genomic RNA. The only external viral protein that governs the viral tropism [105,106].

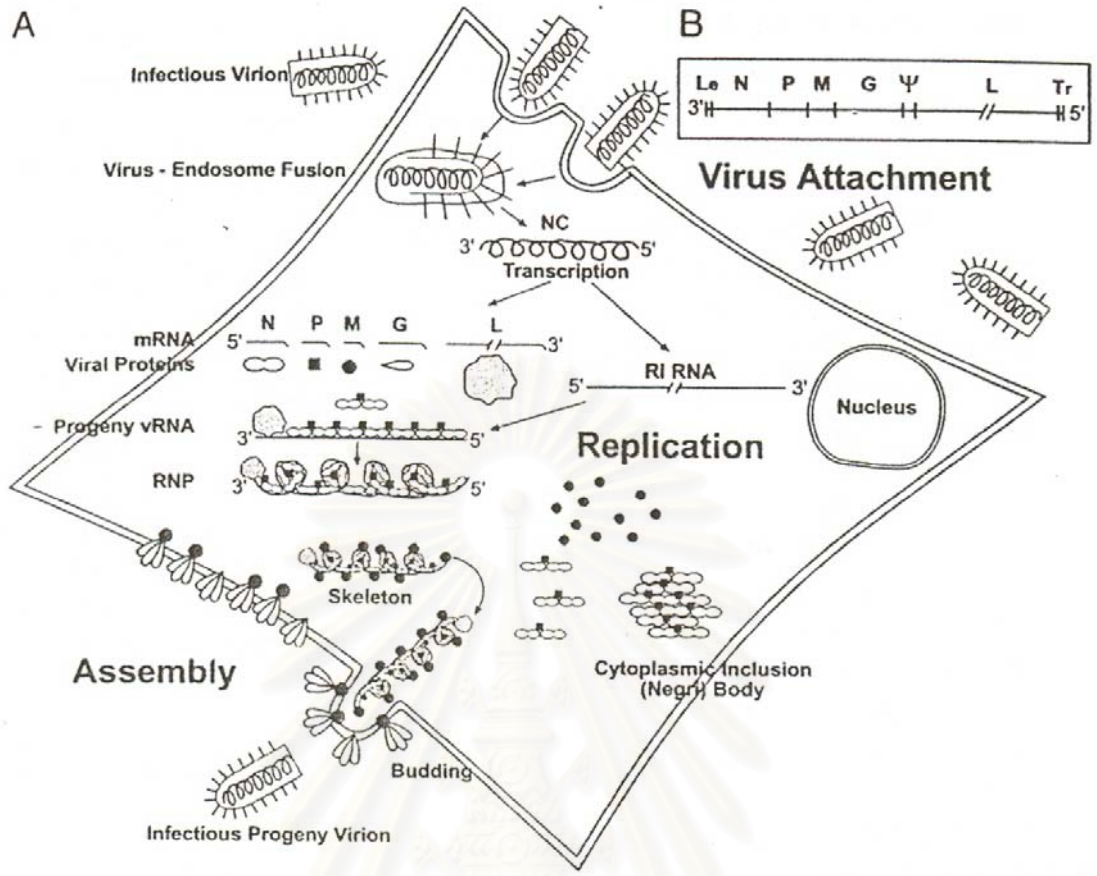


Figure 2 : Rabies virus life cycle in the cell and organization of the rabies virus genome.

3. <u>Transmission</u>

Transmission of rabies virus usually begins when infected saliva of a host is passed to an uninfected animal. Various routes of transmission have been documented and include contamination of mucous membranes (i.e., eyes, nose, mouth), aerosol transmission, and corneal transplantations. The most common mode of rabies virus transmission is through the bite and virus-containing saliva of an infected host.

Following primary infection, the virus enters an eclipse phase in which it cannot be easily detected within the host. This phase may last for several days or months. Investigations have shown both direct entry of virus into peripheral nerves at the site of infection and indirect entry after viral replication in nonnervous tissue (i.e., muscle cells). During the eclipse phase, the host immune defenses may confer cell-mediated immunity against viral infection because rabies virus is a good antigen . The uptake of virus into peripheral nerves is important for progressive infection to occur.

After uptake into peripheral nerves, rabies virus is transported to the central nervous system (CNS) via retrograde axoplasmic flow. Typically this occurs via sensory and motor nerves at the initial site of infection. The incubation period is the time from exposure to onset of clinical signs of disease. The incubation period may vary from a few days to several years, but is typically 1 to 3 months. Dissemination of virus within the CNS is rapid, and includes early involvement of limbic system neurons. Active cerebral infection is followed by passive centrifugal spread of virus to peripheral nerves. The amplification of infection within the CNS occurs through cycles of viral replication and cell-to-cell transfer of progeny virus. Centrifugal spread of virus may lead to the invasion of highly innervated sites of various tissues, including the salivary glands. During this period of cerebral infection, the classic behavioral changes associated with rabies develop [107].

4. <u>Exposures and incubation period</u>

The infectious cycle of rabies virus is perpetuated mainly through animal bites with the deposition of rabies virus-laden saliva into subcutaneous tissues

and muscle. With respect to human rabies, worldwide, dogs are by far the most common and important rabies vector; bats are most important in the United States and Canada, although there is also a reservoir in terrestrial animals. Other types of nonbite exposures, including contamination of an open wound, scratch, abrasion, or mucous membrane by saliva or central nervous system (CNS) tissue from an infected animal, occur commonly but are rarely responsible for transmission of rabies virus. Handling and skinning of infected carcasses and perhaps consumption of raw infected meat have resulted in transmission of rabies virus [108-110]. Rarely, inhalation of aerosolized rabies virus in caves containing millions of bats [111], or in laboratories [112,113] has resulted in human rabies. At least 13 cases of rabies have resulted from transplantation (human-to-human) of rabies virus infected corneas, lung, kidney, liver, pancreas, vascular conduit [114-116].Transplantation of other tissues or organs has been documented to be associated with transmission of rabies virus. [117,118].

Human rabies usually develops 20-90 days after exposure, although occasionally disease develops after only a few days [119], and rare cases have occurred after a year or more following exposure. The incubation period (from exposure to onset of disease) in rabies has a greater length and variability than in most other infectious disease, which may cause considerable emotional stress to the patient. Very long incubation periods raise the possibility of another unrecognized or forgotten exposure, particularly in rabies-endemic areas. Severe multiple bites and facial bites are associated with shorter incubation periods [120], although attempts to find a clear correlation between the site of the bite and the incubation period have not been successful [121].

5. Clinical forms of disease

Classical features in human rabies can be distinctively classified into 2 separate types, encephalitic or furious and paralytic or dumb rabies [14]. About 80% of patients develop an encephalitic (also called furious) form of rabies, and about 20% experience a paralytic form of disease. Another form “nonclassic or atypical rabies” [15,16] lacks the usual rabies characteristic symptoms and signs thus, make diagnosis extremely difficult.

In furious rabies, patients have episodes of generalized arousal or hyperexcitability that are separated by lucid periods [122], and these features reflect brain involvement with the infection. Intermittent episodes may occur with confusion, hallucinations, agitation, and aggressive behavior, which typically last for periods of 1-5 minutes [15,120,123]. About 50-80% of patients develop hydrophobia, which is a characteristic and the most widely recognized manifestation of rabies. Hydrophobia is not a feature of other neurologic diseases. The term hydrophobia is derived from the Greek meaning "fear of water." Patients initially may experience pain in the throat or difficulty swallowing.

Paralytic rabies also has been called dumb rabies. Patients may be literally dumb or mute due to laryngeal muscle weakness, but the term dumb rabies usually refers to the quieter clinical features and prominent weakness rather than specifically to the presence of anarthria [124,125]. The development of paralytic rabies does not appear to be related to the anatomic site of the bite [126] and the incubation period is similar to that in furious rabies.

The pathogenetic basis for the two different clinical forms of rabies has not been determined. In a small series, there were no marked differences in the regional distribution of rabies virus antigen or in the inflammatory changes [126]. However, at the time of death, the distribution of viral infection may be much more widespread and not closely reflect the distribution at the time of the patient's presentation with paralytic rabies. It is curious that an earlier serum neutralizing antibody response was observed in patients with furious rabies than in those with paralytic rabies [26].

6. Laboratory diagnosis

A diagnosis of rabies should be considered in any patient who presents with encephalopathy of unknown cause. The first signs and symptoms of rabies are often nonspecific. Without a clear history of animal bite, rabies often is not suspected until late in the clinical illness. A delayed diagnosis may increase the number of persons potentially exposed to rabies by contact with the patient. An early diagnosis may eliminate the expense and discomfort of unnecessary diagnostic tests and medical treatment of the patient.

Once considered, a diagnosis of rabies is not always easy to confirm. Antemortem diagnosis of rabies is one of the most difficult procedures attempted by a laboratory and should be performed only by experienced laboratories. The risks of performing a brain biopsy are unacceptable for the routine use of these samples for diagnosis. If rabies is suspected, a complete set of samples should be collected for testing by all currently used diagnostic procedures. Because of the implications of a

positive test, a finding of rabies must be confirmed in more than one tissue or sample. Because antibody is produced late in a lyssavirus infection, and virus may be absent or present at very low levels in peripheral nerves and tissues, samples taken for antemortem diagnosis cannot definitively rule out rabies. If a suspicion of rabies persists despite negative findings, repeated sampling may be necessary [127].

The standard test for rabies testing is FAT. This test has been throughly evaluated for more than 40 years, and is recognized as the most rapid and reliable of all the tests available for routine use. This test requires brain tissue from animals and human suspected of being rabid. The test can only be performed post-mortem (after the animal or human is dead). Several tests are necessary to diagnose rabies ante-mortem (before death) in humans. Tests are performed on samples of saliva, serum, spinal fluid, and skin biopsies of hair follicles at the nape of the neck [107].

Other tests for diagnosis and research, such as electron microscopy (EM), histologic examination, immunohistochemistry (IHC), RT-PCR, and isolation in cell culture are useful tools for studying the virus structure, histopathology, molecular typing, and virulence of rabies viruses. Molecular tests, such as reverse transcriptase polymerase chain reaction (RT-PCR), real time PCR and nucleic acid sequence based amplification (NASBA), have been becoming established techniques for rabies diagnosis.

NASBA technology has been used for the detection of primarily RNA targets. This technology generates RNA amplicons with polarity opposite that of the input RNA. No amplification of background DNA will occur using this technology. This method is an isothermal-based RNA amplification technique, which employs three different enzymes active at 41 ^{O}C: avian myeloblastosis virus reverse

transcriptase (AMV-RT), RNase H and T7 RNA polymerase. NASBA technique has been applied to RNA viruses, such as HIV type1/2, HBV, HCV, HAV, FIV, SIV, enteroviruses, influenza, dengue, SLEV, West Nile and SARS coronavirus. Recently, it has been shown that NASBA can reliably detected rabies RNA in various biological specimens and be extremely useful in antemortem diagnosis of human rabies [60,61].

Development of NASBA, a user-defined RNA amplification assay using NucliSens Basic Kit reagents and ECL detection comprises four procedures: nucleic acid release, nucleic acid isolation, nucleic acid amplification, nucleic acid detection.

Nucleic acid release, the specimen is added to NucliSens lysis buffer containing guanidithiocyanate and triton X-100 / performance control RNA (PC RNA), by means of which the complete procedure can be validated, is added to an extra tube of lysis buffer to which optionally the same background matrix can be added as is contained in the specimens to be tested. Any viral particles and cells present in the specimen disintegrate releasing nucleic acids, whilst any RNases and DNases present in the specimen are inactivated.

Nucleic acid isolation, under the high salt conditions, nucleic acid in the lysis buffer, including the PC-RNA, binds to the silicon dioxide particles (13). These particles, acting as the solid phase, are washed several times. Finally, the nucleic acid is eluted from the silica.

Nucleic acid amplification, the NASBA reaction utilizes three enzymes, two specific oligonucleotide primers, nucleoside triphosphates and appropriate buffer components. One of these oligonucleotide primers contains a 5'-terminal T7 RNA polymerase promoter sequence in addition to a stretch of nucleotides that is

complementary to a sequence on a target RNA. The second oligonucleotide primer encompasses a short sequence which is identical to a segment of the target RNA and is located upstream of the region where the T7 promoter-containing oligonucleotide can anneal. In the Basic Kit, primer P2 also contains an additional S'-terminal stretch of 20 nucleotides that is not related to the target RNA, but is used for detection. The three enzymes involved in the amplification reaction are Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT), Escherichia coli RNase H and T7 RNA polymerase, each acting continuously on its appropriate substrate(s). Following a single RNA target molecule through the NASBA process illustrates how amplification of the target RNA is achieved by the concerted action of these three enzymes (**Figure 3**). The reaction starts with hybridization of the oligonucleotide primer containing the T7 RNA polymerase binding site to the target RNA. AMV-RT elongates the primer, creating a cDNA copy of the RNA template and forming a RNA/DNA hybrid. RNase H recognizes this hybrid as substrate and hydrolyses the RNA portion of the hybrid leaving single-stranded DNA to which the second oligonucleotide can anneal, thereby again forming a substrate suitable for reverse transcriptase extension. This extension finally renders the promoter portion of the nucleic acid sequence double stranded, and transcriptionally active. Recognizing the now functional promoter, T7 RNA polymerase produces multiple copies of RNA transcripts which are antisense to the original target RNA sequence. Each newly synthesized antisense RNA molecule can itself act as a template and be converted to a DNA intermediate with a functional T7 promoter in a way similar to the original target RNA. However, in this case the oligonucleotide primers anneal in reverse order because the newly generated RNA molecules are opposite in orientation to the original target and the resulting DNA

intermediate is only partly double-stranded. In this manner, many RNA copies are generated from each RNA target that re-enters the reaction resulting in the exponential synthesis of RNA products. The NASBA reaction continues in a self-sustained manner under isothermal conditions, thus achieving dramatic amplification in a short period of time. Amplification of approximately 10^6 to 10^9-fold is obtained within 90 minutes.

Nucleic acid detection, end-point detection of the presence of RNA amplicons generated in a NucliSens amplification reaction is based on the electrochemiluminescence (ECl) principle employing the NucliSens Reader (**Figure 4**) (19-21). Two oligonucleotides are used, of which one is immobilized onto paramagnetic beads and serves as the capture oligonucleotide. The second oligonucleotide is complexed to a ruthenium chelate and forms the Eel probe. The capture oligonucleotide is specific for the RNA amplicon of interest (i.e. control RNA amplicon or target RNA amplicon). In contrast, the ECl probe is generic since it is complementary to the amplicons 3'- terminal end which is transcribed from a stretch of nucleotides that is identical in every P2 primer and precedes the target specific segment of the primer. An aliquot of the amplification reaction is added to a hybridization solution containing both the capture oligonucleotide-saturated beads and the ECl probe oligonucleotide. After incubation, the paramagnetic beads carrying the hybridized amplicon ECl probe complexes are captured on the surface of an electrode by means of a magnet. Voltage applied to this electrode triggers the electrochemiluminescence (ECl) reaction. The light emitted by the hybridized ruthenium-labeled probes is proportional to the amount of amplicons generated in the corresponding amplification reaction. An ECl signal above the cut-off level reflects

the presence of target RNA in the original sample. The ECl signal for the performance control RNA can be used to determine the validity of the assay run.

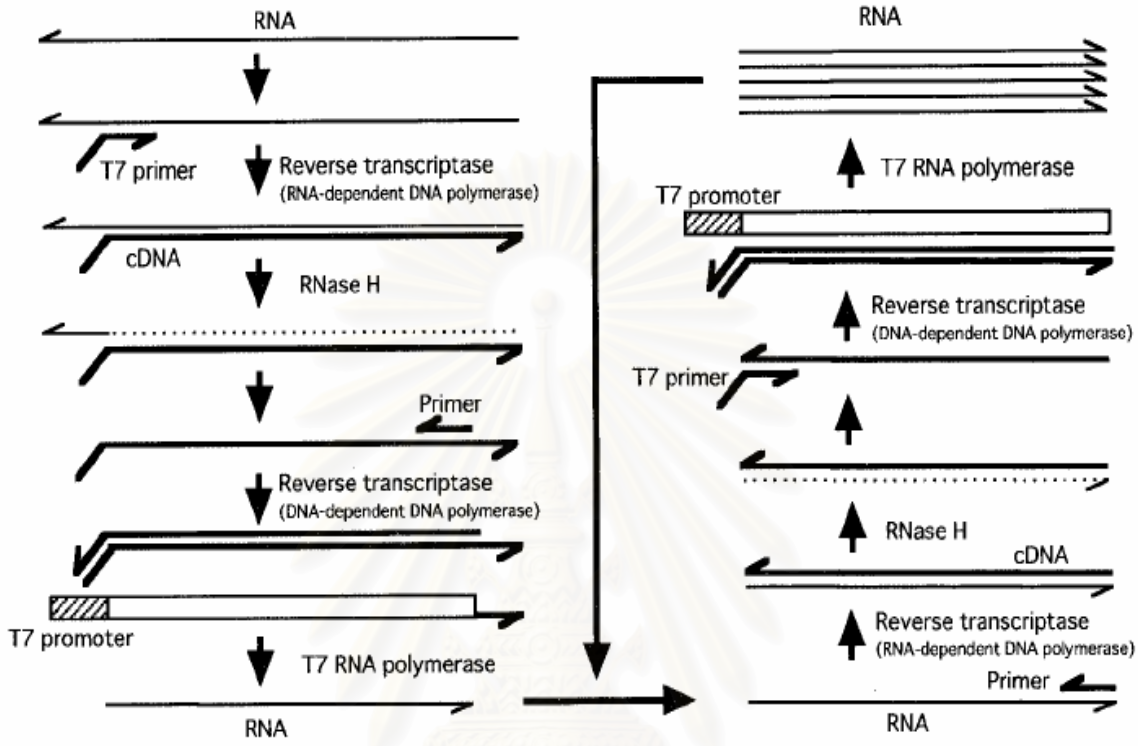


Figure 3 : Scheme for the amplification of RNA and cDNA by the NASBA reaction.

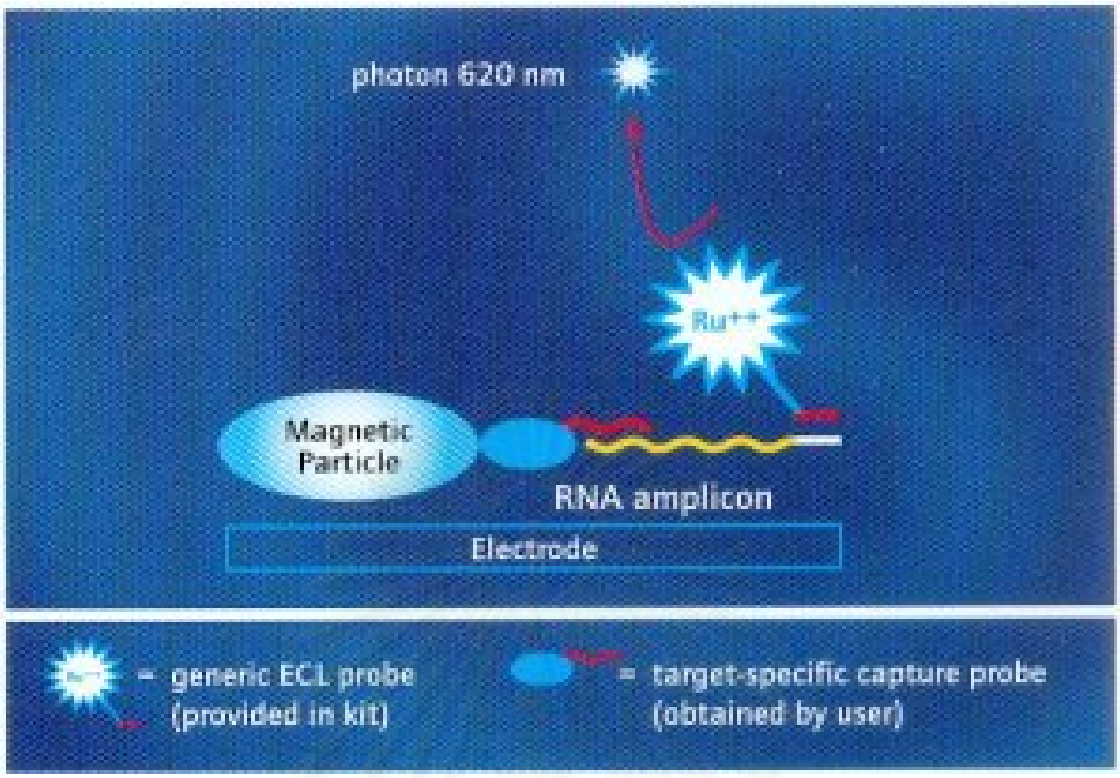


Figure 4 : Scheme for the detection of RNA by the electrochemiluminescence technique.

7. <u>Design and use of performance and sample controls</u>

Quality control of NucliSens Basic Kit runs can be performed with two types of controls: the performance control (PC), which is a single control for every run and/or the sample control (SC), which is a control for every sample (**Figure 5).** Both controls monitor the entire procedure, nucleic acid release and isolation, amplification and detection. Controls monitoring individual parts of the procedure are not addressed in this section.

Performance control (PC), the function of the PC is to monitor the entire procedure (nucleic acid release, isolation, amplification and detection) and the performance of the reagents. It is added as an additional sample to a set of samples

that is under investigation and should be treated in exactly the same manner. In designing a PC there are two options. In the most ideal situation, the PC contains RNA that is identical or similar to the target RNA tested in the biological samples (called wild-type performance control; WT-PC). The WT-PC can either be a biological sample, which is positive for the target RNA or a negative sample to which (for example) *in vitro* generated target RNA has been added. To mimic a biological sample as closely as possible, such an artificial performance control should consist of the same matrix as the samples under investigation. Nuc1eic acid purified from a WT-PC is amplified with the same Basic Kit primer pair as the nucleic acid extracted from the biological samples under investigation. Moreover, WT-PC and biological samples are analyzed with the same Basic Kit detection format (target RNA-specific capture probe and Basic Kit generic ECl probe). Another option is to use RNA, which is not related to the target RNA (called non-WT PC). Although this RNA is detected using a specific set of primers and capture probe, it is still suitable to monitor the different steps in the Basic Kit procedure and the performance of the generic reagents. A non-WT PC has been included in the Basic Kit, together with a specific primer pair for amplification and a specific capture probe linked to magnetic particles for detection.

Sample control (SC), the function of the SC is to monitor the entire procedure (nucleic acid release, isolation, amplification and detection) and the performance of the reagents for each individual biological sample. In addition to the PC it, therefore, also addresses sample-specific effects like inhibitory factors. In **Figure 5**, the various possibilities for this SC are depicted schematically.

In sample control such as intrinsic SC, the easiest option is to use RNA already present in the specimen which is co-isolated with the target RNA. Choosing an

intrinsic RNA which is derived from a cellular housekeeping gene and, as a consequence, is transcribed in most if not all cell types, results in a Sample Control which is broadly applicable. An example of such a universal Sample Control is the human messenger RNA encoding the U1 small nuclear ribonucleoprotein (snRNP)-specific A protein (U1A mRNA). Well-established primer and capture probe sequences, as well as amplification conditions for this target RNA are given below. The use of an intrinsic RNA as SC can also be regarded as indicative for the integrity of the RNA in the original sample, especially if a low abundance messenger like U1A mRNA is used. Substantial degradation of RNA in a particular specimen is reflected by the absence of amplifiable SC RNA.

If no intrinsic RNA is available or in case the amount of SC RNA needs to be known exactly, a specific RNA of known primary sequence can be added to the samples being analyzed. Although such an extrinsic SC reveals no information with regard to integrity of the RNA in the original sample, it is a perfect tool to monitor the entire procedure (nucleic acid release, isolation, amplification and detection) and the performance of the reagents for each individual biological sample.

Other one extrinsic sample control or extrinsic SC, the most ideal composition for an extrinsic SC is a target RNA based molecule (WT-based) consisting of a non-target RNA sequence flanked by segments of the target RNA which contains the recognition sites for the Basic Kit amplification primers. As such, the extrinsic SC can be amplified with the same primers used for the amplification of the target RNA and, thus, can be co-amplified in a single reaction. The extrinsic SC amplicon should preferably be longer than the target RNA amplicon to prevent a more efficient amplification of the Sample Control. In the subsequent detection procedure,

SC amplicons can be distinguished from the target RNA-based amplicons using a specific capture probe hybridizing to the unique non-target RNA-based region. In analyte specific NucliSens assays, this type of extrinsic target RNA-based SC are provided and are referred to as system controls or internal control (IC). Since an extrinsic target RNA-derived sample control (system control) requires the construction of a recombinant DNA molecule from which the sample control RNA can be transcribed, an easier approach is to use a nontarget RNA-related sample control (extrinsic non-WT derived SC). For this type of SC, any RNA with an acceptable length for efficient silica· based extraction (i.e. minimally about 1000 nucleotides) can be used in principle. However, such a SC cannot be amplified with the same primer pair as used for the target RNA. Therefore, either extra amplification reactions are required or multiplex amplification must be performed (multiple primer pairs in a single reaction). Since the combination of multiple primer pairs in multiplex reactions can have a negative impact on the sensitivity of the individual primer pairs, the latter option is not recommended. In this study, the extrinsic non-WT has been used for IC in IC-NASBA.

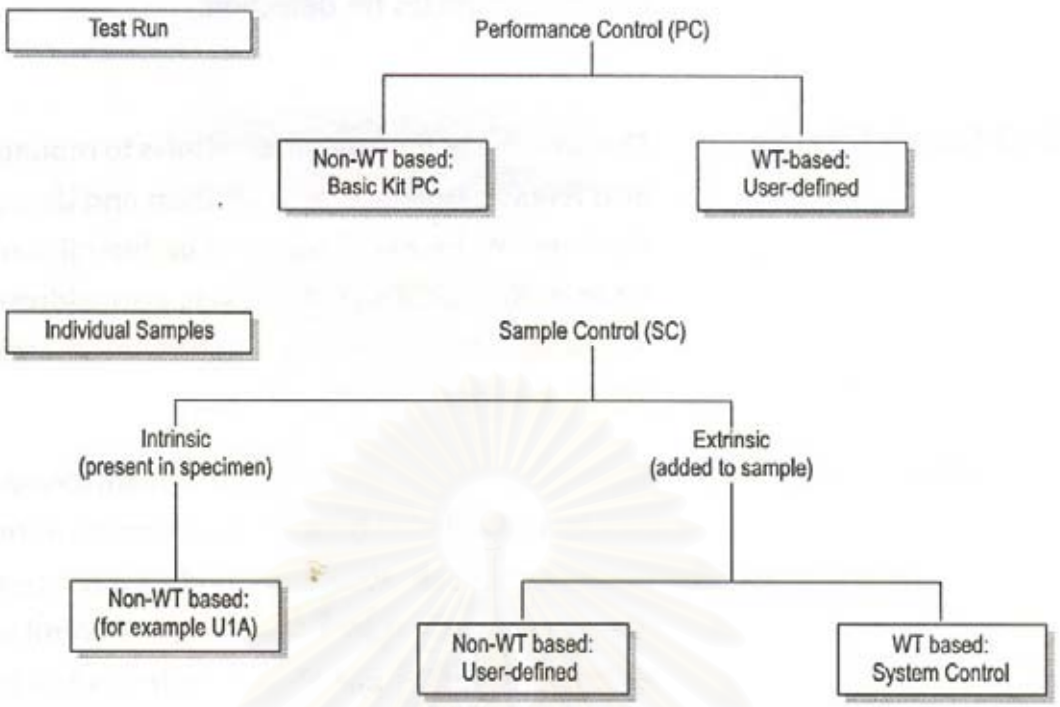


Figure 5 : Schematic overview of control levels and control options at assay run or sample level for the NucliSense Basic kit.

8. Mutagenesis

Site-specific mutagenesis of DNA is a very important tool in genetic engineering. Changing the DNA sequence can facilitate the study of the structure function relationships of DNA, RNA, or protein coded by the DNA sequence. A variety of methods have been applied for the introduction of specific base change at predetermined site in DNA sequences [128-136], the most powerful among which is overlap extension by polymerase chain reaction (OE-PCR), described by Higuchi et al. (1988) [128]. In some case, it is desirable to introduce multiple different substitutions at a particular position or at several positions in a gene and to determine the

consequences of these changes on protein function or to optimize the expression. However, traditional OE-PCR can introduce mutations at only one site at a time; and efficiencies drop drastically when a few sites are targeted simultaneously. New method has developed a rapid, efficient and high-fidelity modification (M) of OE-PCR (i.e. MOE-PCR) which allows one to generate multiple site-directed mutations in a given DNA fragment [130].

The concept of overlap extension allows PCR to be used for both introducing mutations into the centre of a fragment and for creating recombinant DNA molecules. The process of overlap extension depends on the fact that sequences added to the 5'- end of a PCR primer become incorporated into the end of the product molecule. By adding the appropriate sequences, a PCR amplified segment can be made to 'overlap' or share sequences with another segment. In a subsequent reaction, the overlap serves as a primer for extension by DNA polymerase, which creates a recombinant molecule. An overview of the method and its applications is given in **Figures 6-7**.

The estimated T_d [137] as a guide in deciding how long to make priming and overlap regions. The estimated T_d is an empirically derived formula which usually provides a fairly accurate guess of the temperature at which an oligonucleotide will bind to 50% of a target sequence on a solid support in a standard salt buffer (5 x SSPE). It is calculated as follows:

((# G's + # C's) x 4) +((# A's + #T's) x 2)

This number is the approximate denaturation temperature of the oligo-nucleotide in degrees Celsius.

One word of caution: the estimated T_d is not an accurate estimation of the annealing temperature of an oligo in PCR buffer which contains, among other things, Mg^2+, which is not included in 5 x SSPE. Also, one might be tempted to place the oligo in a very GC-rich region in order to achieve the 50°C temperature with as few residues as possible. To do so would violate a commonly accepted standard of primer design, which is to have a decent balance of nucleotides in the primers.

Primer sequences should always be checked to be sure that they do not form any obvious hairpin loops, and that they do not hybridize to the other primer in the same reaction. It is also important that the most 3' four or five bases match the template if a sequence is to act as a primer. Mismatches too close to the 3'- end will prevent priming. In this study, the MOE-PCR method was used to construct rabies modified fragment containing 25 changed and 42 inserted nucleotides in N gene of rabies virus for internal control (IC) in IC-NASBA.

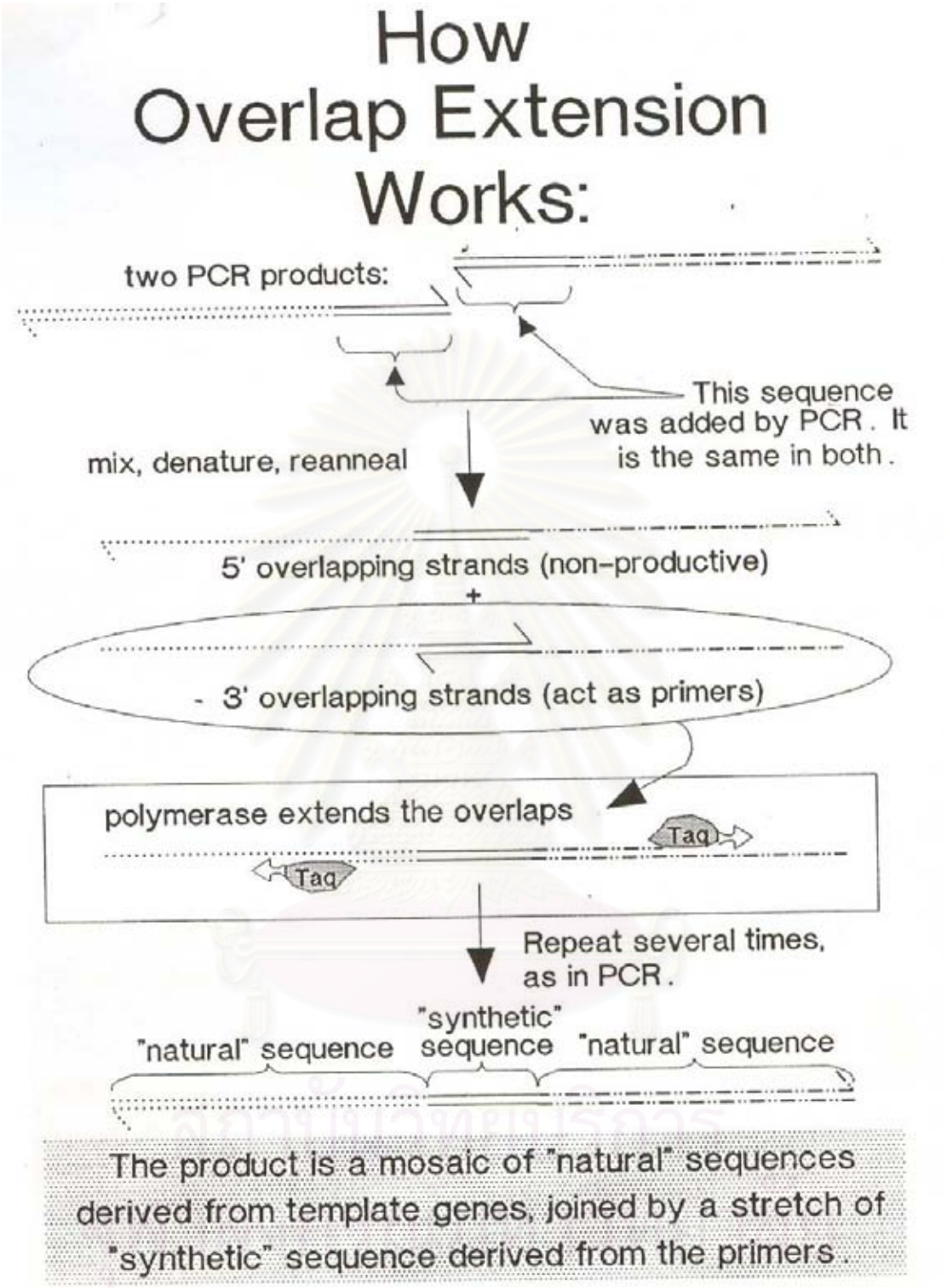


Figure 6 : Outline of the principle of overlap extension PCR to create a recombinant molecule.

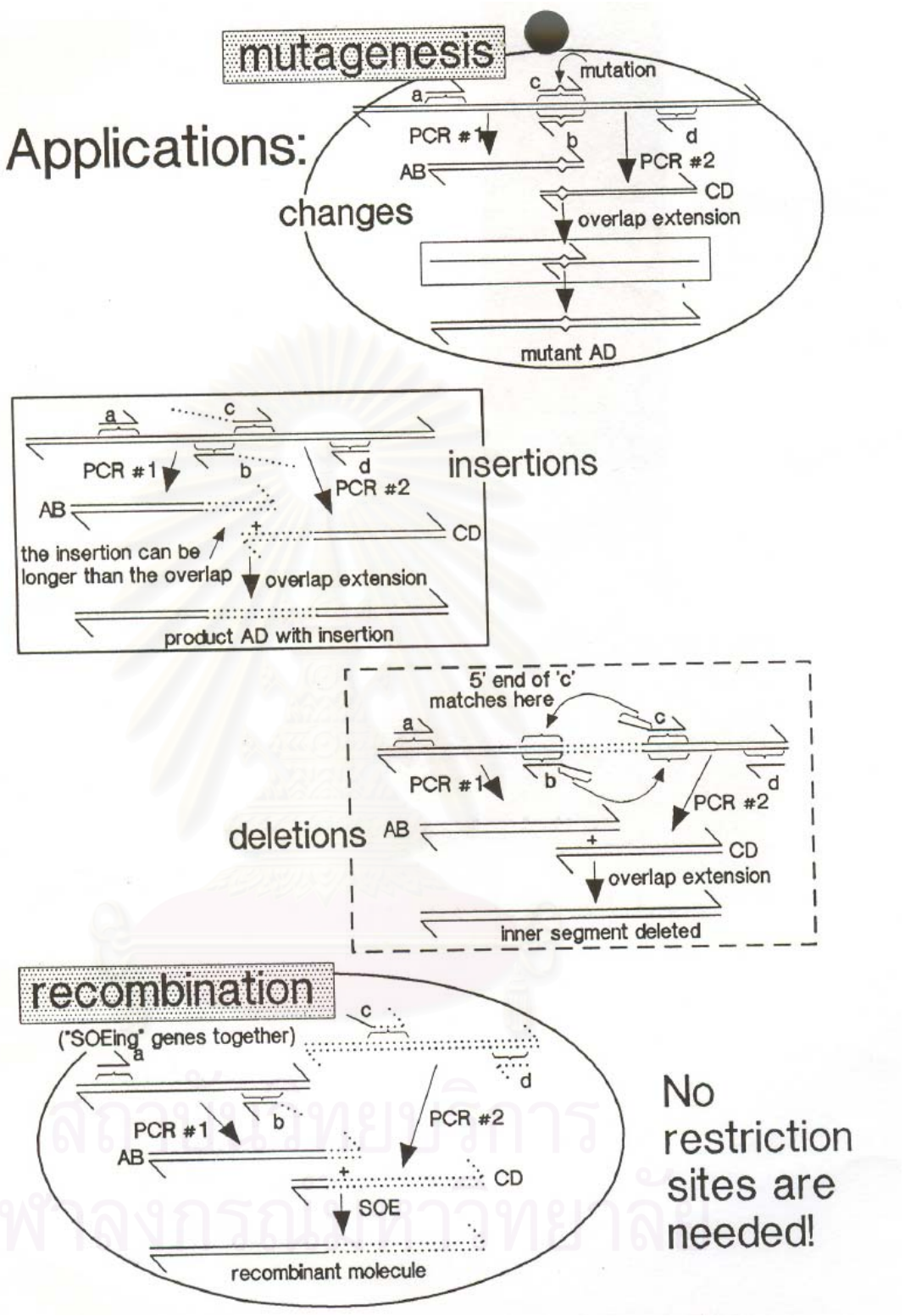


Figure 7 : Outline of some current applications of overlap extension PCR for the mutagenesis and recombination of DNA molecule.

9. IC-NASBA

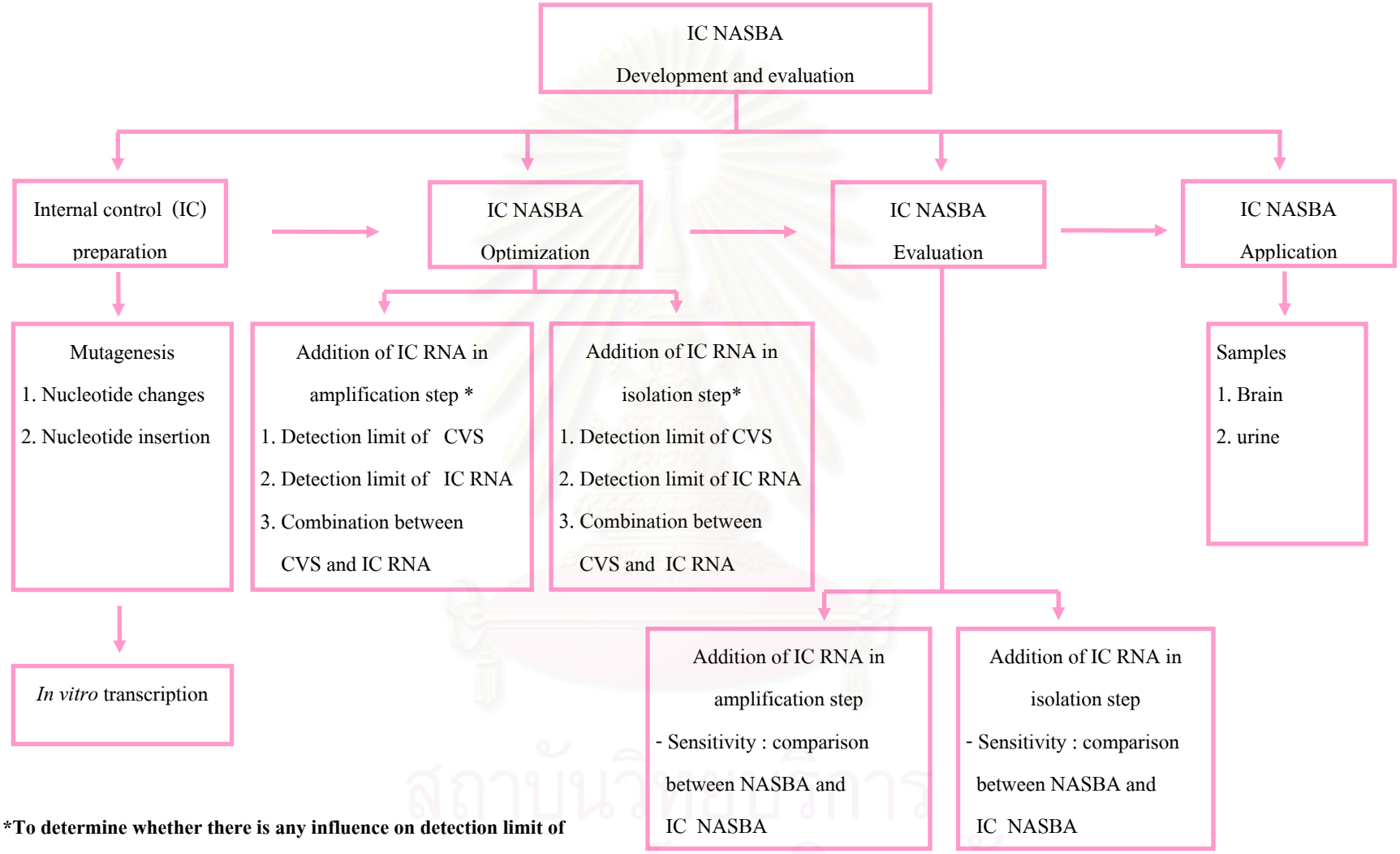
Aims of the present study were to evaluate nucleic acid sequence based amplification with internal control RNA (IC-NASBA) for diagnosis of rabies, and to compare the sensitivity between NASBA and IC-NASBA. Advantages of using IC-NASBA, for example, it can be used to avoid false negative result, thus, increasing accuracy in the diagnosis of rabies. IC-NASBA system has been successfully applied to many RNA viruses, including enteroviruses and rhinoviruses [138,139].

The principle of IC-NASBA and NASBA are similar. IC-NASBA theory which relies on using internal control (IC) or extrinsic non-WT can be described as the following steps: first, IC can be amplified with the same primers used for the amplification of the target RNA and, thus, can be co-amplified in a single reaction. Second, IC amplicon should preferably be longer than the target RNA amplicon to prevent a more efficient amplification of the IC. Finally, IC can be distinguished from the target RNA-based amplicons using a specific capture probe hybridizing to the unique non-target RNA-based region. For this type of IC, any RNA with an acceptable length for efficient silica-based extraction (i.e. minimally about 1000 nucleotides) can be used in principle. In this study, MOE-PCR method was used to construct N gene of rabies virus with 25 changed and 42 inserted nucleotides as IC for IC-NASBA. Comparison of sensitivity and specificity was also made when IC was added at initial prior to the step of extraction or after extraction step but prior to amplification.

CHAPTER III

MATERIALS AND METHODS

A conceptual framework of the experimental designs to develop and evaluate protocol to detect rabies virus RNA using NASBA with internal control RNA (IC-NASBA) was presented in **Figure 8**.



***To determine whether there is any influence on detection limit of IC RNA and CVS. Test performed in amplification and isolation to determine detection limit of CVS and IC RNA and when IC RNA and CVS are present together.**

Figure 8 : Conceptual framework of the experimental designs.

1. Samples

1.1 Challenge virus standard (CVS) : for development and evaluation step

Challenge virus standard (CVS) rabies virus from infected baby hamster kidney (BHK) cells was used in the development and evaluation steps. RNA was extracted from tissue culture medium harvested from BHK cells infected with fixed CVS rabies virus (Gift of Dr. Pkamatz Khawplod, Thai Red Cross Society)

1.2 Biological specimens : in the application step

1.2.1 Brain specimens

Brain specimens were from naturally rabies infected dogs from Quarantine and Diagnostic Unit, Queen Saovabha Memorial Institute. These were confirmed for the presence of rabies virus by using direct fluorescent antibody (DFA) test and mouse inoculation test (MIT). All specimens were determined the blind specimen.

1.2.2 Urine specimens

Urine specimens in this study were previously confirmed to contain inhibitor by nested RT-PCR [32]. All specimens were determined the blind specimen.

2. Internal control RNA preparation

2.1 Mutagenesis : by modified overlap extension PCR (MOE-PCR)

The MOE-PCR method was used to construct rabies modified fragment containing 25 changed and 42 inserted nucleotide in N gene fragment of rabies virus for IC DNA. The whole processes were shown in **Figures 9 - 10**.

2.1.1 <u>Nucleotide changes method</u>: target for specific IC probe

The primers use for amplification of pre-nucleotide change fragment were designed according to GenBank accession number M13215 with some modifications as shown in **Table 1**.

Table 1: Oligonucleotide sequence primers for 25 changed nucleotides by MOE-PCR

Primer Set	Primer Combination	Sense Primer	Anti-sense Primer	PCR Fragment
1	CN11 (A) and Mutant-1R (B)	TGCAGACAGGATA GAGCAGA	**GAGTCCTAATAG ACAGAGTAATCG** **A**ATAGATGCTCA ATCC	192
2	Mutant-4F (C) and LISEBL2R (D)	**TCGATTACTCTG TCTATTAGGACT** **C**TGTCACTGCTTA TG	GATCTCTTCCTCG AAGTTCTT	121
3	CN11 (A) and LISEBL2R (D)	TGCAGACAGGATA GAGCAGA	GATCTCTTCCTCG AAGTTCTT	313

Note : Nucleotide changes in bold and consensus sequences in italic.

2.1.1.1 Modification overlap extension PCR (MOE-PCR)

Two PCR reactions (AB and CD) were performed to synthesize AB and CD products. Fifty µl of PCR reaction contained 1X PCR buffer (Promega), with sense and anti–sense primers (A and B primers for PCR 1 and C and D for PCR 2) to a final

concentration of 1 µM each, $MgCl_2$ to 3 mM, dNTP to 0.2 mM, 2.5 U Taq DNA Polymerase and 5 µl DNA template were added. All the mixtures were placed in a Thermal Cycler with the following program: incubation at 94°C for 10 minutes, amplification was carried out for 10 cycles with the following temperature cycling parameters: 94°C for 1 minute of denaturation; 38°C for 1 minute of annealing; and 72°C for 1 minute of extension, followed by 35 cycles of temperature cycling parameters: 94°C for 1 minute of denaturation, 50°C for 1 minute of annealing; and 72°C for 1 minute of extension. The final amplification cycle included an addition of 7 minutes extension at 72°C. The PCR products were separated in a 2% agarose gel and visualized by ethidium bromide staining.

The final product AD was made by hybridizing the overlapping strands from the two fragments of AB and CD. The PCR reaction was performed in a 50 µl reaction containing 1X PCR buffer (Promega), sense and anti–sense primers to a final concentration of 1 µM each, $MgCl_2$ to 3 mM, dNTP to 0.2 mM, 2.5 U Taq DNA Polymerase and 10 µl DNA template of the PCR product AB+CD mix were added. All the mixtures were placed in a Thermal Cycler with the following program : incubation at 94°C for 10 minutes, amplification was carried out for 35 cycles with the following temperature cycling parameters: 94°C for 1 minute of denaturation; 58°C for 1 minute of annealing; and 72°C for 1 minute of extension. The final amplification cycle included an addition of 7 minutes extension at 72°C (**Figure 10**). The PCR products were separated in a 2% agarose gel and visualized by ethidium bromide staining.

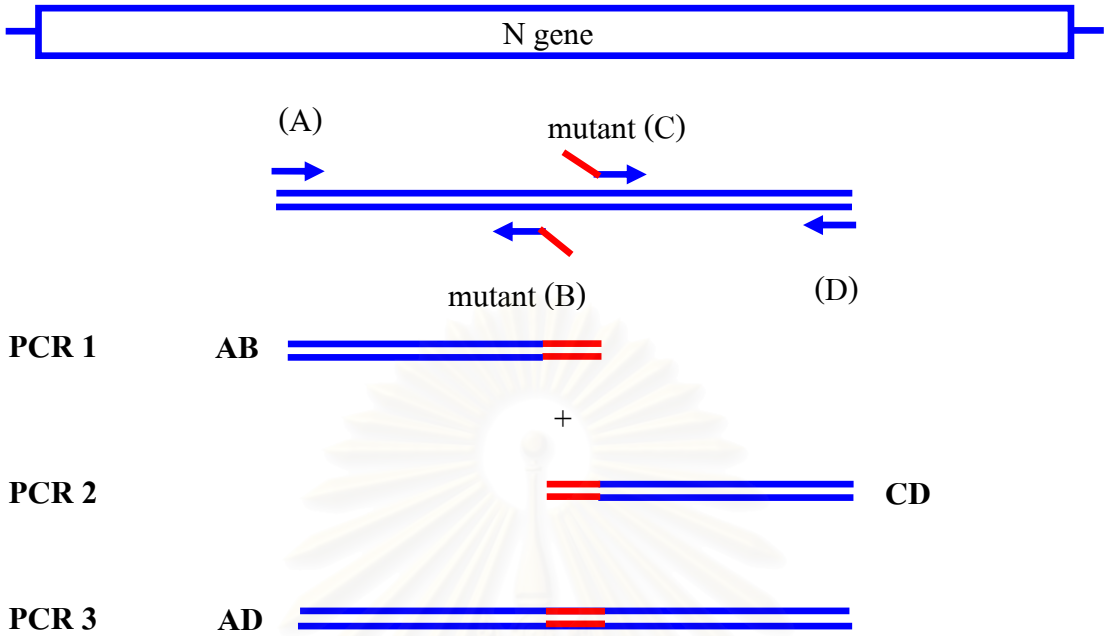


Figure 9: The principle of MOE-PCR for 25 changed nucleotides in N gene fragment of rabies virus.

2.1.1.2 DNA Cloning: AD PCR product was cloned in to pGem®-T plasmid for template in nucleotide insertion as following steps.

2.1.1.2.1 DNA purification

AD PCR products of IC DNA were separated by gel cutting and purified by QIAquick® Gel Extraction Kit (Qiagen, Chatsworth, CA). Three volumes of Buffer QG to one volume of gel (100 mg ~ 100 µl) were added and the tube was placed in a 50°C water bath incubator. After agarose gel was completely dissolved, one gel volume of isopropanol was added, mixed and applied to the QIAquick column. After centrifugation at 10,000 g for 1 minute, the flow-through solution was discarded. The DNA fragments was washed with Buffer PE and centrifuged for 1 minute. Buffer EB

(10 mM Tris·Cl, pH 8.5) or ddH_2O was added to elute DNA and was then centrifuged for 1 minute, stored at – 20°C.

2.1.1.2.2 Ligation of PCR products into plasmid vector

The ligation of gel purified IC DNA was carried out in a 10 µl reaction mixture containing pGem®-T (Promega, Madison, WI, USA) vector and IC DNA in the molar ratio 1:3, 2 units of T_4 DNA ligase and 1X buffer (provided). The pGem®-T vector was approximate 3.0 kb and supplied at 50 ng/µl. The amount of the DNA insert was calculated from the following equation:

$$\frac{\text{ng of vector x size (kb) of insert}}{\text{size (kb) of vector}} \times \text{insert : vector molar ratio} = \text{ng of insert}$$

The ligation reaction was carried out at room temperature for 1 hour at 4°C for 16-18 hours and the ligation products were used to transform *Escherichia coli* competent cells prepared by $CaCl_2$ method.

2.1.1.2.3 Preparation of *E. coli* competent cells by $CaCl_2$ method

A single colony of *E. coli* was inoculated into 3 ml of LB broth and incubated at 37°C with 200 rpm shaking for 16-20 hours. The overnight culture was diluted 1:100 into 100 ml of new LB broth and incubated at 37 °C until an OD_{600} of 0.4-0.5. The cell culture was chilled on ice for 10 minute prior to pipetting the 10 ml aliquots of the culture into 15 ml sterile polypropylene tubes. The cells was pelleted by centrifugation

at 4,000 rpm for 10 minute at 4°C, the pellet was suspended in 5 ml of ice-cold 0.1 M $MgCl_2$. After centrifugation at 4,000 rpm for 10 minutes at 4°C, the pellet was suspended in 5 ml of ice-cold 0.1 M $CaCl_2$ and left on ice for 30 minutes to establish competency. After centrifugation at 4,000 rpm for 10 minutes at 4°C, the pellet was resuspended in 750 µl of 15% (v/v) glycerol and 0.1 M $CaCl_2$. The cells were kept in 200 µl aliquots at –80°C until required.

2.1.1.2.4 Transformation of *E. coli* competent cells

One hundred microlitters of Top10 *E. coli* competent cells were mixed with 2 µl of ligation products and immediately placed on ice for 30 minutes. The cells were subjected to heat-shock at 42°C for 1 minute and placed on ice for an additional 3 minutes. The transformed cells were mixed with 900 µl of LB broth and incubated at 37°C for 1 hour with shaking at 200 rpm. Finally, 100 µl of the transformed culture was spread on an LB agar plate containing 50 µg/ml ampicillin, 40 µl of stock solution of X-gal (20 mg/ml in dimethylformamide) and 8 µl of a solution of isopropylthio-β-D-galactoside (IPTG) (100 mg/ml) and incubated at 37°C overnight. After transformation, the white colonies containing IC DNA were selected and extracted by QIAprep miniprep (QIAGEN,).

2.1.1.2.5 Plasmid DNA extraction by QIAGEN Plasmid Mini Kit

Plasmid DNA extraction was performed by QIAGEN Plasmid Mini Kit according to manufacturer's protocols (QIAgen). Briefly, a single white colony of bacteria was inoculated in 5 ml LB broth and incubated at 37°C with 200 rpm shaking for 16-20 hr. The cells were harvested in 15 ml centrifuge tubes by centrifugation at

3,000 rpm for 5 minutes and then resuspended in 250 µl of P1 buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0). To the cell suspension, 250 µl of P2 buffer (1% SDS, 0.2 N NaOH) was added and mixed by invert. Then, 350 µl of N3 buffer (3 M potassium acetate, 11.5% glacial acetic acid) was added to the mixture and mixed by invert. The mixture was pelleted by centrifugation at 13,000 rpm for 10 minutes. The plasmid DNA was recovered from the supernatant. The supernatant was moved to a column. The content was centrifuged at 13,000 rpm for 1 minute. The pellet was washed with 500 µl of PB buffer, centrifuged at 13,000 rpm for 1 minute and washed again with 750 µl of PE buffer, centrifuged at 13,000 rpm for 1 minute. The DNA pellet was resuspended in 30 µl of EB buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) or sterile water. Then the plasmid DNA were digested with *Eco*R I and separated on agarose gel again to check for successful cloning.

2.1.1.2.6 Digestion of restriction endonucleases and analysis

About 500 ng of plasmid DNA was digested with 5 units of *Eco*R I according to manufacturer's protocols (New England Biolabs), 1X reaction buffer (provided), and sterile distilled water added to a final volume of 20 µl. The digestion was incubated at 37°C for 3 hours. After digestion, agarose gel electrophoresis was performed and analyzed to screen for plasmids, which contain IC DNA.

2.1.1.2.7 DNA sequencing for check sequence and direction of IC DNA

Positive colonies were sequenced using ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction kit with Amplitaq® DNA Polymerase FS Version 2 (Applied Biosystems, Foster city, CA). The PCR reaction was carried out in a 10 µl

reaction containing 4 µl of terminator ready reaction mix, 200 nM of primer and 500 ng DNA template. After incubation at 95°C for 5 minutes, amplification was carried out for 25 cycles with the following temperature cycling parameters; 95°C for 10 seconds of denaturation, 50°C for 5 seconds of annealing and 60°C for 4 minutes of extension. The DNA was then precipitated by the addition of 1/10 volume of 3 M sodium acetate and 2 volume of absolute ethanol and incubated at –20°C overnight. After centrifugation at 12,000 rpm for 25 minutes, the pellet was washed with 500 µl of 70% ethanol, centrifuged at 12,000 rpm for 8 minutes and air dried. The DNA pellet was resuspended in 10 µl Template Suppression Reagent (Perkin) and sequencing was proceeded using an automated sequencer (Applied Biosystems, Foster city, CA) according to manufacturer's protocol, to confirm the sequence of PCR product using T7 primer.

2.1.1.2.8 Alignment and computational searching sequences analysis

The nucleotide sequences obtained from the clones of interest were compared against nucleotide sequences in all database by using BLAST (Basic Local Alignment Search Tool, Version 2.0, developed by Altschul et al. (1990) program via the world wide web [140]. The sequences were consisted of 2 region, conserve region and mutant region. Alignments of IC sequences were made using CLUSTAL X multiple alignment program (EMBL).

2.1.2 <u>Nucleotide insertion method</u>:

Since IC RNA might interfere with the PCR of CVS, nucleotide insertion was designed in order to reduce the efficiency of IC RNA in amplification step. The primers

used for amplification of pre nucleotide insertion were designed according to GenBank accession number M13215 with some modification as shown in **table 2**. The MOE-PCR method was used to cloning mutant DNA of AD for template (master mix and PCR profiles as described previously) **(Figure 11)**. This synthesized DNA was cloned into plasmid pGem®-T (Promega, Madison, WI, USA) and check sequences. After alignment of IC DNA, the sequences were consisted of 3 region, conserve region, changes region and insertion region.

Table 2: Oligonucleotide sequence primers for 42 inserted nucleotide by MOE-PCR.

Primer Set	Primer Combination	Sense Primer	Anti-sense Primer	PCR Fragment
1	CN11 (A) and Mutant-2R (E)	TGCAGACAGGATA GAGCAGA	**ACCGGATGTGCT CACAGAACTGCT GATGTTTCCTGA ACTGCA**CTCCAA TTAGCACACATC	141
2	Mutant-3F (F) and LISEBL2R (D)	**TGCAGTTCAGGA AACATCAGCAGT TCTGTGAGCACA TCCGGT**TACTATA CCGAACTTCAG	GATCTCTTCCTCG AAGTTCTT	256
3	CN11 (A) and LISEBL2R (D)	TGCAGACAGGATA GAGCAGA	GATCTCTTCCTCG AAGTTCTT	355

Note: Nucleotide insertion in bold and consensus sequences in italic.

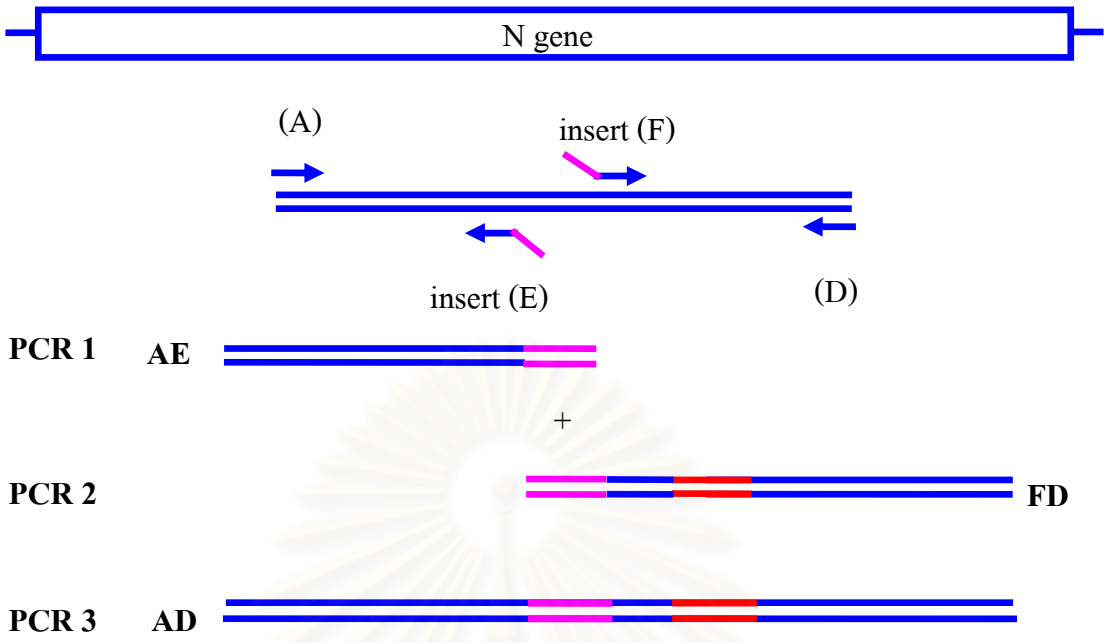


Figure 10: The principle of MOE-PCR for 42 inserted nucleotide in N gene fragment of rabies virus.

2.2 RNA synthesis by *in vitro* transcription

For generation of *in vitro* RNA, plasmids were linearized with Xmn I followed by SP6 RNA polymerase transcription [141]. *In vitro* transcription was performed using the SP6 RiboMAX™ Longe Scale RNA Production Systems (Promega) according to the manufacturer's instruction. Briefly, the reaction mixture contained SP6 Transcription 5X Buffer (Promega), 2.0 µg Enzyme Mix (SP6), 10 mM of rNTPs , 3 ug of linear DNA template plus Nuclease-free water to 50 ul total volume. The reactions were incubated for 3 hours at 37 °C. The DNA template was removed by digestion with DNase following the transcription reaction. RQ1 RNase free DNase added to a concentration of 1 unit per microgram of template DNA and incubated for 15 minutes at

37 ^{o}C. The reaction was diluted to 200 µl prior to RNA purification by RNA cleanup protocol of RNeasy kit (Qiagen) using procedures recommended by the manufacturer.

After the removal of the template and unincorporated nucleotides, the RNA concentration was quantitated by ultraviolet light absorbance. One A260 unit equals approximately 40 µg/m/ of RNA 1:50 dilution of RNA were prepared and read absorbanced at wavelength of 260 nanometers. The copy number of anti–sense RNA was calculated based on the concentration measured and its molecular weight. IC RNA was sized 1.5 Kb. The PCR products were separated in a 1% agarose gel and visualized by ethidium bromide staining.

3. <u>IC-NASBA Optimization</u> : Two options of IC RNA adding were performed.

3.1 <u>Addition of IC RNA in amplification step</u>

NASBA was performed using the Nuclisens Basic Kit (bioMe´rieux, Durham, NC) and detection system (bioMe´rieux). Each NASBA assay included a deionized H_2O blank as a negative control.

3.1.1 NASBA

3.1.1.1 Nucleic acid isolation

Nucleic acids were isolated from 100 µl CVS supernate in NASBA lysis buffer essentially as described by Boom et al. (1990) [142]. Nucleic acids were allowed to bind to activated silica for 10 minutes at room temperature and were mixed every 2 minutes. The silica was washed twice with wash buffer (5 M guanidinium thiocyanate, 50 mM Tris pH 6.4), two times with 70% ethanol and once with acetone. Finally, the

silica was dried at 56°C and nucleic acids were eluted with 50 µl elution buffer (1 mM Tris pH 8.5) at 56°C during 10 minutes.

3.1.1.2 Nucleic acid amplification

NASBA reactions were carried out as described by Kievits et al. (1991) in a 10 µl reaction mixture containing NASBA buffer and primer mix (**Table 3**) was added to 5 µl of template containing 1 µl of IC RNA and 4 µl of isolated or diluted RNA. Final concentrations were as follows: (a) NASBA buffer; 40 mM Tris pH 8.5, 12 mM MgCl2, 80 mM KCl, 1 mM of each dNTP, 2 mM of each NTP; (b) 0.2 µM of each primer. The mixture was heated to 65°C for 5 minutes and afterwards cooled at 41°C for 5 minutes. Then 5 µl of an enzyme mix containing T7 RNA polymerase (40 U), avian myeloblastosis reverse transcriptase (8 U), RNase H (0.2 U) were added and the mixture was incubated at 41°C for 90 minutes in water bath. The final volume was 20 µl. In other specimen, nucleic acids were isolated from 4 µg of brain tissue and 1 ml of urine in NASBA lysis buffer.

Table 3: Sequences of primers and probes for NASBA

Virus (gene target)	**Primer and probe**	**Sequence 5'-3'**	**Position**	**Function**
rabies virus	P1 (sense)	ATCGTGGAGCACC ATACTCTCA	611-632	NASBA P1 primer T7 RNA polymerase tail
	P2 (antisense)	TACCAGCCCTGAA CAGTCTTCA	790-769	NASBA P2 primer ECL detection tail
	WT Probe	ACTGTGCCCACTC TGATTGCTGAAT	756-732	Rabies WT capture probe
	Mutant Probe	GAGTCCTAATAGA CAGAGTAATCGA	-	Rabies mutant capture probe

3.1.1.3 Nucleic acid detection

For optimal detection, the amplification products for nucleoprotein of rabies virus were diluted 1:100 in detection reagent (1.0 mM Tris pH 8.5, 1.3 mM 2-methylisothiazolone HCl). Five microliters diluted NASBA product were incubated with IC and WT probe of specific biotin labeled probe bound to streptavidin coated magnetic beads and a ruthenium-labeled probe in a total volume of 25 µl of 5 × SSC (0.75 M NaCl and 75 mM sodium citrate) for 30 min at 41°C. Assay buffer (100 mM tripropylamine, pH 7.5) was added and the tubes were placed in an electrochemiluminescence (ECL) reader instrument (NASBA System, Biomerieux, Boxtel, The Netherlands). Determination of cut-off level was done by protocols previously described in Wacharapluesadee S. et al., 2001. The principle method of IC-NASBA: adding IC in amplification step **(Figure 11)**.

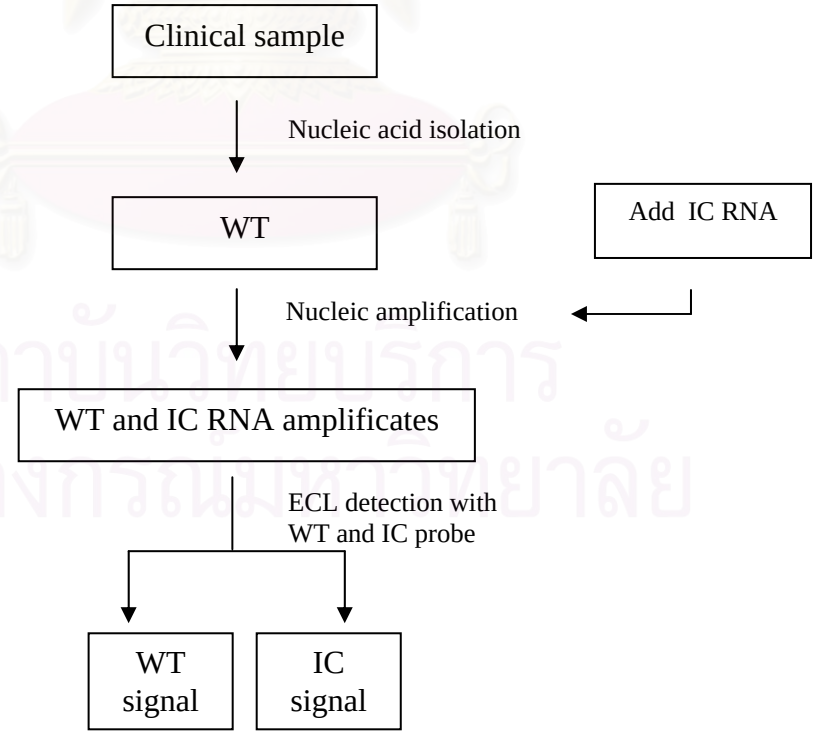


Figure 11: Schematic flow-chart of the one-tube IC-NASBA with addition internal control (IC) RNA before nucleic acid amplification in the NASBA reaction .

3.1.2 Detection limit of CVS RNA

Detection limit of CVS was performed by 10- fold dilution series in amplification steps.

3.1.3 Detection limit of IC RNA

Detection limit of IC was performed by 100- fold dilution series in amplification step.

3.1.4 Combination of CVS and IC RNA

CVS and IC RNA with previously determined concentrations from 3.1.2 + 3.1.3 were mixed. The optimized IC RNA concentration was to be obtained.

3.2 <u>Addition of IC RNA in isolation step</u>

3.2.1 NASBA

3.2.1.1 Nucleic acid isolation

Using standard protocol as in 3.1.1.1 and that IC RNA was added directly into the specimens.

3.2.1.2 Nucleic acid amplification

Using standard protocol as 3.1.2.1 and that IC RNA was not added in to the NASBA reactions.

3.2.1.3 Nucleic acid detection

Using protocol as described above.

The principle method of IC-NASBA: adding IC in isolation step **(Figure 12)**.

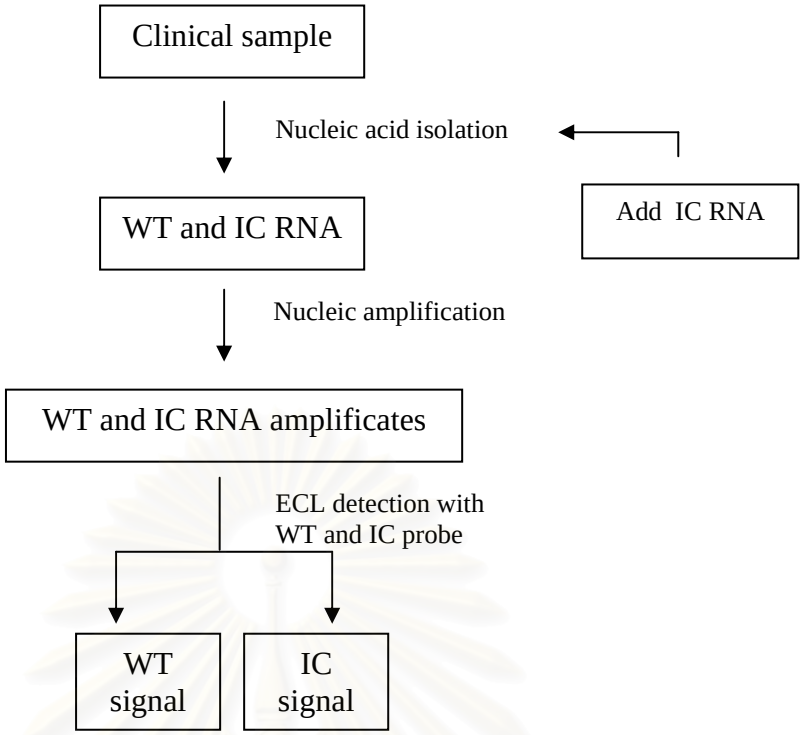


Figure 12: Schematic flow-chart of the one-tube IC-NASBA with addition of internal control (IC) RNA prior to nucleic acid isolation in the lysis buffer.

3.2.2 Detection limit of CVS RNA

Detection limit of CVS was performed by 10- fold dilution series in isolation steps.

3.2.3 Detection limit of IC RNA

Detection limit of IC was performed by 100- fold dilution series in isolation steps.

3.2.4 Combination of CVS and IC RNA

CVS and IC RNA with previously determined concentrations from 3.2.2 + 3.2.3 were mixed. The optimized IC RNA concentration was to be obtained.

4. IC-NASBA evaluation

4.1 Addition of IC RNA in amplification step

Comparison of sensitivity between NASBA and IC -NASBA.

4.2 Addition of IC RNA in isolation step

Comparison of sensitivity between NASBA and IC-NASBA.

5. IC-NASBA application

5.1 Twenty samples of rabies infected and non-infected brains were tested with IC-NASBA.

5.2 Twenty urine samples were tested with IC-NASBA. These urine samples were already proven to contain inhibitor by nested RT-PCR.

6. Data Analysis

Data analysis was performed, the results were as follows:

6.1 A signal was detected in WT channel.

Positive result: The specimen contained rabies virus RNA.

In this case, the detection of a signal in the IC channel was dispensable, since high initial concentrations of rabies RNA (positive signal in the WT channel) could lead to a reduced or absent ECL signal of the IC in the IC channel (competition).

6.2 No signal in WT channel but IC channel was detected.

There was no rabies virus RNA in this specimen and this could be considered as true negative.

6.3 <u>No signal was detected in WT or IC channel.</u>

This was inconclusive. This sample might contain inhibitor(s) which could inhibit the amplification of IC RNA.

CHAPTER IV

RESULTS

1. Internal control RNA preparation

1.1. Nucleotides change

1.1.1 Modified overlap extension PCR (MOE-PCR)

The MOE-PCR method was used to construct rabies modified fragment containing 25 changed nucleotides in N gene fragment of rabies virus for probe design in IC-NASBA. The AB PCR product of CN-11 (A) and Mutant 1R (B) primers showed PCR of product size 192 bp (lane1). The CD PCR product of Mutant 4F (C) and LISEBL 2R (D) primers showed PCR of product size 121 bp (lane2). The final AD PCR product was made by hybridizing the overlapping strands from the two fragments of AB and CD. The AD PCR product using CN-11 (A) and LISEBL 2R (D) primers showed PCR product size of 313 bp (lane3) (**Figure 13**).

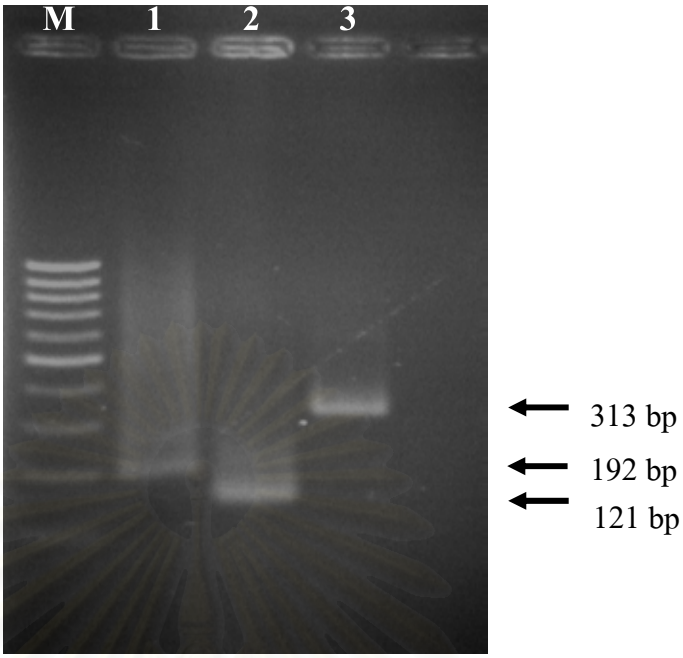


Figure 13: An ethidium bromide stained agarose gel showing the PCR products of IC using CN-11 (A), Mutant 1R (B), Mutant 4F (C) and LISEBL 2R (D) primers in MOE-PCR. Lane M, 100 bp DNA ladder; lane1, AB primer; lane2, CD primer; lane 3, AD primer.

1.1.2 Cloning and Sequencing of nucleotide changed product

1.1.2.1 Digestion of restriction endonucleases and analysis of the pGem®-T vector containing 25 changed nucleotides sequences.

After purification the IC PCR product (**Figure 11**) and pGem®-T vector was ligated and transformed to the *E. coli* competent cells. The selected white clones were inoculated into 5 ml of LB broth and incubated at 37°C for 16-20 hours. After that, plasmid DNA extractions were digested with *Eco*R I, and then fractionated on agarose gel electrophoresis to screen the clones which containing of 356 bp insertions (**Figure 14**).

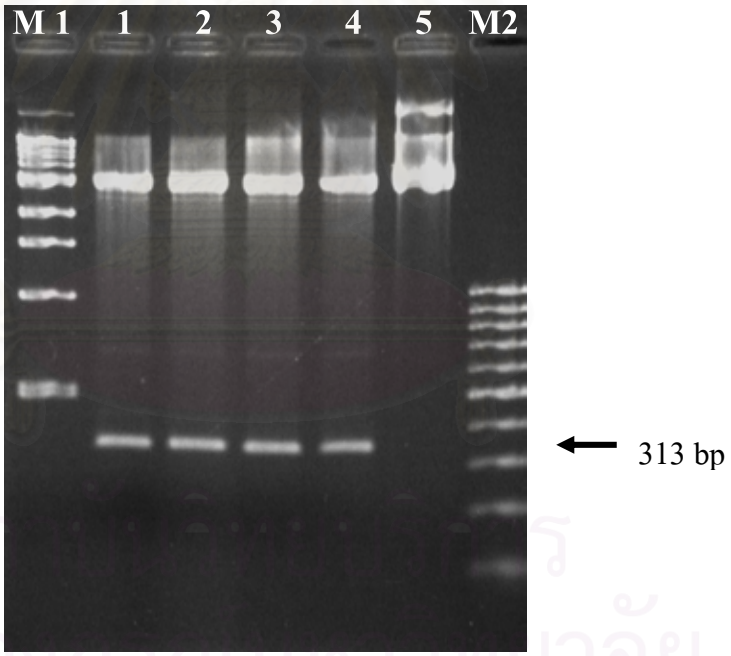


Figure 14: An ethidium bromide stained agarose gel showing the digestion of ligated pGem®-T vector containing 25 changed nucleotides sequences. Lane M1; 1 Kb DNA ladder, lane 1, 2, 3, 4; pGem®-T vector with IC DNA. lane 5; non-digested IC in pGem®-T vector, lane M2; 100 bp DNA ladder.

1.1.2.2 Nucleotide Sequence Alignment

The nucleotide sequences of CVS and IC DNA from clones were aligned using CLUSTAL X. The result showed that IC DNA sequences contained 2 regions; rabies conserved region designed primer in Nuclisens amplification and designed changed region which were used for probe design (**Figure 15**).

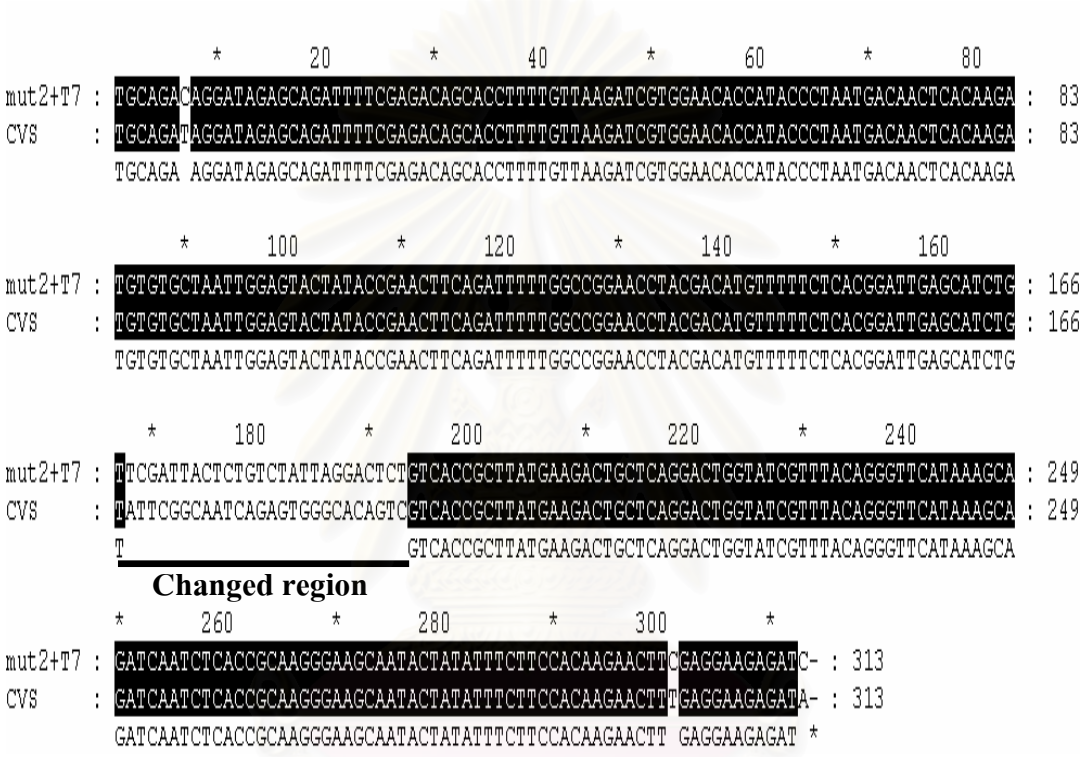


Figure 15: Nucleotide sequences alignment of CVS and IC DNA containing 25 changed nucleotides (mut2) from clones. Identical nucleotides were highlighted. Those underlined indicated the regions, which were designed to the probe.

1.2 Nucleotides insertion

1.2.1 Modified overlap extension PCR (MOP-PCR)

The MOE-PCR method was used to add 42 nucleotides in N gene fragment of rabies virus fragment from1.1 to reduce the efficiency of IC RNA in amplification step. The AE PCR product of CN-11 (A) and Mutant 2R (E) primers showed PCR product size of 141 bp (lane1). The FD PCR product of Mutant 3F (F) and LISEBL 2R (D) primers showed PCR product size of 256 bp (lane2). The final AD PCR product was made by hybridizing the overlapping strands from the two fragments of AE and FD. The AD PCR product using CN-11 (A) and LISEBL 2R (D) primers showed PCR product size of 355 bp (lane3) (**Figure 16**).

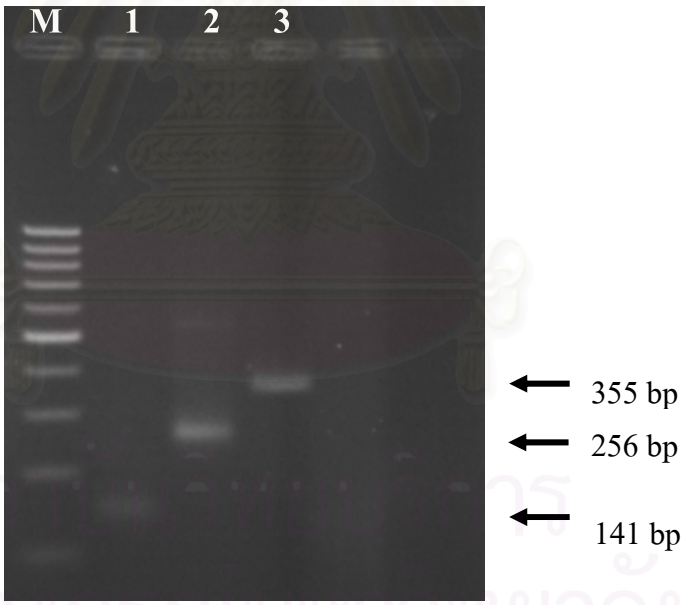


Figure 16: An ethidium bromide stained agarose gel showing the PCR products of IC using CN-11 (A), Mutant 2R (E), Mutant 3F (F) and LISEBL 2R (D) primers in MOE-PCR. Lane M, 100 bp DNA ladder; lane1, AE primers; lane 2, FD primers; lane 3, AD primers.

1.2.2 Cloning and Sequencing

1.1.2.1 Digestion of restriction endonucleases and analysis of the pGem®-T vector containing 25 changed and 42 inserted nucleotides sequences.

After purification of the IC PCR product (**Figure 13**) and pGem®-T vector was ligated and transformed to the *E. coli* competent cells, the selected white clones were inoculated into 5 ml of LB broth and incubated at 37°C for 16-20 hours. After that, plasmid DNA extractions were digested with *Eco*R I, and then fractionated on agarose gel electrophoresis to screen the clones which contained 374 bp insertions (**Figure 17**).

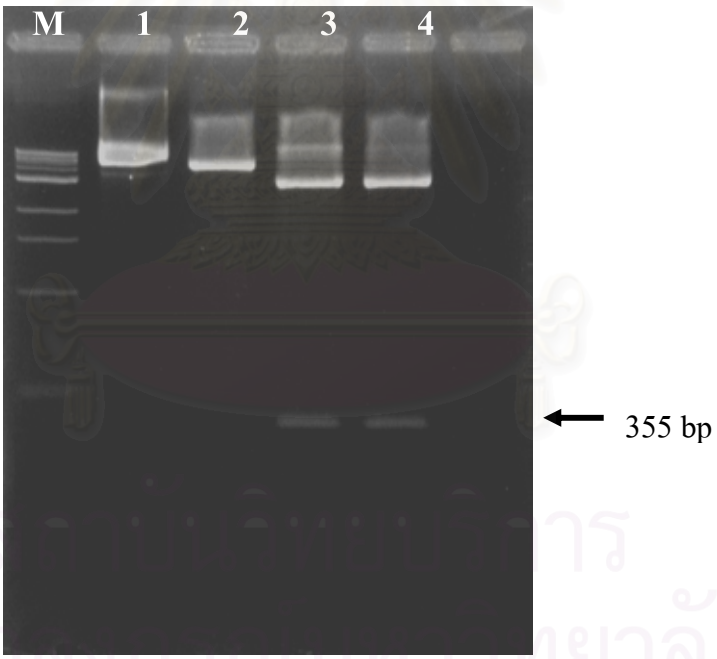


Figure 17: An ethidium bromide stained agarose gel showing the digestion of ligated pGem®-T vector containing 25 changed and 42 inserted nucleotides sequences. Lane M; 1 Kb DNA ladder, lane 1; non-digested IC in pGem®-T, lane 2; pGem®-T vector without inserted DNA, lane 3 and 4; pGem®-T vector with IC DNA.

1.1.2.2 Nucleotide Sequence Alignment

The nucleotide sequences of CVS and IC DNA with added 42 nucleotides were aligned using CLUSTAL X. The results showed that IC DNA sequences contained 3 regions ; conserved region was used to designs primer in nucleic acid amplification, inserted region was used to increase size of PCR product and changed region was used to design specific probe from IC RNA (**Figure 18**).

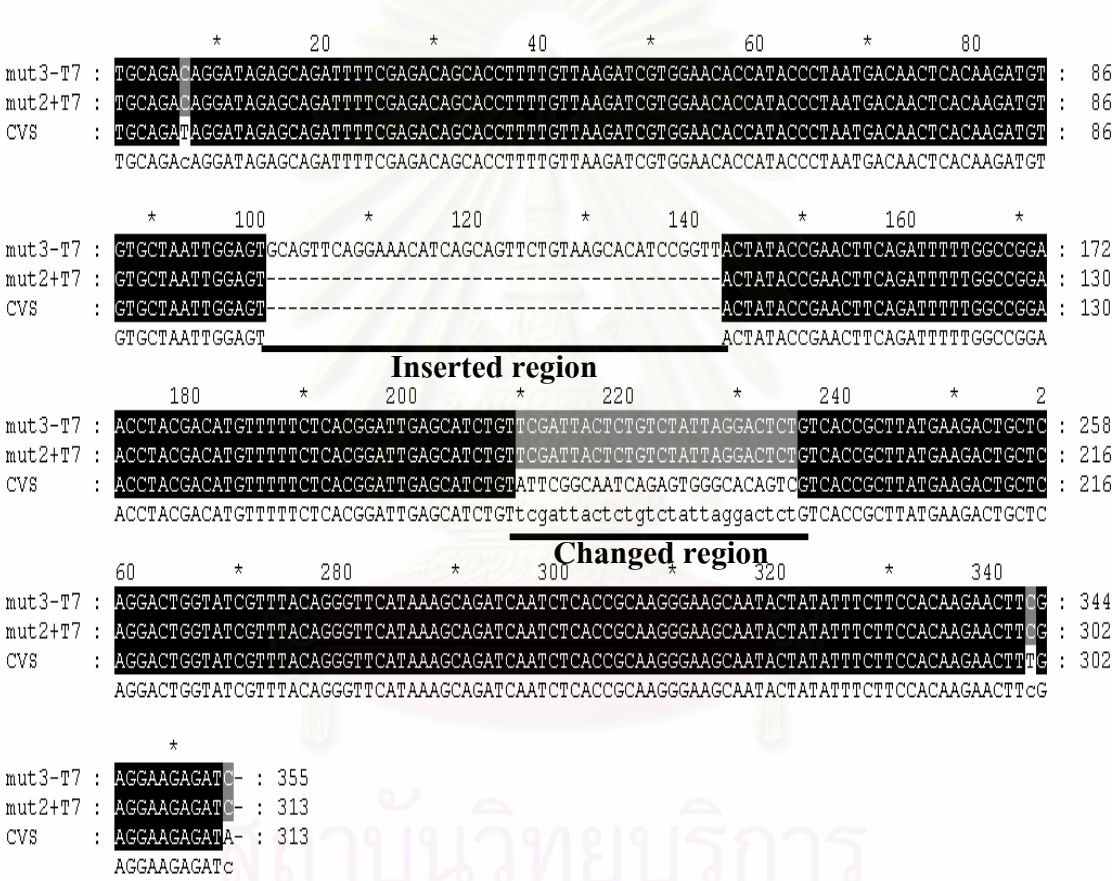


Figure 18: Nucleotide sequences alignment of CVS, IC DNA containing 25 changed nucleotide (mut2) and IC DNA containing 25 changed and 42 inserted nucleotides (mut3) from clones. Identical nucleotides were highlighted. Those underlined indicated the insertion and mutant regions. Mutant region was designed for probe.

1.3 *In vitro* transcription

CN-11 (A), Mutant 2R (E), Mutant 3F (F) and LISEBL 2R (D) primers were used in MOE-PCR to construct rabies modified fragments containing 25 changed and 42 inserted nucleotides in N gene fragment of rabies virus for IC DNA.

The ligation of gel purified IC DNA into the pGem®-T cloning vector was done according to the manufacturer's instructions (Promega). Clones were checked for directionality by sequence using an internal T7 reverse primer site located on the plasmid.

These plasmids were used to act as template in *in vitro* transcription. For generation of *in vitro* RNA, plasmids were linearized with Xmn I followed by SP6 RNA polymerase. The final product size of the RNA was 1.6 Kb.

After the removal of the template and unincorporated nucleotides, the RNA concentrations were quantitated by ultraviolet light absorbance. One A260 unit was equal to approximately 40 μg/ml of RNA. 1:50 dilutions of RNA were prepared and read absorbance at a wavelength of 260 nanometers. The copy number of anti–sense RNA was calculated based on the concentration measured and its molecular weight. The concentration of IC RNA was 1,614 μg/μl **(Figure 19)**

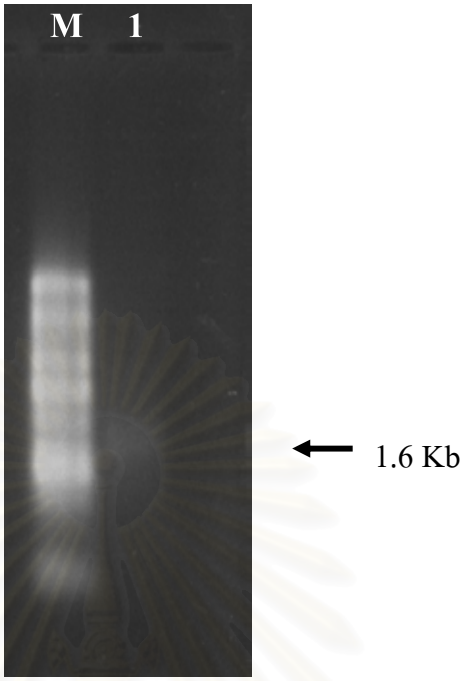


Figure 19: An ethidium bromide stained agarose gel showing the PCR product of IC RNA in *in vitro* transcription. Lane M, RNA marker; lane 1, IC RNA.

2. IC-NASBA optimization

2.1 Addition of IC RNA in amplification step

2.1.1 Detection limit of CVS

To determine the detection limit, CVS was diluted in 10-fold dilutions. The detection limit was 0.04 pg of rabies virus specific RNA at 1:10^7dilution **(Table 4)**. The amplicon size was 180 bp.

Table 4: Titration of CVS for detection of ECL signal

No.	Isolated CVS RNA	Signal	Result
1	Negative control	1	-
2	CVS 10^{-3}	2,333,629	+
3	CVS 10^{-4}	2,264,556	+
4	CVS 10^{-5}	1,738,392	+
5	CVS 10^{-6}	1,825,789	+
6	CVS 10^{-7}	2,041,798	+
7	CVS 10^{-8}	41	-

Note : +, positive; -, negative.

2.1.2 Detection limit of IC

To determine the detection limit, IC RNA was diluted in 100-fold dilutions. The detection limit was 25×10^{-4} fg per reaction (**Table** 5). The amplicon size was 220 bp.

Table 5: Titration of IC RNA for detection of ECL signal

No.	IC RNA (fg / reaction)	Signal	Result
1	Negative control	1	-
2	25×10^{-1}	1,157,614	+
3	25×10^{-3}	987,595	+
4	25×10^{-4}	995,260	+
5	25×10^{-5}	123	-
6	25×10^{-7}	74	-
7	25×10^{-9}	60	-

Note : +, positive; -, negative.

2.1.3 Combination of CVS and IC RNA

The optimal quantity of IC RNA which did not have any interfering effect on the detection of CVS was determined by adding IC 25×10^{-3} and IC 25×10^{-4} of IC RNA to CVS concentrations of CVS 10^{-6} and CVS 10^{-7}, respectively. These reactions were mixed for evaluation of the competition between IC and CVS RNA.

The best result for internal control was shown with a concentration of IC of 25×10^{-4} fg/NASBA reactions in IC-NASBA (**Table 6**).

Table 6: Titration of mixture between IC and CVS for detection ECL signal

No.	Mixture CVS and IC RNA	WT Signal	IC Signal	Result	Valid
1	Negative control	1	905,463	-	V
2	CVS 10^{-6} + IC 25×10^{-3}	365,094	957,414	+	V
3	CVS 10^{-6} + IC 25×10^{-4}	2,015,609	294,012	+	V
4	CVS 10^{-7} + IC 25×10^{-3}	20,825	698,550	+	V
5	CVS 10^{-7} + IC 25×10^{-4}	856,123	693,135	+	V

Note : +, positive; -, negative; V, valid.

2.2 Addition of IC RNA in isolation step

2.2.1 Detection limit of CVS

To determine the detection limit, CVS was diluted in 10-fold dilutions and then extracted by Boom technique. The detection limit was at $1:10^{6}$ dilution (**Table 7**). The amplicon size was 180 bp.

Table 7: Titrations of CVS for detection of ECL signal

No.	CVS RNA (supernate)	WT Signal	Result
1	Negative control	1	-
2	CVS 10^{-3}	2,311,610	+
3	CVS 10^{-4}	2,167,729	+
4	CVS 10^{-5}	1,867,189	+
5	CVS 10^{-6}	453,330	+
6	CVS 10^{-7}	1	-
7	CVS 10^{-8}	41	-

Note : +, positive; -, negative.

2.2.2 Detection limit of IC

To determine the detection limit, IC RNA was diluted in 10-fold dilutions and then extracted by Boom technique. The detection limit was 25×10^{-2} fg per reaction (**Table 7**). The amplicon size was 220 bp.

Table 8: Titration of IC RNA for detection of ECL signal

No.	IC RNA conc. (fg / reaction)	IC Signal	Result
1	Negative control	1	-
2	25×10^{-1}	1,256,231	+
3	25×10^{-2}	686,885	+
4	25×10^{-3}	28	-
5	25×10^{-4}	1	-

Note : +, positive; -, negative.

2.1.3 Combination of CVS and IC RNA

The optimal quantity of IC RNA which did not interfere with the detection of CVS was determined by adding IC 25×10^{-1} and IC 25×10^{-2} of IC RNA to CVS concentrations; CVS 10^{-4} ,CVS 10^{-5} and CVS 10^{-6}, respectively. This reaction was mixed to test for competition between IC and CVS RNA.

This result showed that concentration of IC of 25×10^{-1} fg/NASBA reaction interfered with the signal of CVS. (**Table 9**).

Table 9: Titration of mixture between IC and CVS for detection ECL signal

No.	Mixture CVS and IC RNA	WT Signal	IC Signal	Result	Valid
1	Negative control	1	890,986	-	V
2	CVS 10^{-4} + IC 25×10^{-1}	753,760	336,578	+	V
3	CVS 10^{-5} + IC 25×10^{-1}	44,137	330,768	+	V
4	CVS 10^{-6} + IC 25×10^{-1}	1	1	+/-	IV
5	CVS 10^{-4} + IC 25×10^{-2}	1,068,242	1	+	V
6	CVS 10^{-5} + IC 25×10^{-2}	686,318	1	+	V
7	CVS 10^{-6} + IC 25×10^{-2}	1	1	+/-	IV

Note : +, positive; -, negative; -/+, specimens assigned to contain inhibitor and need to re-analyzed; V, valid; IV, invalid.

3. IC-NASBA evaluation

3.1 Adding IC in nucleic acid amplification

Comparison of the sensitivity between NASBA and IC-NASBA was performed. The sensitivity of NASBA and IC-NASBA was comparable in amplification step (**Table 10**).

Table 10: Comparison between the sensitivity of NASBA and IC-NASBA

No.	CVS RNA	NASBA		IC-NASBA (IC conc. 25×10^{-4} fg / reaction)			
		WT Signal	Result	WT Signal	IC Signal	Result	Valid
1	CVS 10^{-3}	2,333,629	+	2,310,734	45	+	V
2	CVS 10^{-4}	2,264,556	+	2,194,934	48,796	+	V
3	CVS 10^{-5}	1,738,392	+	1,267,622	376,974	+	V
4	CVS 10^{-6}	1,825,789	+	2,015,609	294,012	+	V
5	CVS 10^{-7}	2,041,798	+	20,825	693,135	+	V
6	CVS 10^{-8}	41	-	ND	ND	ND	ND

Note : +, positive; -, negative; V, valid; ND, not performed.

3.2 Adding IC in nucleic acid isolation step

Comparison of the sensitivity between NASBA and IC-NASBA was made. The sensitivity of NASBA and IC-NASBA were at $1:10^6$ and $1:10^7$ dilution, respectively (**Table 11**).

Table 11: Comparison between the sensitivity of NASBA and IC-NASBA

No.	CVS RNA	NASBA		IC-NASBA (IC conc. 25×10^{-1} fg/ reaction)			
		WT Signal	Result	WT Signal	IC Signal	Result	Valid
1	CVS 10^{-2}	ND	ND	ND	ND	ND	ND
2	CVS 10^{-3}	ND	ND	ND	ND	ND	ND
3	CVS 10^{-4}	ND	ND	753,760	336,578	+	V
4	CVS 10^{-5}	1,138,746	+	44,137	330,768	+	V
5	CVS 10^{-6}	453,330	+	1	1	+/-	IV
6	CVS 10^{-7}	1	-	ND	ND	ND	ND

Note : +, positive; -, negative; -/+, specimens assigned to contain inhibitor and need to re-analyzed; V, valid; IV, invalid; ND, not performed.

5. IC-NASBA application

All data was described below with the reaction adding IC in nucleic acid amplification for IC-NASBA. IC-NASBA application was tested in brain tissues and urines sample.

5.1 Brain tissues

Twenty samples of rabies infected and non-infected brains were tested with IC-NASBA (**Table 12**). IC RNA could be demonstrated in all, except 3 samples which contained high virus quantities.

Table 12: Brain tissues were tested with IC-NASBA

No.	Brain tissues		IC-NASBA			
		FAT	WT signal	IC signal	Result	Valid
1	Negative control	ND	1	899,980	-	V
2	A	-	89	569,465	-	V
3	B	+	2,094,034	35,786	+	V
4	C	+	1,267,622	276,974	+	V
5	D	+	915,609	294,012	+	V
6	E	+	13,760	336,578	+	V
7	F	+	69,637	330,768	+	V
8	G	+	2,236,980	50	+	V
9	H	+	605,602	196,583	+	V
10	I	-	1	993,135	-	V
11	G	+	202,812	25,968	+	V
12	K	+	89,024	3,056	+	V
13	L	+	1,803,761	5	+	V
14	M	-	121	686,885	-	V
15	N	-	1	856,320	-	V
16	O	+	56,981	5,763	+	V
17	P	+	1,101,074	1	+	V
18	Q	+	161,307	5,697	+	V
19	R	+	46,493	11,514	+	V
20	S	-	215	820,185	-	V
21	T	+	1,000,416	20,996	+	V

Note : +, positive; -, negative; V, valid; ND, not performed.

5.2 Urine samples

Fifteen urine samples were tested with IC-NASBA. These urine samples were previously shown to contain inhibitor by nested RT-PCR (**Table 13**). Results as tested by this method were strictly in accordance with those using nested PCR.

Table 13: Urines were tested with IC-NASBA

No.	Brain tissues	Nested RT-PCR	IC-NASBA			
		Presence of inhibitor	WT signal	IC signal	Result	Valid
1	Negative control	-	1	956,013	-	V
2	U1	-	1	750,455	-	V
3	U2	+	1	1	+/-	IV
4	U3	-	1	532,989	-	V
5	U4	-	1	659,459	-	V
6	U5	+	1	1	+/-	IV
7	U6	-	1	589,716	-	V
8	U7	-	1	893,500	-	V
9	U8	+	1	1	+/-	IV
10	U9	-	1	969,289	-	V
11	U10	-	1	669,890	-	V
12	U11	+	1	1	+/-	IV
13	U12	-	1	631,498	-	V
14	U13	-	1	957,136	-	V
15	U14	-	1	856,890	-	V
16	U15	+	1	1	+/-	IV

Note : +, inhibitor; -, non inhibitor; -/+, specimens assigned to contain inhibitor and need to re-analyzed; V, valid; IV, invalid.

CHAPTER V

CONCLUSION AND DISCUSSION

False – positive and negative results are major concerns in nucleic acid amplification tests. The former is due to contamination whereas the latter is usually associated with the presence of inhibitors in the clinical specimens. A high percentage of false-negative results have been previously reported especially when nucleic amplification test has been applied to urine specimens. It has been later shown that enzyme inhibitors are responsible in such cases. Therefore, internal controls for detection of inhibitors are indispensable.

In the case of rabies, the test sensitivity using NASBA on urine is lower than saliva raising the question whether this may be due to enzyme inhibitors. Our previous result on the study of Nipah virus, another RNA virus in the same order of mononegavirale, showed that a significant number of bat's urine samples contained inhibitors [32]. This IC-NASBA was developed to avoid false negative results and to increase diagnostic accuracy.

In this study, MOE_PCR was used to construct rabies modified fragment in N gene, containing 25 changed and 42 inserted nucleotides. This was used as IC. When being compared of the sensitivity when IC was added to each reaction tube at isolation or amplification step, it was clearly shown that adding in amplification step is superior. When IC RNA was added in isolation step, it was found that there was IC RNA loss which might be due to the extraction procedure. Moreover, IC RNA interfered with WT signal resulting to at least 2 log less in sensitivity than when adding

IC in amplification. In spite of a better outcome when IC was added in amplification step , there is evidence of competition between the IC RNA and the CVS or WT RNA, thus, requiring a careful titration of the IC quantity. 25×10^4 fg per reaction of IC RNA is the optimal amount since this did not interfere the detection sensitivity as compared to NASBA. The detection limit of rabies virus specific RNA was 0.04 pg at $1:10^7$ dilution.

The results of IC-NASBA could be interpreted as valid or invalid. Negative result is judged when only the IC RNA amplicon was detected and positive result when IC and WT RNA or only WT RNA could be detected. Specimens assigned to contain inhibitor and need to re-analyzed whenever none of the amplicons could be detected.

Testing of IC-NASBA system with rabies infected brains and controls, the sensitivity was 100% and IC RNA could be demonstrated in all except 3 samples which contained very high virus quantity. Results on urine known to contain enzyme inhibitors were strictly in accordance with those using nested PCR.

In conclusion, IC-NASBA has been proven to be useful in terms of avoiding false negative result and sensitivity was not compromised. It should also be a preliminary step to NASBA quantification assay.

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APPENDIX

APPENDIX

CHEMICAL AGENTS AND INSTRUMENTS

A. Laboratory supplies

Beaker (Pyrex)

Combs (BIO-RAD, USA)

Cylinders (Pyrex, England)

Disposable gloves(Latex, Thailand)

Flask (Pyrex)

Microcentrifuge (Eppendorf, USA)

Microcentrifuge tube (Axygen, USA)

Parafilm (American National Can, USA)

Pipette boy (Tecnomara, Switzerland)

Pipette rack (Autopack,USA)

Pipette tip (Axygen, USA)

Plastic wrap

Polypropylene conical tube (Elkay, USA)

Reagent bottles: 250 ml, 500 ml, 1000 ml (Duran®, Germany)

B. Equipment

Autopipette (Eppendorf, Germany)

Chemi Doc (BIO-RAD, USA)

DNA Thermal cycler 2400 (Perkin Elmer, Cetus USA)

Electrophoresis Chamber set (BIO-RAD, USA)

Freezer -20°C (SANYO, Japan)

Heat block (Bockel)

Incubator (Heraeus)

pH meter (Eutech Cybernataics)

Power supply model

Refrigerator 4^{o}C (SANYO, Japan)

Spectrophotometer (BIO-RAD, USA)

Thermometer (Precision, Germany)

Timer

Vortex (scientific Industry, USA)

Water bath

C. General Reagents

Absolute ethanol (Merck)

Acetic acid (Merck)

Agar (Scharlau)

Agarose (USB, Spain)

Ampicillin (M&H manufacturing)

Bromphenol blue (Sigma, USA)

Disodium ethylenediamine tetraacetic acid: EDTA (Merck)

Ethidium bromide (Sigma)

Glycerol (Phamacia Amersham)

Hydrochloric acid (Merck)

IPTG (USB)

Isoamyl alcohol (Merck)

Phenol (Sigma, USA)

RNA marker

Sodium acetate (Merck)

Tris base (USB)

100 bp DNA ladder (Fermntus)

D. Reagents of PCR

10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4,15 mM $MgCl_2$) (Promega)

Deoxynucleotide triphosphates (dNTPs) (Promega)

Oligonucleotide primer (BSU)

Taq DNA polymerase (Promega)

E. Restriction endonucleases

Appendix 1: Restriction enzymes with their recognition sites, recommended buffer and manufacturer.

Enzymes	**Recognition sequence**	Buffer	**Manufacturer**
XmnI	5'..GAANN^NNTTC..3'	Buffer B	Promega

NOTE: ^ represent the cleavage site of restriction enzyme

F. Reagent kit

1. QIAGEN Plasmid Mini Kit, (QIAGEN, Germany)
2. RiboMAX. Large Scale RNA Production Systems.SP6 and T7, (Promega, USA)
3. ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit with Amplitaq® DNA Polymerase FS, (Perkin-Elmer, USA)

G. Buffer and Reagent preparation

1. 5X TBE buffer, 1 liter

Tris base	54	g.
Boric Acid	27.5	g.
0.5 M EDTA, pH 8.0	20	ml.
Add DW up to 1 liter.		

2. 5x MOPS buffer, 1 liter

MOPS	263	g.
$NaCH_2COOH$	0.41	g.
0.5 M EDTA pH 8	20	ml.
Add DW up to 1 liter.		

H. Determination of the cut-off level in IC-NASBA

The cut-off level was performed by using the NucliSens Basic Kit user software (bioMe´rieux, Durham, NC). To determine the cut-off for ECL detection, the counts for 100 different individual truly negative samples were measured. In each run, a tube with the reference solution was included. The measured counts were recalculated in reference to the same signal of the reference solution, i.e., 20,000 counts. The average and three times the standard deviation of the 100 measurements were calculated. The cut-off level is expressed relative to the signal of the reference solution, according to the following formula: (three times the standard deviation + average count)/20,000. The results of the NASBA assay were considered positive when the signal was above a cut-off of 0.01 times the signal of the reference solution[46].

BIOGRAPHY

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