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THESIS

IDENTIFICATION OF EFFECTIVE RNA INTERFERENCE TARGET SITES WITHIN HEPATITIS B POST-TRANSCRIPTIONAL REGULATORY ELEMENT

NUTTKAWEE THONGTHAE

A Thesis Submitted in Partial Fulfillment of
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Hepatitis B virus (HBV) infection causes chronic diseases, including acute liver cirrhosis and hepatocellular carcinoma (HCC). More than two billion people worldwide have been infected, thus effective antiviral drugs or novel treatments are pressingly needed. This study was performed to identify the effective RNAi target sites within the highly conserved region of HBV RNA known as hepatitis B post-transcriptional regulatory element (HBV PRE) by using bioinformatics and experimental approaches. To identify potential siRNA target sites, eleven predicting siRNA targets were employed and the results were clearly demonstrated into nine clusters. Three potential siRNA target sites were selected from the major clusters and called 1317-1337, 1357-1377 and 1644-1664. The features of these selected siRNA target sites were then characterized. The feature analysis revealed that only siRNA target site 1317-1337 did not form actual hairpin structure of any tested genes and it contained the most preferential bases. Based on the results of luciferase assay and real-time PCR analysis, only the siRNA expression plasmids driven by H1 promoter (pShPRE1317-1337, pShH1-1357 and pShH1-1644) showed to have potent inhibitory effects on the expression of luciferase, core, surface and X transcripts. However, the siRNA 1317-1337 had the best inhibitory effect. Therefore, the results suggested that secondary structure and the base preference were the key features for being the effective siRNAs. In addition, only the siRNA 1357-1377 significantly inhibited level of cccDNA. Importantly, all three selected siRNA target sites did not induce the expression of STAT1 and OAS1 and did not cause cell cytotoxicity. Consequently, the study successfully identified effective siRNA target sites that may further develop as anti-viral drugs for inhibiting HBV replication.

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Student's signature Thesis Advisor's signature

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Nuttkawee Thongthae

May 2014

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LIST OF ABBREVIATIONS

AS	=	antisense strand
bp	=	base pairs
chr.	=	chromosome
cccDNA	=	covalently closed circular DNA
CMV	=	Cytomegalovirus
^{o}C	=	degree Celsius
DNA	=	deoxyribonucleic acid
dNTP	=	deoxynucleoside triphosphate
dsRNA	=	double-stranded RNA
E. coli	=	*Escherichia coli*
EDTA	=	ethylenediaminetetra acetic acid
FBS	=	fetal bovine serum
Luc^{+}	=	Firefly luciferase
g	=	gram
HBc	=	hepatitis B core
HBIG	=	hepatitis B immune globulin
HBs	=	hepatitis B surface
HBV	=	hepatitis B virus
HBV PRE	=	hepatitis B virus post-transcriptional regulatory element
IFN	=	interferon
L	=	liter
LB	=	Lurai Bertani
mRNA	=	messenger RNA
µg	=	microgram
µL	=	microliter
µM	=	micromolarity
miRNA	=	microRNA
mg	=	milligram
mL	=	milliliter
mM	=	millimolarity

LIST OF ABBREVIATION (Continued)

M	=	molarity
ng	=	nanogram
nL	=	nanoliter
nM	=	nanomolarity
nt	=	nucleotide
OAS1	=	2 '-5' oligoadenylated synthetase 1
ORF	=	open reading frame
PBS	=	phosphate buffer saline
PCR	=	polymerase chain reaction
pgRNA	=	pregenomic RNA
RLuc	=	*Renilla* luciferase
RNA	=	ribonucleic acid
RISC	=	RNA-induced silencing complex
RNAi	=	RNA interference
SS	=	sense strand
STAT1	=	signal transducers and activators of transcription 1
ssDNA	=	single-stranded DNA
shRNA	=	short hairpin RNA
siRNA	=	short interfering RNA
SRE-1	=	splicing regulatory element-1
TLR	=	Toll-like receptor
TAE	=	Tris-acetate-EDTA
WHO	=	World Health Organization

IDENTIFICATION OF EFFECTIVE RNA INTERFERENCE TARGET SITES WITHIN THE HEPATITIS B POST-TRANSCRIPTIONAL REGULATORY ELEMENT

INTRODUCTION

Hepatitis B virus (HBV) is a member of *Hepadnaviridae* family and *Orthohepadnavirus* genus. Its genome is the partially circular double-stranded DNA of about 3,200 nucleotides. HBV infection causes chronic diseases, including acute liver cirrhosis and hepatocellular carcinoma (Ganem and Prince, 2004). From World Health Organization (WHO) report (2012), more than two billion people worldwide have been infected by HBV, which is transmitted through contact with the blood or bodily fluids of an infected person, and about 240 million people chronically infected with HBV. Moreover, about 600,000 people die every year due to the acute or chronic consequences of HBV infection, whereas South East Asia has been reported to be the most prevalence of HBV infection (Chen *et al.*, 2000). In addition, the most predominant HBV genotypes found in Thailand are genotype B and C (Tangkijvanich *et al.*, 2005). Notably, HBV infections have always been the critical problems of public health in Thailand, which need to be improved urgently.

Although HBV vaccination is 95% effective in preventing of new HBV, it is useless for chronic patients. Even if the chronic patients can be treated by licensed antiviral drugs, such as interferon-α, lamivudine and adefovir dipivoxil, these drugs have been reported to cause emergence of drug-resistant variants (Karayiannis, 2003; Tillmann, 2007). Therefore, novel antiviral drugs or alternative treatments are pressingly needed. Currently, RNA interference (RNAi) technology has potentially provided approachable way for developing specific gene silencing therapeutics against abnormal metabolisms, cancers, viral replication (Kariko *et al.*, 2004; Guo *et al.*, 2011; Yang *et al.*, 2011), and HBV replication (Panjaworayan *et al.*, 2010).

At present, there are many available RNAi-predicting programs such as siDirect 2.0 (Naito *et al*., 2009) and siRNA Target Designer 1.6 (Promega, 2005), which differ in criteria and algorithms. However, there is no recognition of which is the gold standard program. Currently, conserved regions of a viral genome have been proposed as effective RNAi target sites because they can minimize viral escape that may occur due to selection pressure of RNAi on pre-existing resistant mutant of virus (Wu *et al.*, 2005).

This study therefore aimed to identify the effective siRNA target sites within the highly conserved region of HBV RNA known as hepatitis B virus post-transcriptional regulatory element (HBV PRE) by using bioinformatics. The study also determined to test inhibitory effects of selected RNAi target sites by using quantitative real-time PCR (delta delta Ct method). Moreover, the study also tested the analysis of siRNA-induced interferon response and cell viability. The results of this study would provide effective siRNA target sites that could be further developed as the therapeutics against HBV infection.

OBJECTIVES

1. To identify the effective siRNA target sites within HBV PRE.

2. To construct the siRNA expression plasmids that are driven by RNA polymerase II and III promoters.

3. To test the inhibitory effects of siRNA expression plasmids by using quantitative real-time PCR (delta delta Ct method).

4. To determine the siRNA-induced interferon response and cell viability.

LITERATURE REVIEW

Hepatitis B virus

Hepatitis B virus is the sphere shape of about 42 nanometres, which consists of an outer lipoprotein envelope and an icosahedral nucleocapsid core antigen (HBcAg). The envelope composes of different surface antigen (HBsAg) that are large-, medium- and small surface antigens. The nucleocapsid encloses the viral DNA genome and DNA polymerase that has reverse transcriptase activity same as retroviruses (Howard, 1986) (Figure 1).

Currently, HBV is classified into ten genotypes, which are genotype A – J based on sequence difference in the whole genome of greater than 8% (Nordel *et al*., 1994; Tatematsu *et al*., 2009; Yu *et al*., 2010).

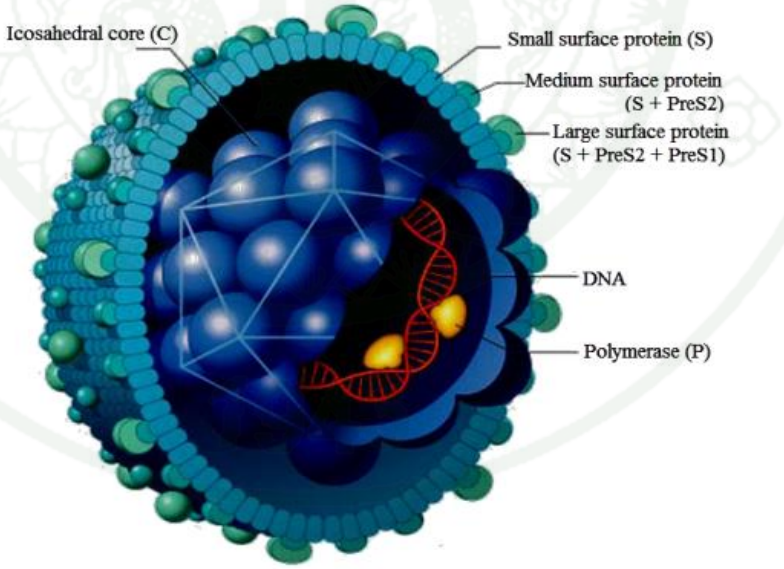

Figure 1 The structure of HBV and its elements, which are surface proteins, nucleocapsid core, viral DNA genome and DNA polymerase.

Source: Zimet *et al.* (2001)

Each HBV genotype has different geographical distributions (Bartholomeusz and Schaefer, 2004). Moreover, HBV are also divided into four major serological subtypes, *adw*, *adr*, *ayw* and *ayr*. This classification is based on the immunoreactivity of an antibody to a limited number of amino acid substitutions in the HBsAg. There is no correlation between the serotypes and genotypes (Magnius and Norder, 1995).

HBV genome

The HBV genome is the partially circular double-stranded DNA of about 3,200 nucleotides (3,020-3,320 nucleotides for minus strand and 1,700-2,800 nucleotides for plus strand) (Kay and Zoulim, 2007). The negative sense or minus strand (non-coding) is complementary to the viral transcript, whereas the transcription is processed on positive sense or plus strand (coding) (Seeger and Mason, 2000). There are four main open reading frames (ORFs) that transcribe for four transcripts, which are called pregenomic RNA (pgRNA), large surface mRNA (preS1), N-terminally truncated S (preS2/S) and X mRNA (X). These transcripts encode for the nucleocapsid protein (C), or core antigen (HBcAg), polymerase (P), large surfaces or surface antigen (HBsAg), medium/small surface antigen and X protein, respectively (Beck and Nassal, 2007). The HBV envelope antigen (HBeAg) is produced by proteolytic mechanism of the precore protein. All four primary HBV transcripts have the overlapped organisation and have the polyadenylation sites at the same position (Figure 2). Notably, the function of X protein is not clear, but it is related to a development of liver cancers (Li *et al.*, 2010).

Hepatitis B virus post-transcriptional regulatory element (HBV PRE)

The length of HBV PRE sequence is about 534 base pairs located at the position 1151-1684. Since the HBV genome is the circular DNA, the HBV PRE sequence is therefore found in all HBV transcripts (Figure 2).

Several reports have been suggested that the functions of HBV PRE involve in the nuclear export (Huang and Yen, 1995), the preservation of mRNA stability

(Ehlers *et al.*, 2004) and the regulation of mRNA splicing (Heise *et al.*, 2006). However, the core function of HBV PRE is still arguable. By using genomic comparative analysis of HBV, Panjaworayan *et al.* (2007) indicated that HBV PRE contains many conserved regions. Further analysis of regulatory elements has indicated that HBV PRE is likely to contain several elements that may play differently roles such as nuclear export and splicing (Panjaworayan *et al.*, 2010).

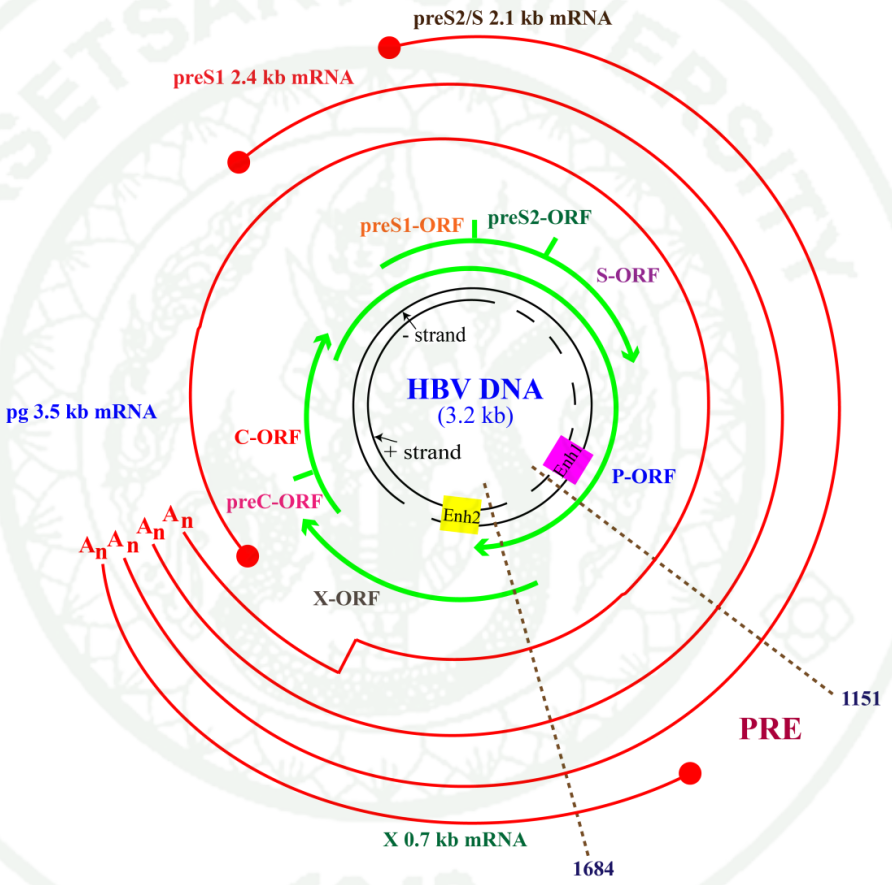

Figure 2 The HBV genome is the partially circular double-stranded DNA of about 3,200 base pairs (black line). There are four mRNAs, pg, preS1, preS2/S and X (red line) and also four ORFs, C, S, P and X (green line). In addition, the conserved region (HBV PRE) locates at the nucleotide position from 1,151 to 1,684 that is found in all HBV transcripts.

HBV life cycle and its replication

HBV is distantly related to retroviruses as it uses reverse transcription as a part of its replication process. At first, the viral particle penetrates into the susceptible cells by binding to the specific surface receptors. After the cell membrane is fused with the viral membrane, the viral nucleocapsid core is released into cytoplasm (Summers and Mason, 1982). The core dissociates and viral genomic DNA is then transferred to the cell nucleus. The viral DNA polymerase repairs the partially double-stranded DNA into the completely double-stranded DNA and the ends are then ligated by host enzyme. The viral genomic DNA is therefore transformed into covalently closed circular DNA (cccDNA) that serves as a template for transcription of its major viral transcripts. All viral transcripts are transported to the cytoplasm and are subsequently used as the templates for translation (Will *et al.*, 1987). The largest transcript (pgRNA), which is longer than the viral genome, is used to create the new copies of the genome, core protein and the viral DNA polymerase. The subgenomic RNAs, preS1, preS2/S and X, are used as the templates for large-, medium/small surface and X proteins, respectively. The progenies are assembled and then released from the cell or returned to the nucleus and re-cycled to produce even more copies (Beck and Nassal, 2007). The pgRNA is then transported back to the cytoplasm, where the viral polymerase synthesises genomic DNA via its reverse transcriptase activity (Figure 3).

Prevention and treatments

At present, several vaccines have been developed for an effective prevention of HBV infection. These rely on the use of part of the viral surface proteins. Current vaccines are made using a synthetic recombinant DNA technology from DNA samples of people who have had long-standing HBV infection (Hepatitis B Foundation, 2009). For prevention of HBV infection, the first dose of HBV vaccines and hepatitis B immune globulin (HBIG) are given to newborn within 12 hours of birth. This prevention can reduce the risk of transmission. Then, a second dose of hepatitis B vaccine is given at 1–2 months later. Finally, a third dose is delivered

after 6 months (Libbus and Phillips, 2009). Besides, the WHO has recommended to use the immunoprophylaxis for the newborn by multiple injections of small doses of HBIG (Shi *et al.*, 2010a), or oral antiviral drug known as lamivudine for HBV carrier mothers at the last three months of pregnancy (Shi *et al.*, 2010b).

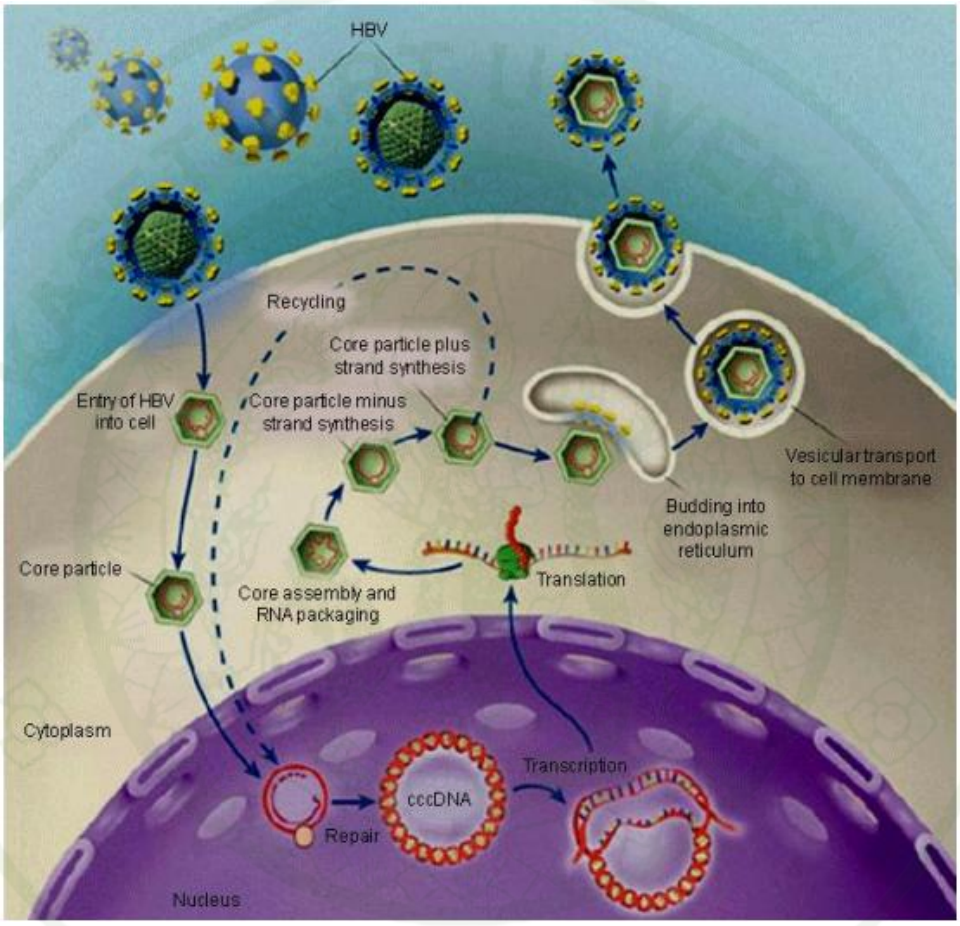

Figure 3 The replication process of HBV.

Source: Ganem and Prince (2004)

In addition, chronically infected patients always have persistent elevation of serum alanine aminotransferase (a marker of liver damage) and HBV DNA levels (Lai and Yuen, 2007). Although, available antiviral drugs can inhibit the viral replication and subsequently minimise liver damage, none of them can completely cure the infection. Currently, there are two general groups of antiviral drugs known as

nucleos(t)ide analogues and immunomodulators. At present, there are five nucleos(t)ide analogues namely lamivudine (Epivir), adefovir (Hepsera), tenofovir (Viread), telbivudine (Tyzeka) and entecavir (Baraclude) and the two immune system modulators, which are interferon alpha-2a (IFN-α) and pegylated IFN-α -2a (Pegasys) (Dienstag, 2008). By taking nucleos(t)ide-analogs for a long-term, the emergence of viral escape and drug-resistant variants is highly appeared (Karayiannis, 2003). Furthermore, the immunomodulators group, such as IFN-α, is more expensive and associated with many severe side effects. Novel antiviral drugs or alternative treatments are therefore pressingly needed. In addition, it has been recently reported that RNAi mechanism effectively inhibits HBV replication (Ren *et al.*, 2006; Sun *et al.*, 2007; Panjaworayan *et al.*, 2010). Therefore, RNAi technique appears as the powerful approach for new treatment of viral infection, including HBV.

RNA interference (RNAi)

RNAi mechanism is divided into two major groups, which are microRNA (miRNA) and small interfering RNA (siRNA). In 1998, it was firstly reported to down-regulate specific genes expression in *Caenorhabditis elegans* (Fire *et al.*, 1998). The siRNA pathway is found in many eukaryotes, including mammals.

1. RNAi mechanism

Many kinds of RNA molecules such as long double-stranded RNAs (dsRNAs), short hairpin RNAs (shRNAs), transposon elements, RNA viruses and exogenously introduced siRNAs are involved in RNAi pathway (Rana, 2007). Long dsRNA and shRNA molecules (that also could be expressed by DNA vectors in target cells) are initially cleaved into short duplexes by the RNase III family endoribonuclease, which is called Dicer. The short RNA duplexes, namely siRNAs are about 21 nucleotides (nt) long with a 2 nt overhang at the 3' end of each strand and a monophosphate at the 5' end. Next, the helicase unwinds siRNA duplex at the easier end. The sense (passenger) strand (SS) that has the same sequence as the target mRNA will be cleaved and degraded. The antisense (guide) strand (AS) that has the

complementary sequence to the target mRNA, is then loaded into an RNA-protein complexes termed RNA-induced silencing complexes (RISCs). Alternatively, exogenous siRNAs can be directly loaded into RISCs without Dicer processing (Li and Rana, 2012). The RISC completely recognizes and cleaves the target mRNA at 10-11 nt upstream of the 5' end of the AS. This action is mediated by the activity of Argonaute2 (Ago2), which is one of the main components of RISC and contains an enzymatically competent RNase H-like domain. After that, the RISC is looped and recognizes other target mRNA (Miyoshi *et al.*, 2010). On the other hand, miRNA- or siRNA-protein complex that complement to the target mRNA, imperfectly cannot cleave the target mRNA, but it suppresses the protein translation (Schwarz *et al.*, 2003; Zeng *et al.*, 2003). However, both of effective miRNAs and siRNAs can lead to gene silencing (Figure 4).

2. Thermodynamic properties

In order to obtain effective siRNAs, the good siRNAs must be rationally designed to contain appropriate characteristics. Up to date, many reports have found some new key features that are crucial for RNAi mechanism. One essential characteristic is the thermodynamic property. Several studies have suggested that duplex unwinding is the important step for both dsRNAs and pre-miRNA hairpin precursors (Bernstein *et al.*, 2001; Nicholson and Nicholson, 2002). Khvorova *et al.* (2003) showed that thermodynamic profiling of the 5' end of AS exhibited a lower internal stability that associated with duplex unwinding. This result corresponded with other studies (Ui-Tei *et al.*, 2004; Ichihara *et al.*, 2007). On the other hand, the lower internal stability of the 5' end of SS inhibits RNAi effect (Ui-Tei *et al.*, 2004), because a low internal stability at the 5' end of SS mediates the SS to be loaded into RISC instead of AS (Schubert *et al.*, 2005). In addition, Khvorova *et al.* (2003) suggested that the internal stability of the 9-14 nt region from the 5' end of AS plays important role in the mRNA turnover by fasilitating dissociation of RISC from the target mRNA so that it can be recycled for a new reaction (Figure 5).

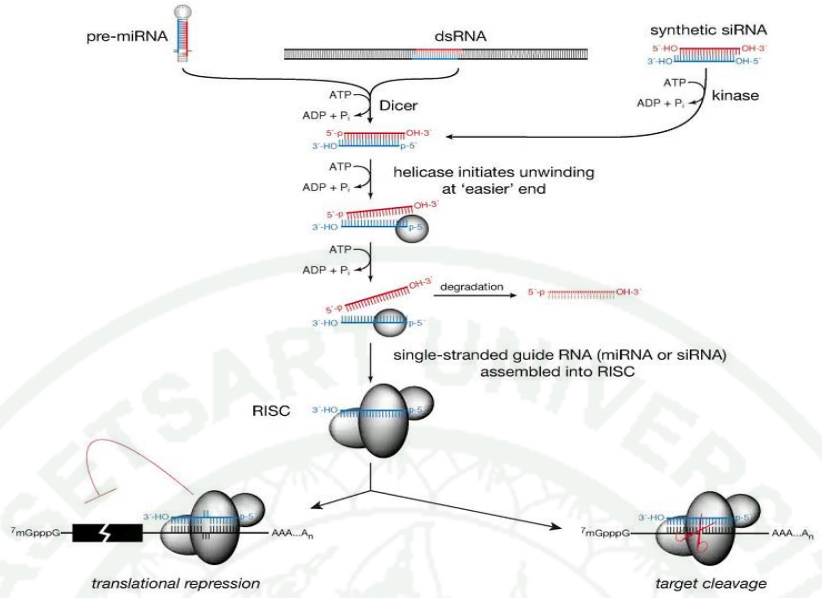

Figure 4 The RNAi mechanism. It is involved in hybridization of an effective siRNA and a specific target mRNA that leads to be gene silencing.

Source: Schwarz *et al.* (2003)

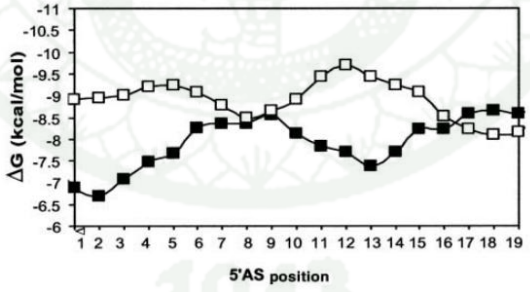

Figure 5 Calculated average internal stability profiles for a set of 37 siRNAs targeting human and mouse genes. The functional siRNAs have a low internal stability at the 5' end and 9-14 nt region of the AS (closed box indicates functional siRNAs and opened box indicates nonfunctional siRNAs).

Source: Khvorova *et al.* (2003)

Moreover, the 5' phosphorylation of the AS is necessary for an efficient RISC loading. All siRNAs generated from long dsRNAs or shRNAs by Dicer contain 5' monophosphates, whereas exogenous synthesized siRNAs always have 5' hydroxyl groups (Figure 4). Therefore, the loading of synthesized siRNAs into the functional RISC may require 5' phosphorylation of AS (Li and Rana, 2012). There is an evidence supports that RNAi activity can be repressed by chemical modification of the 5' end of AS with amino groups and 3-carbon, which can block phosphorylation (Chiu and Rana, 2002). On the other hand, chemical modification of the 5' end of SS has no effect to RNAi efficiency, because its A-form helix structure of siRNA duplex is maintained (Rana, 2007).

3. GC contents and base preferences

Furthermore, the percentage of GC content can affect unwinding of siRNA duplex. An optimal GC content should be about 30-52% (Elbashir *et al.*, 2002; Reynolds *et al.*, 2004). A high GC content results in a packed duplex, which is harder to be dissociated. In addition, there are many reports suggested on base preferences of good siRNAs, such as base A at nucleotide position 3, T at nucleotide position 10, not G at nucleotide position 13 and A at nucleotide position 19 of sense strand (Reynolds *et al.*, 2004). However, base preferences reported from several studies are not agreeable among each other (Hsieh *et al.*, 2004; Reynolds *et al.*, 2004; Takasaki *et al.*, 2004; Katoh and Suzuki, 2007).

4. Secondary structures

Elbashir *et al.* (2002) suggested that the secondary structure of target mRNA had no effect on RNAi efficiency. Later on, many reports indicated that the secondary structure of target mRNA may interfere RISC formation (Yoshinari *et al.*, 2004; Schubert *et al.*, 2005). Moreover, the secondary structure of siRNA also affect the RNAi activity. This is because the ideal siRNA duplex can adopt an A-form helix, which differs from the general B-form helix of DNA molecules. This helical structure leads to a more rigidly packaged RNAi molecule with a narrower and deeper

major groove, thus it is more stable than the B-form helix (Chiu and Rana, 2002). In addition, the bulge structure of A-form helix may be twisted and then distorts the major groove. Rana (2007) found that bulge structures of the AS completely abrogated the RNAi activity. In addition, the more stable secondary structures of AS obstructs the RISC formation (Reynolds *et al.*, 2004) (Figure 6).

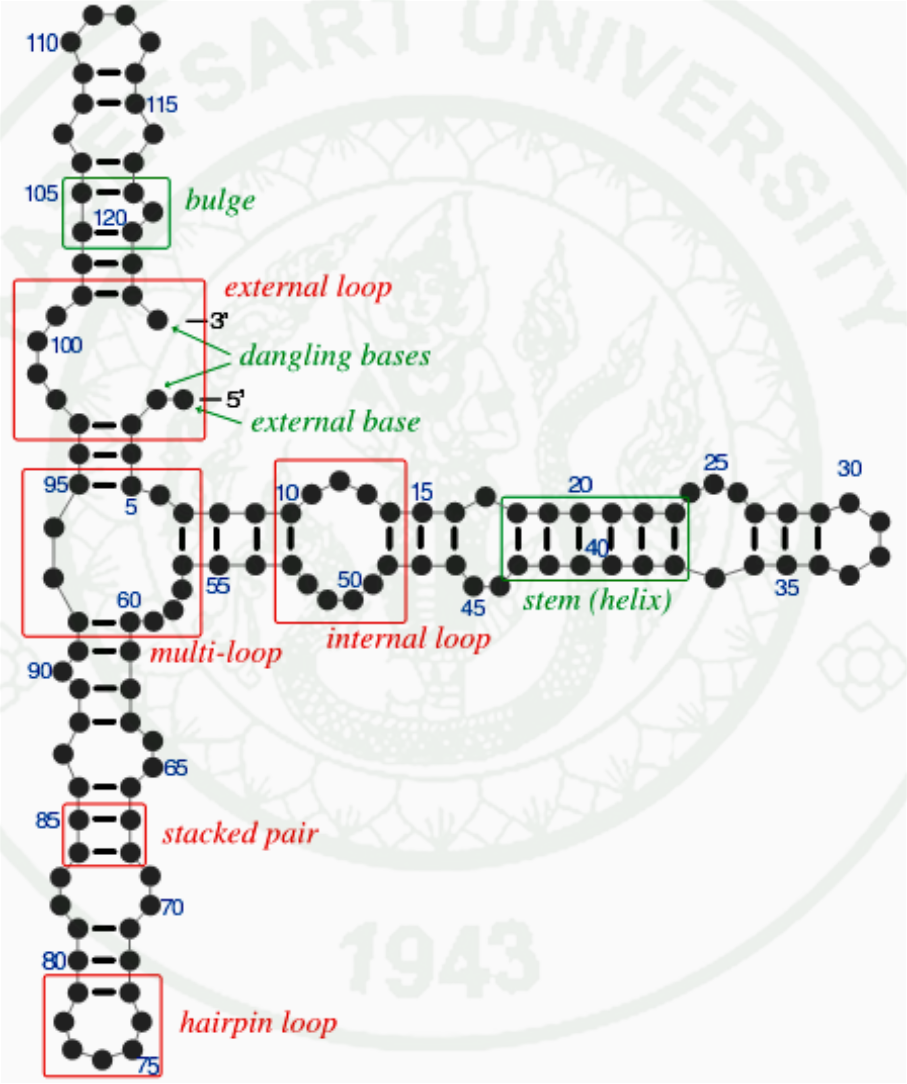

Figure 6 The various secondary structures such as bulge, stem, stacked pair and hairpin loop.

Source: Andronescu *et al.* (2005)

5. The prediction of RNAi target sites

Currently, there are several siRNA predicting programs for identifying siRNA target sites such as siExplorer (Katoh and Suzuki, 2007), siDirect (Naito *et al.*, 2009) and AsiDesigner (Park *et al.*, 2008). Each program predicts siRNA target sites based on different algorithms. Therefore, each program considers different criteria such as consensus sequence, thermodynamic stability, percentage of GC content, base length and secondary structures. The details of available programs are summarized in Table 1. However, there is still no agreement on which the best siRNA-predicting program is. In addition, conserved regions of viral genome have been currently proposed to be effective RNAi target sites. Since these sites contain minimum mutation; thus, they can minimize viral escape occurring from selection pressure (Wu *et al.*, 2005).

6. RNAi applications

At present, RNAi technique is used for studying gene function, gene expression and regulation. Moreover, it has been potential used for development of specific gene silencing therapeutics, including abnormal metabolisms, cancers and viral replication (Kariko *et al.*, 2004; Ely *et al.*, 2008; Guo *et al.*, 2011). Several studies have evidently supported that RNAi technique can effectively inhibit HBV replication or down-regulate expression of the specific HBV gene such as precore, surface, X and HBV PRE (Giladi *et al.*, 2003; Hamasaki *et al.*, 2003; Kayhan *et al.*, 2007; Panjaworayan *et al.*, 2010).

In order to perform effective RNAi technique in research, several procedures have been developed. The major development is chemical synthesis of siRNA duplexes and RNAi expression plasmids by expressing pri-miRNAs or shRNAs under RNA polymerase II or RNA polymerase III. Synthetic siRNA duplexes are usually transported into cells by cationic liposomes, whereas RNAi expression plasmids rely on liposomes or viral vectors (Figure 7).

Table 1 List of available siRNA predicting programs and characteristics. ⊗ Indicates absence of the feature, whereas ☑ indicates presence of the feature.

Programs	Base length	Consensus sequence	GC (%)	Thermo-dynamic stability	Based on research results	Avoid secondary structure	Reference
AsiDesigner	23	⊗	30-62	☑	⊗	☑	Park *et al.* (2008)
BLOCK-iT™ RNAi Designer	25	⊗	35-55	⊗	⊗	⊗	Invitrogen (2008)
Dicer Substrate RNAi Design	27	⊗	30-50	☑	Reynolds	⊗	Kim *et al.* (2005)
DSIR	21	$AAN_3AN_5TN_4C(G/C)NN$	⊗	☑	⊗	☑	Vert *et al.* (2006)
siDesign Center	19	⊗	30-64	⊗	⊗	⊗	Thermo Fisher Scientific (2008)
siDirect 2.0	21	$NN(G/C)N_{17}(A/U)$	32-53	☑	Ui-Tei Reynolds	⊗	Naito *et al.* (2009)

Table 1 (Continued)

Programs	Base length	Consensus sequence	GC (%)	Thermo-dynamic stability	Based on research results	Avoid secondary structure	Reference
siExplorer	19	NNAANAANNTNAA NAWNAA	30-50	☑	⊗	⊗	Katoh and Suzuki (2007)
siRNA Target Designer 1.6	21	$AAN_{17}TT$, NAN_{19} and NARN15YNN	35-60	☑	⊗	⊗	Promega (2005)
siRNA Target Finder	21	⊗	30-60	⊗	Elbashir	⊗	Ambion (2008)
siRNA Target Finder	21	⊗	30-60	☑	Khvorova Reynolds Ui-Tei	☑	GenScript (2008)
T7 RNAi Oligo Designer	21	$NNGN_{17}C$	30-50	☑	⊗	⊗	Dudek and Picard (2004)

Nevertheless, synthetic siRNA duplexes have some limitations, for example: rapid liver clearance, lack of target specificity, and high cost (Sioud and Sorensen, 2003; Jones, 2009; Li and Shen, 2009). On the other hand, RNAi expression plasmid offers many advantages, including low cost, stable duration and long term-response (if stably transfected and delivered). In addition, DNA-based viral expression cassettes have been reported as the effective procedures for HBV treatment (Sun *et al.*, 2007) (Figure 7).

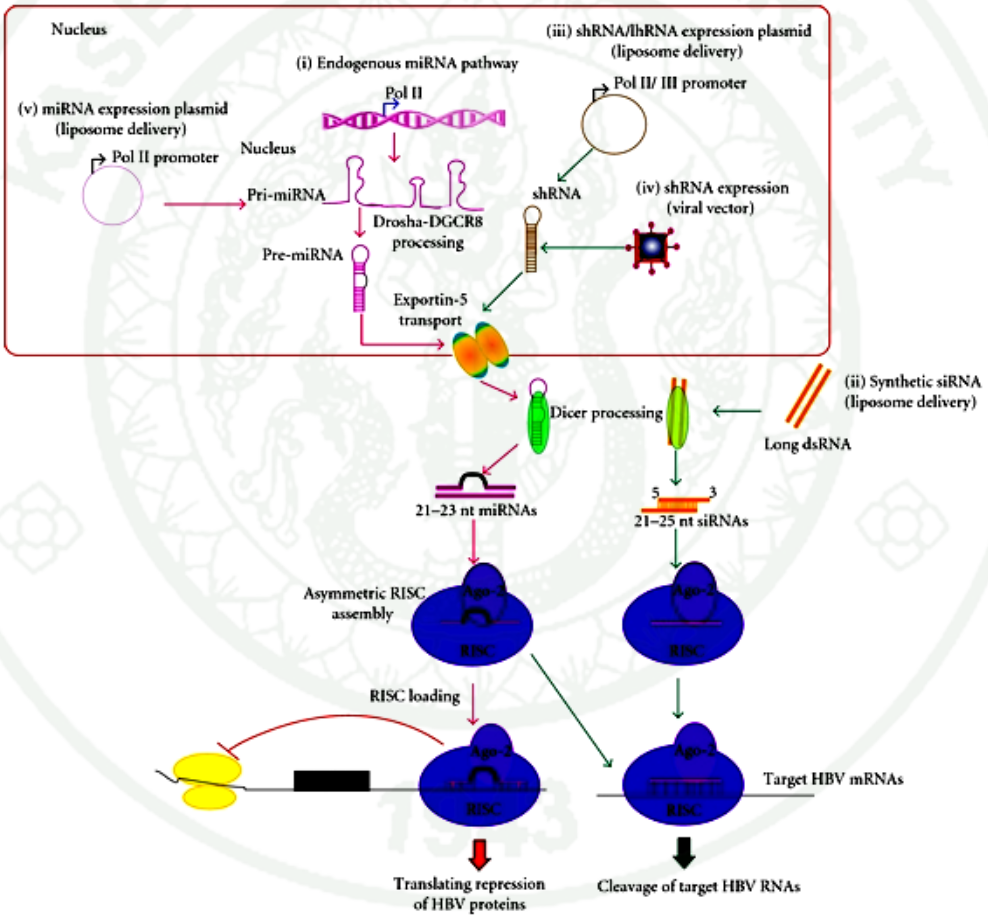

Figure 7 RNAi pathways in HBV research. Diagram of the miRNA and siRNA pathway (i). Current RNAi-based procedures and their deliveries (ii)–(v).

Source: Panjaworayan and Brown (2011)

The cytotoxicity and off-target effect

Both *in vitro* and *in vivo* studies have demonstrated that siRNAs can be potent activators of an immune response such as IFNs and inflammatory cytokines (Hornung *et al.*, 2005; Judge *et al.*, 2005; Sioud, 2005). The main factor for the IFN responses is the activation of the dsRNA-dependent protein kinase R (PKR) that leads to a general inhibition of protein synthesis. Nevertheless, siRNAs can evade PKR activation when they are shorter than 30 base pairs (Figure 8). Moreover, there are other receptors such as Toll-like receptor 3 (TLR3) for dsRNA that is involved in siRNA-induced IFN response (Kariko *et al.*, 2004). The activation of immune cells by siRNAs is reported to be sequence-dependent manner and each strand of siRNA duplex can stimulates cytokine production and involves TLR7 and TLR8 same as ds-siRNAs (Hornung *et al.*, 2005; Judge *et al.*, 2005; Sioud, 2005). These endosomal receptors, TLR7 and TLR8, have been proposed to recognize U-rich and GU-rich of siRNAs sequence and they can activate siRNA compartmentalisation in endosomes. Furthermore, pCpG-siRNAs have been found to stimulate IFN responses via TLR9 (Figure 8). Notably, the detection of IFN response is commonly performed for determination of cytotoxicity of siRNA. In addition, siRNA that has low specificity causes off-target effect and leads to non-specific mRNA degradation.

To determine cytotoxicity of siRNAs, down-stream genes regulated by IFNs are quantitatively evaluated. Three classes of IFN have been identified, which are type I to III. They are classified accordingly to their receptor complexes. The type II IFN composes of a single IFNγ gene product that binds to the IFNγ receptor (IFNGR) complex. The IFNγ plays the major role in immune system that responds to pathogens other than viruses. The type III IFNs consists of three IFNλ gene products that are signaled by the combined IFNλ receptor 1 (IFNLR1) and interleukin-10 receptor 2 (IL-10R2) complex. Recently, the type III IFN is hypothesized to regulate the antiviral activity and has been proposed to be the ancestral type I IFNs (Levraud *et al.*, 2007).

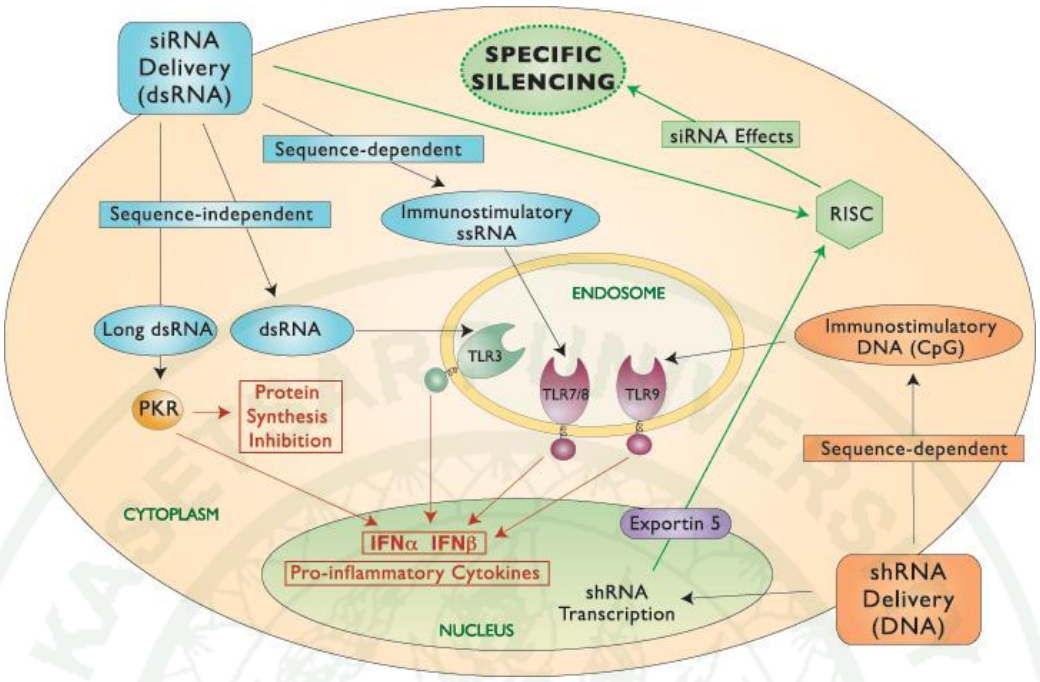

Figure 8 Diagram shows the IFN response that is stimulated by various siRNAs via PKR and different TLRs.

Source: InvivoGen (2006)

The type I IFNs in humans consists of 13 IFNα subtypes, including IFNβ, IFNκ, IFNε, IFNo, IFNτ and IFNδ. They engage the ubiquitously expressed IFNα receptor (IFNAR) complex, which consists of IFNAR1 and IFNAR2 (Figure 9). The function of type I IFNs is well known as a robust host response against viral infection (O'Connell *et al.*, 2004). Signal transduction is initiated by pre-associated Janus tyrosine kinases (JAK1 and TYK2), which phosphorylated the IFNAR1 chain enabling recruitment and then phosphorylation of the Signal Transducers and Activators of Transcriptions (STATs). STAT heterodimers (STAT1 and STAT2) associated with the IFN regulatory factor 9 (IRF9), resulting in the formation of the IFN-stimulated transcription factor 3 (ISGF3). This complex translocates into nucleus to bind the IFN-stimulated regulated element (ISRE) and then induces IFN-stimulated genes such as the 2'-5' oligoadenylated synthetase (OAS), Mx and protein kinase R (PKR) to cause antiviral immunity (Sadler and Williams, 2008) (Figure 9).

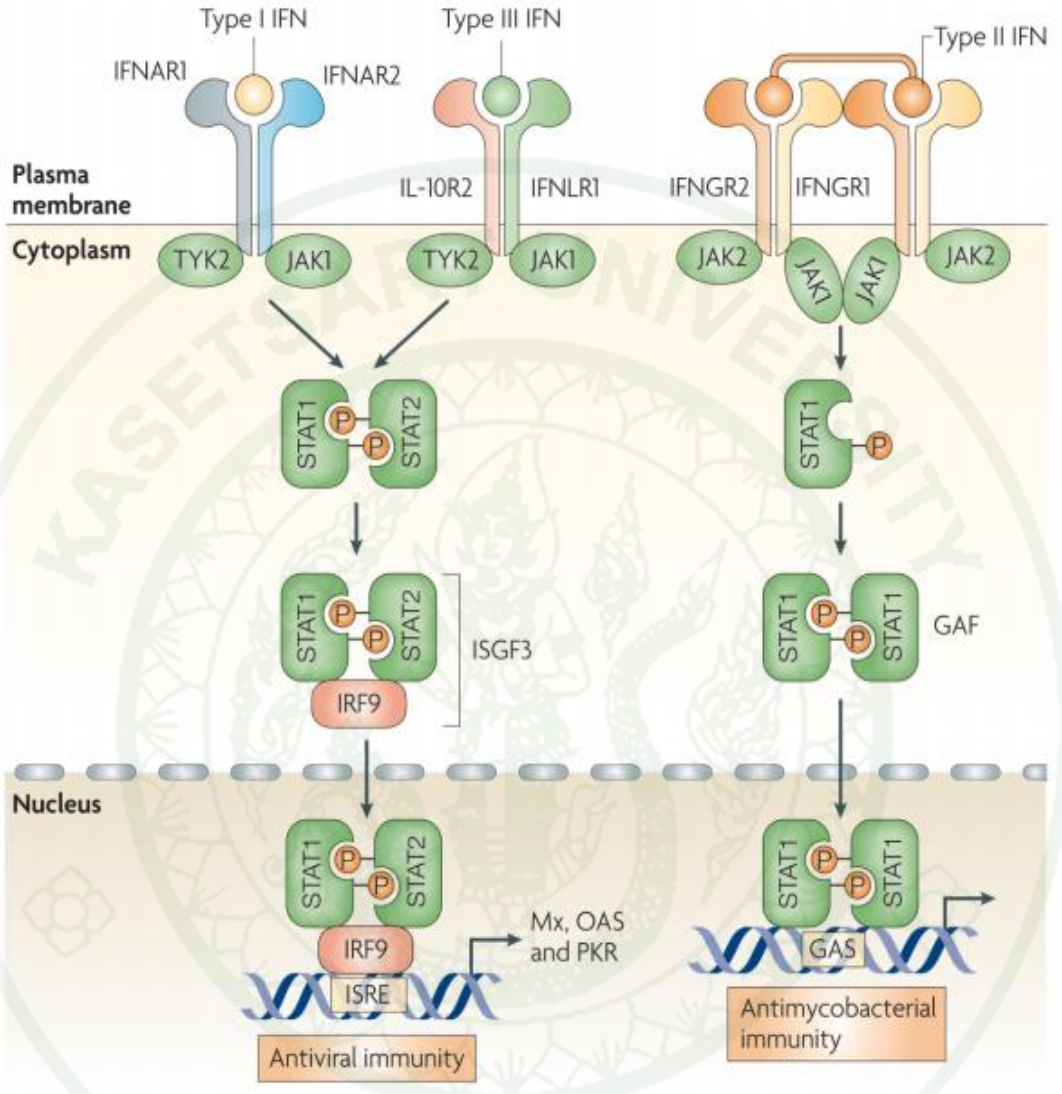

Figure 9 IFN-signaling is mediated by three receptor complexes. Signal transduction is initiated by pre-associated JAK1, JAK2 and TYK2. They then cause the phosphorylated STAT1 and STAT2 to form ISGF3 and GAF. Finally, these complexes are transported into nucleus and then act against the antiviral or antimycobacterial immunities.

Source: Sadler and Williams (2008)

Furthermore, the 2'-5' oligoadenylated synthetase (OAS) and ribonuclease L (RNaseL) pathway is involved in antiviral immune response. The OAS proteins accumulate in the cytoplasm as inactive monomers. Upon activation by viral dsRNA, the enzyme forms a tetramer that can synthesizes 2', 5'-linked phosphodiester bonds to inactive RNaseL monomer (Rebouillat and Hovanessian, 1999). Then, binding of 2', 5'-oligoadenylated to RNaseL triggers dimerization of RNaseL monomers leading to be a latent form of RNaseL. In this way, OAS in combination with RNaseL constitutes an antiviral RNA decay pathway (Clemens and Vaquero, 1978) (Figure 10).

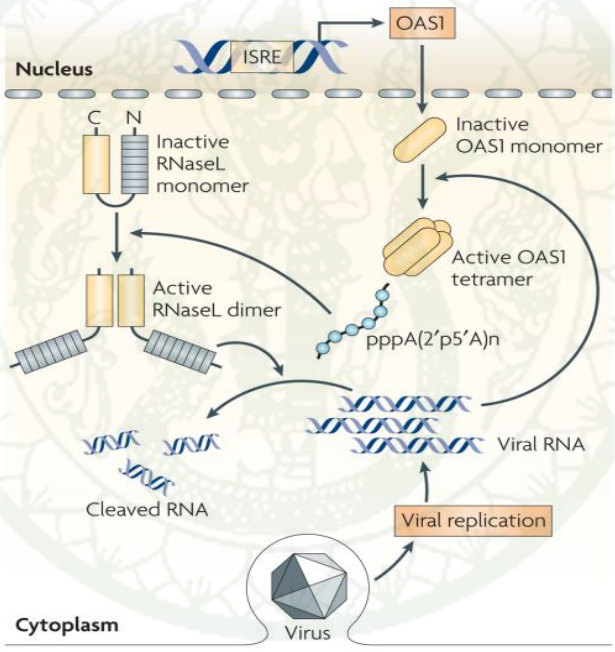

Figure 10 Diagram shows the co-activity of OAS and RNaseL that is initially activated by viral RNA and consequently to be the viral RNA degradation.

Source: Sadler and Williams (2008)

Notably, STAT1 and OAS1 are commonly detected to determine the cytotoxicity or IFN response that may occur from siRNAs (Sun *et al.*, 2010).

MATERIALS AND METHODS

Materials

Reagents

Reagents were obtained as outlined in Table 2.

Table 2 List of reagents and manufacturers.

Reagents	Manufacturers
(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Invitrogen
1× DNA annealing solution	Ambion
5× Passive lysis buffer (5× PLB)	Promega
6× DNA loading dye	Fermentas
Agar C	Pacific Science
Agarose, low EEO	Research organic
Amonium persulfate ($(NH_4)_2SO_4$)	Univar
Amplicilin sodium salt	Bio Basic INC.
Antimicotic antibiotic	Gibco, Invitrogen
Bovine serum albumin (BSA)	BAA
Bromophenol Blue	Fluka
Calcium chloride ($CaCl_2$)	Univar
Chloroform ($CHCl_3$)	Merck
Dinucleoside triphosphate mix (dNTPs)	GeneON
Disodium hydrogen phosphate (Na_2HPO_4)	Univar
Dithiothreitol (DTT)	Bio Basic INC.
Dulbecco's modified eagle medium (DMEM)	Gibco, Invitrogen
Dimethyl sulfoxide (DMSO)	Amresco
Ethanol (C_2H_6O)	Merck

Table 2 (Continued)

Reagents	Manufacturers
Ethylenediaminetetra acetic acid (EDTA)	Bio Basic INC.
Ethidium bromide	Bio Basic INC.
Fetal bovine serum (FBS)	Gibco, Invitrogen
GeneRuler™ DNA Ladder Mix	Fermentas
Glucose	APS Ajax
Glycerol	APS Ajax
Hypure Nuclease free water	Hyclone
Isopropanol (C_3H_8O)	Merck
Kanamycin	Meji
Lipofectamine™ 2000	Invitrogen
Magnesium chloride ($MgCl_2$)	Carlo Erba
Magnesium sulfate ($MgSO_4$)	APS Ajax
Peginterferon alfa-2a, Pegasys®	Roche (Gift from Prof. Yong Poovorawan, CU)
Peroxide solution	GE Healthcare
Potassium chloride (KCl)	Univar
Potassium dihydrogen phosphate (KH_2PO_4)	Univar
Sodium chloride (NaCl)	Amresco
Sodium hydroxide, pellets (NaOH)	J.T. Baker
Sterile, nuclease-free water	Amresco, Inc.
Tris-(hydroxymethy)-aminomethane	Scharlau
Trizol	Invitrogen
Trypsin-EDTA	Gibco, Invitrogen
Tryptone Powder	Pacific Science
Tween 20	Sigma
White polystyrene, 96 well assay plate	Costar
Yeast Extract	Pacific Science

Enzymes

Table 3 List of enzymes and manufacturers.

Enzymes	Manufacturers
*Bam*HI	Fermentas
DNaseI	New England BioLabs, Inc.
HelixCript™ Thermo Reverse Transcriptase	Nanohelix
*Hind*III	Fermentas
RBC ThermoOne™ Real-time PCR premix (2×)	RBC Bioscience
T4 DNA ligase	Fermentas
Taq DNA polymerase	RBC Bioscience

Commercial Kits

Table 4 List of kits and manufacturers.

Kits	Manufacturers
AxyPrep Plasmid Miniprep Kit	Axygen
Dual-Luciferase® Reporter Assay System	Promega
Gel/PCR DNA Fragments Extraction Kit	Geneaid
Genomic DNA Extraction Kit	RBC Bioscience

Bacterial strain

Escherichia coli (*E. coli*; New England Biolabs, Inc.) strain DH5α was used as a general cloning host.

Mammalian tissue cells

HepG2 cells (human hepatocellular carcinoma) were used for testing to the effects of RNAi expression plasmids as a general transfection-mammalian host.

Plasmids

Table 5 List of plasmids.

Plasmids	Sources
pBasic/fPRE	(Panjaworayan *et al.*, 2010)
pCMV-RLuc	Gift from Sunchai Payungporn, CU
pHBV-48	Gift from Wu H. (Wu *et al.*, 1991)
pShH1-1357	Constructed in this study
pShH1-1644	Constructed in this study
pShCMV-1317	Constructed in this study
pShCMV-1357	Constructed in this study
pShCMV1644	Constructed in this study
pShPRE1317-1337	(Panjaworayan *et al.*, 2010)
p*Silencer* 3.0-H1	Ambion, Inc.
p*Silencer* 4.1-CMV neo	Ambion, Inc.
p*Silencer* 4.1 GAPDH	Ambion, Inc.
p*Silencer* GAPDH	Ambion, Inc.

Primers

Table 6 List of primers.

Primers	Sequences	Notes
M13F(-40)	GTTTTCCCAGTCACGAC	For sequencing of
2_rev	AGGCGATTAAGTTGGGTA	p*Silencer* 3.0-H1
CMV4_1neoF	AGGCGATTAAGTTGGGTA	For sequencing of
CMV4_1neoR	CGGTAGGCGTGTACGGTG	p*Silencer* 4.1-CMV neo
HBV_CCC_F	ACTCTTGGACTCCCAGCAATG	For detection of
HBV_CCC_R	CTTTATAAGGGTCAATGTCCA	HBV cccDNA

Table 6 (Continued)

Primers	Sequences	Notes
HBV_Surface_F	GTGTCTGCGGCGTTTTATCA	For detection of
HBV_Surface_R	GACAAACGGGCAACATACCTT	HBV S gene
HBV_C_F	CATTGACCCTTATAAAGAATTTGG AGC	For detection of
HBV_C_R	CCAGCAGAGAATTGCTTGCCTGAG	HBV core gene
HBV_X_F	CTCCCCGTCTGTGCCTTCT	For detection of
HBV_X_R	GATCTGGTGGGCGTTCAC	HBV X gene
HumBetaGlobin_F	GTGCACCTGACTCCTGAGGAGA	For detection of
HumBetaGlobin_R	CCTTGATACCAACCTGCCCAG	human beta-globin gene
HumBetaAcT_F	CTGGGCATGGAGTCCTGTGGCATCC	For detection of
HumBetaAcT_R	CGCAACTAAGTCATAGTCCGCCTAG	human beta-actin gene
HumSTAT1_F	ATGTCTCAGTGGTACGAACTTCA	For detection of
HumSTAT1_R	TGTGCCAGGTACTGTCTGATT	human STAT1 gene
HumOAS1_F	GATCTCAGAAATACCCCAGCCA	For detection of
HumOAS1_R	AGCTACCTCGGAAGCACCTT	human OAS1 gene
Oligo-dT_{18}	TTTTTTTTTTTTTTTTTT	For first-strand cDNA synthesis

Table 6 (Continued)

Primers	Sequences	Notes
ShPRE1357_T	GATCCATATACATCGTTTCCATGG TTCAAGAGACCATGGAAACGATGT ATATTTTTTTGGAAA	For construction of pShH1-1357 and pShCMV-1357
ShPRE1357_B	AGCTTTTCCAAAAAAATATACATC GTTTCCATGGTCTCTTGAACCATG GAAACGATGTATAT	
ShPRE1644_T	GATCCGGTCTTACATAAGAGGACT TTCAAGAGAAGTCCTCTTATGTAA GACCTTTTTTGGAAA	For construction of pShH1-1644 and pShCMV-1644
ShPRE1644_B	AGCTTTTCCAAAAAAGGTCTTACA TAAGAGGACTTCTCTTGAAAGTCC TCTTATGTAAGACC	

Analytical equipments

Table 7 List of analytical equipments.

Equipments	Manufacturers
BIOHAZARD class II	Microtech
CO_2 incubator with CO_2 tank	Lishen
Gel documentation	DNR Bio-Imaging Systems
Imaging Analyser	Fusion FX7
Nano Drop Spectrophotometer	NanoDrop®
Olympus CK2	Olympus
Sorvall Legend Micro 17R Centrifuge	Thermo Electron Corporation
Spectrophotometer	Labquip
StepOnePlus Real-Time PCR System	Applied Biosystem
Thermal Cycler	Labnet Leonics

Media and solutions

Table 8 List of solutions and components.

Solutions	Components
50× TAE buffer (pH 8.5)	242 g Tris base, 57.1 mL glacial acetic acid and 18.6 g EDTA. Make up to 1 L
DMEM media	DMEM supplemented with 10% (v/v) heat inactivated FBS and 1% (v/v) antimicotic antibiotic
Freezing media	FBS:DMSO ratio is 9:1
Glucose (2 M stock)	2 M glucose (filter sterilize)
Luria Bertani (LB) broth	1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl (autoclaved)
LB agar plate	1% (w/v) tryptone powder, 0.5% (w/v) yeast extract, 1% (w/v) NaCl and 1.5% (w/v) Agar C. Autoclave prior to addition of 100 µg/mL ampicilin or kanamycin
Mg^{2+} (2 M stock)	2.033 g $MgCl_2.6H_2O$ and 2.265 g $MgSO_4.7H_2O$. Make up to 10 mL (filter sterilize)
Phosphate buffer saline (PBS)-EDTA (pH 7.4)	2.7 mM KCl, 1.4 mM KH_2PO_4, 137 mM NaCl, 4.3 mM Na_2HPO_4 and 0.53 mM EDTA (autoclaved)
SOC media	5 g tryptone, 1.375 g yeast extract, 10 mM NaCl and 10 mM KCl. Make up to 250 mL. After autoclave, add 10 mM of 2 M Mg^{2+} and 20 mM of 2 M glucose stock.

Methods

Conceptual framework

This study was divided into four main parts as illustrated in a flow chart below (Figure 11).

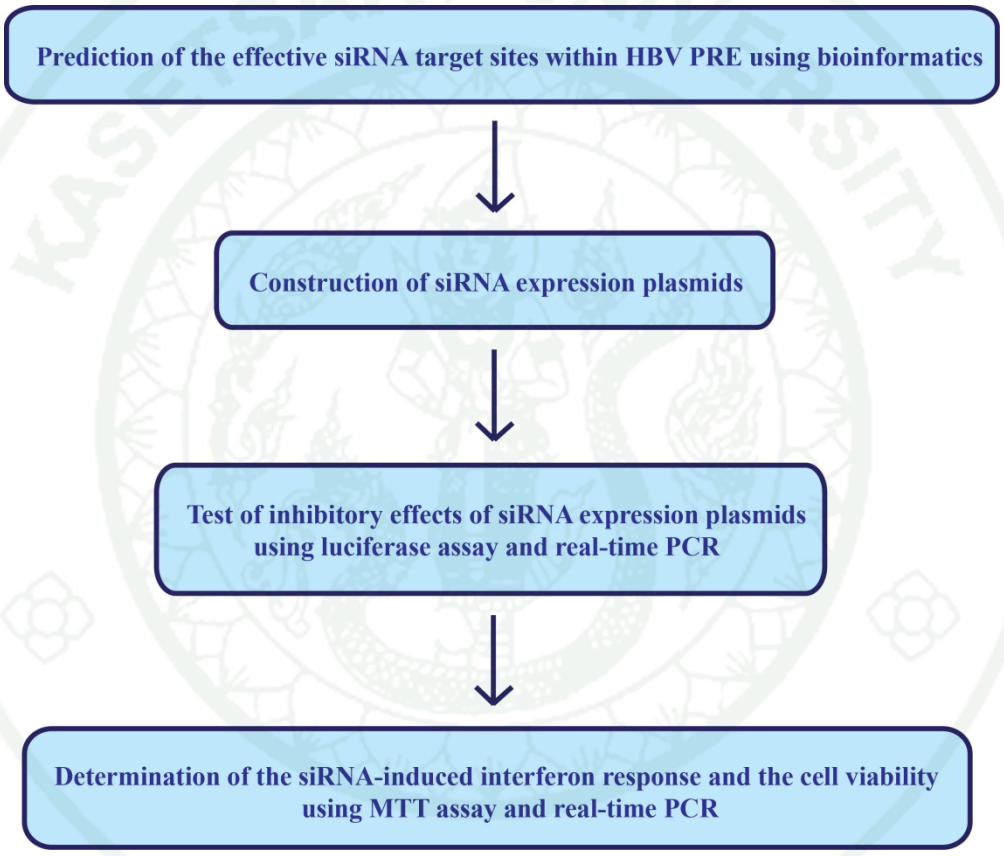

Figure 11 Flow chart of this study.

Prediction of the effective siRNA target sites

1. Prediction of siRNA target sites

HBV PRE sequence (genotype A, Accession No: AM282986) was input into eleven siRNA predicting programs, which were AsiDesigner, BLOCK-iT RNAi

Designer, Dicer Substrate RNAi Design, DSIR, siDESIGN Center, siDirect 2.0, siExplorer, siRNA Target Finder, siRNA Target Designer 1.6 and T7 RNAi Oligo Designer (specific characteristics of each program were described in Table 1). The predicting results were then clustered using Sequencher 4.10.1 program (Gene Codes Corporation). Next, the identified sequences containing position-base preferences (Reynolds *et al.*, 2004) were selected to represent each cluster. Consequently, all selective sequences were determined the similarities to the human genomic and human transcript databases using blastn. Finally, all selective sequences were designed to oligonucleotide sequences, which consist of *Bam*HI, sense sequence, loop, antisense sequence, terminator and *Hind*III sites (Figure 12).

2. Prediction of secondary structures of luciferase and HBV mRNA

Luc^+ (firefly luciferase) conjugating with HBV PRE and four HBV mRNAs (pg, preS1, preS2/S and X; Accession No: AM282986) were obtained from GenBank. Consequently, they were input into mfold program (Zuker, 2003) for prediction of secondary structures (Figure 12).

Contruction of siRNA expression plasmids

1. Plasmid construction

Firstly, one µg/µL of two complementary shRNA oligonucleotides (ShPRE1357_T and ShPRE1357_B; ShPRE1644_T and ShPRE1644_B) were annealed together in 1× DNA annealing solution. The annealing was carried out at 95°C for 5 minutes and then cooled down to room temperature for 1 hour. Finally, all consequent mixtures were ligated with cut p*Silencer* 3.0-H1 and cut p*Silencer* 4.1-CMV neo that had been double-digested with *Bam*HI and *Hind*III prior to ligation.

In addition, the cut pShPRE1317-1337 fragment and cut p*Silencer* 4.1-CMV neo that had been double-digested with *Bam*HI and *Hind*III were ligated together (Figure 13, A-C).

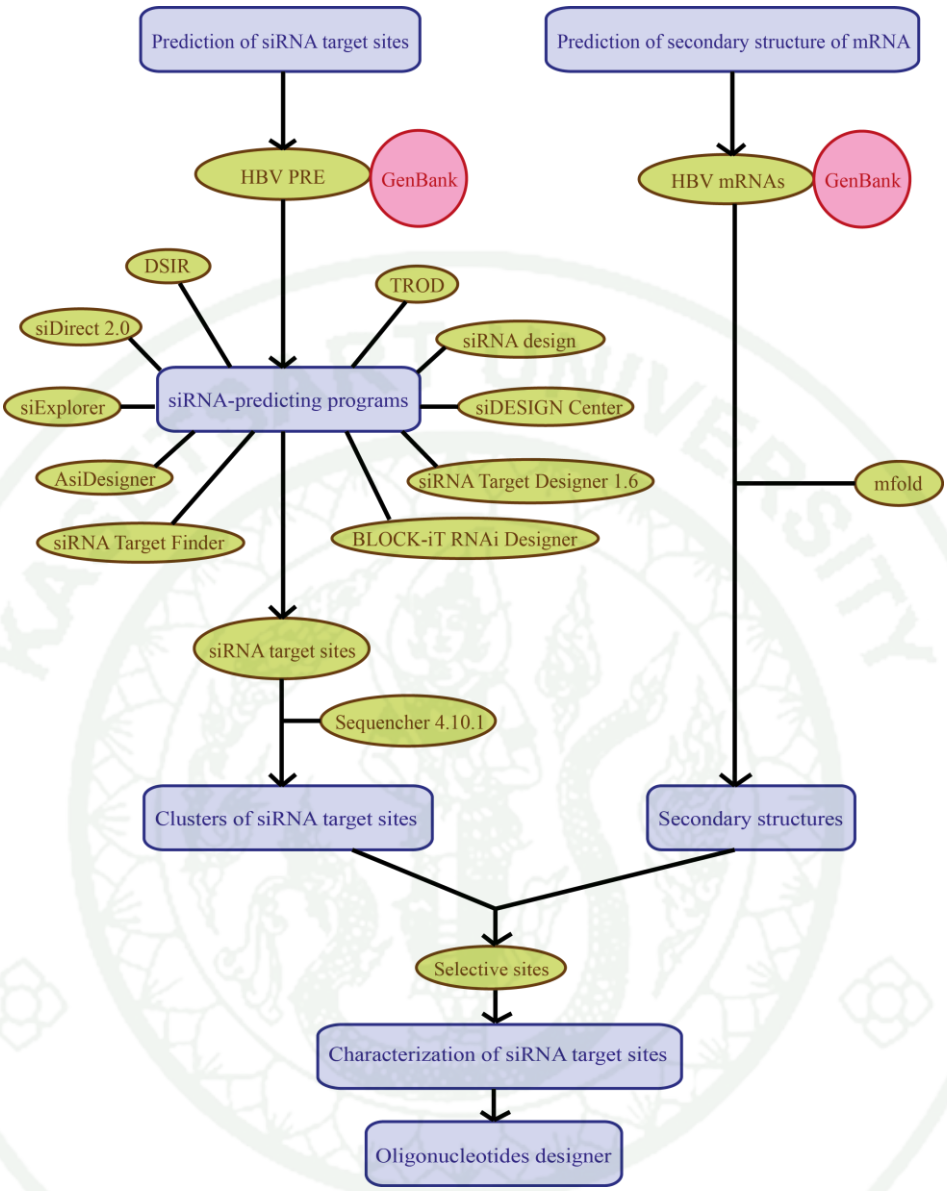

Figure 12 Flow chart shows the steps of this experiment to predict the effective siRNA target sites.

2. Preparation of competent cells

Single colony of *E. coli* DH5α was inoculated in 5 mL of LB broth followed by shaking at 37°C for 16 hours. After that, 200 µL of culture solution was then added in 30 mL of LB broth and shaking at 37°C until O.D. was 0.6 when determine at 600 nm. Next, the culture solution was placed on ice for half hour

followed by centrifugation at 4,100 ×g at 4°C for 15 minutes. The supernatant was discarded and cell pellets were resuspended in 10 mL of 80 mM $MgCl_2$ containing 20 mM $CaCl_2$. Next, the resuspent solution was placed on ice for half hour and then centrifuged at 4,100 ×g at 4°C for 15 minutes. The supernatant was discarded and cell pellets were resuspended in 500 µL of 0.1 M $CaCl_2$ followed by adding of 500 µL of 10% (v/v) glycerol. *E. coli* competent cells were aliquoted and stored at -80°C until used (Figure 13, D).

3. Transformation (heat shock)

At first, 100 ng of plasmid was added into 200 µL of *E. coli* competent cells and placed on ice for half hour. After that, this solution was transferred to a water bath at 42°C for 90 seconds and then immediately placed on ice for 4 minutes Next, the transformed cells were added with 800 µL of SOC medium followed by shaking at 37°C for 1 hour. After incubation, the cells were collected by centrifugation at 9,000 ×g for 1 minute and supernatant was discarded. The cell pellet was resuspended in 200 µL of LB broth and then spread on LB agar plate containing ampicilin or kanamycin (depending on vector). Finally, this plate was incubated at 37°C for 16 hours (Figure 13, E).

4. Plasmid extraction

Single white colony of *E. coli* was inoculated into 5 mL of LB broth containing ampicilin followed by shaking at 37°C for 16 hours. After incubation, the culture solution was centrifuged at 4,100 ×g for collecting the cell pellets. The plasmids were then purified from the cell pellets using the AxyPrep Extraction Miniprep Kit according to the manufacturer's instruction. Finally, plasmids were stored at -20°C until used. In addition, the construct-plasmids were checked using DNA sequencing (Figure 13, F).

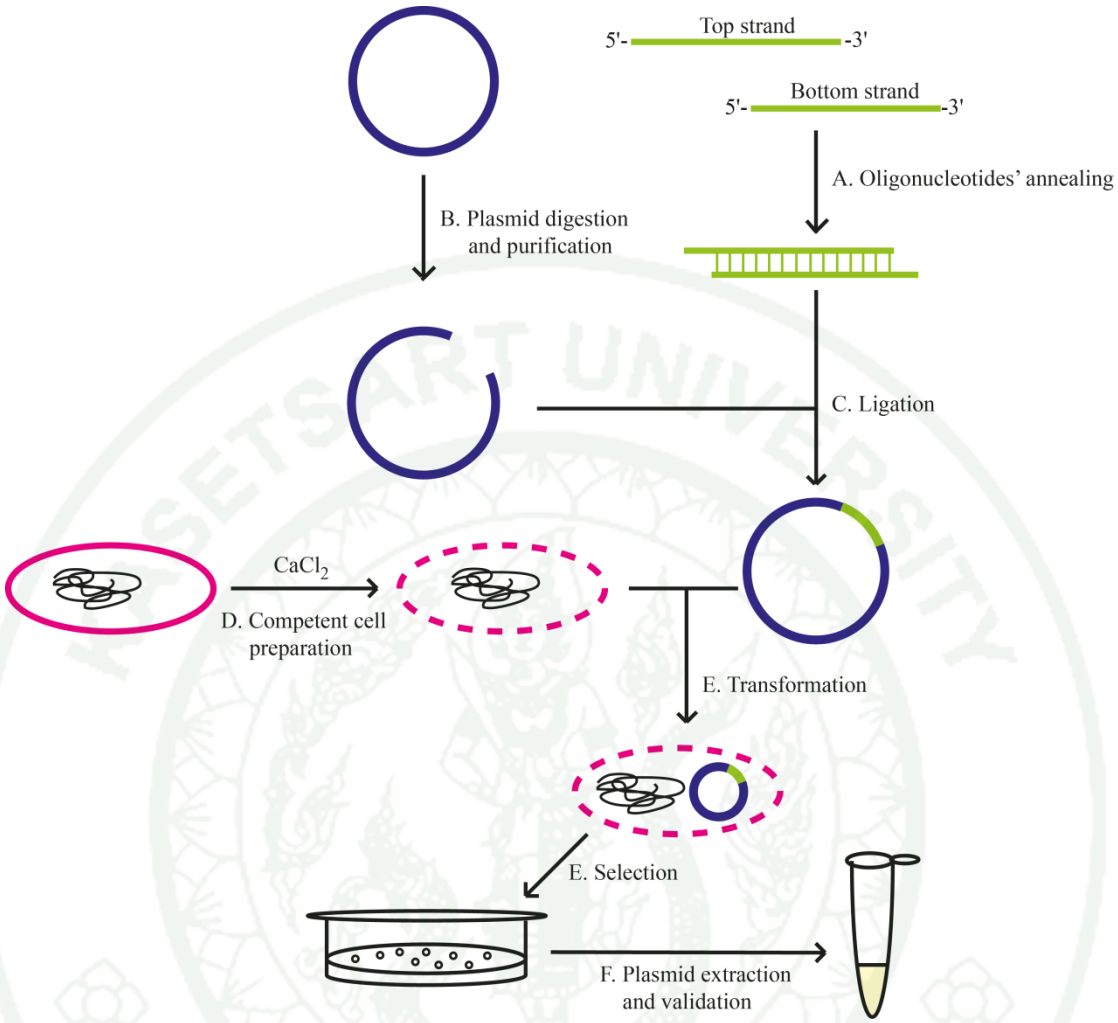

Figure 13 Diagram shows the steps of siRNA expression plasmid's construction.

Test of inhibitory effects of siRNA expression plasmids

1. Cell culture

Human hepatocellular liver carcinoma cells (HepG2) were cultured in DMEM supplemented with 10% (v/v) heat inactivated fetal bovine serum and 1% (v/v) antimycotic antibiotic. All cell lines were cultures in 75 cm^3 sterile tissue culture flasks at 37°C with 5% CO_2.

2. Transfection

For luciferase assay HepG2 cells were seeded on 24-well plates with the approximately 10^5 cells in each well. After 24 hours, the cells were transfected in triplicate with the plasmids using the Lipofectamine™ 2000. In details, 600 ng of pBasic/fPRE (that expressing firefly luciferase, FLuc) was co-transfected with 100 ng of pCMV-RLuc (that expressing *Renilla* luciferase, RLuc) and 300 ng of various siRNA expression plasmids (pShPRE1317-1337, pShCMV-1317, pShH1-1357, pShCMV-1357, pShH1-1644, pShCMV-1644, pShH1-Neg and pShCMV-Neg). The ratio between DNA (µg) and Lipofectamine™ 2000 (µL) was 1:2.5. Lipofectamine™ 2000 was mixed with 50 µL of DMEM medium and strand at room temperature for 5 minutes. The Lipofectamine™ 2000 medium was then gently mixed with plasmid solution to total volume of 100 µL. After incubation at room temperature for 20 minutes, the plasmids containing Lipofectamine™ 2000 mixtures were dropped into each well.

For quantitative real-time PCR analysis, 300 ng of pHBV-48 (that expressing HBV mRNA) was co-transfected with 100 ng of pCMV-RLuc and 600 ng of various siRNA expression plasmids. Notably, the amount of plasmid was optimized for each experiment.

3. Luciferase assay

The cells in each well were washed with PBS-EDTA and then lysed with 100 µL of 1× PLB. Ten µL of each lysate was analysed using Dual-Luciferase® Reporter Assay System Kit according to the manufacturer's instruction. The results were calculated by normalization of FLuc/RLuc.

4. Genomic DNA extraction

The cells in each well were trypsinized and then transferred to the microcentrifuge tubes. Next, the cells were harvested by centrifugation at 6,000 ×g

for 30 seconds. The genomic DNA was then purified from the cell pellets using the Genomic DNA Extraction Kit according to the manufacturer's instruction. The total genomic DNAs were adjusted to final concentration 10 ng/µL before used in next experiment. The genomic DNAs were stored at -20°C.

5. RNA extraction

The cells in each well were directly lysed with 200 µL of TRIzol by pipetting the cells up and down several times. Lysate was then transferred to a microcentrifuge tube and centrifuged at 12,000 ×g for 10 minutes at 4°C. After that, the cleared supernatant was transferred to a new microcentrifuge tube and incubated for 5 minutes at room temperature. Next, 40 µL of chloroform was added and shaked the tube vigorously by hand for 15 seconds. The tube was stood for 5 minutes at room temperature and then centrifuged at 12,000 ×g for 15 minutes at 4°C. After that, the aqueous phase was placed into a new microcentrifuge tube. Next, 100 µL of cool isopropanol was added and then incubated for 10 minutes at room temperature. After that, the tube was centrifuged at 12,000 ×g for 10 minutes at 4°C and then discarded the supernatant. The RNA pellet was washed with 200 µL of 75% ethanol by flicking the tube. Next, the tube was centrifuged at 7,500 g for 5 minutes at 4°C and discarded the wash. The RNA pellet was air dried for 10 minutes and resuspended in 20 µL of RNase-free water by pipetting. The RNA solution was incubated for 10 minutes at 60°C and stored at -70°C.

6. DNase I treatment

The RNA solution of about 2 µg was incubated with 2 units of DNase I in 1× DNase I Reaction buffer for 10 minutes at 37°C (that destroyed DNA contaminant). After that, the reaction was stopped by heating for 10 minutes at 75°C. EDTA should be added to a final concentration of 5 mM to protect RNA from being degraded during enzyme inactivation.

7. The first-strand complementary DNA (cDNA) synthesis

The RNA solution (that had been treated with DNase I) of about 1 µg was mixed with 50 pmoles of Oligo-d(T_{18}), 1 µL of dNTPs mix (each 10 mM) and adjusted final volume to 14 µL. Next, the mixture was incubated for 5 minutes at 65°C and immediately placed on ice. After that, the mixture was mixed with 4 µL of 5× RT Reaction buffer, 1 µL of 0.1 M DTT and 1 µL of HelixCript™ Thermo Reverse Transcriptase (200 units/µL). The mixture was then incubated for 10 minutes at 42°C, followed by 50 minutes at 50°C. The reaction was stopped by heating for 10 minutes at 70°C. The synthesized cDNAs were adjusted to final concentration 300 ng/µL before used in next experiment. The synthesized DNAs were stored at -20°C.

8. Quantitative real-time PCR analysis

The genomic DNAs and total cDNAs were analyzed using real-time PCR. The genomic DNAs (including cccDNAs) and cDNAs were mixed with the specific primers (shown in Table 6) and RBC ThermoOne^{TM} Real-time PCR premix (2×). Beta-globin and beta-actin genes were used as an internal control. Next, all mixtures were carried out in a StepOnePlus Real-Time PCR System under the following conditions (Table 9).

The results were indicated in term of relative quantitative data by comparative threshold (delta-delta Ct) method ($2^{-\Delta\Delta Ct}$). The amount of target gene in the sample, normalized to an endogenous housekeeping gene (reference gene) and relative to the normalized calibrator, was then given by $2^{-\Delta\Delta Ct}$,

$\Delta\Delta Ct = \Delta Ct$ (sample) - ΔCt (calibrator)
ΔCt (sample) = Ct (target gene of sample) - Ct (reference gene of sample)
ΔCt (calibrator) = Ct (target gene of calibrator) – Ct (reference gene of calibrator)
Ratio (folds of difference) of sample: calibrator = $2^{-\Delta\Delta Ct}$

In this study, the reference gene were beta-globin and beta-actin, the target genes were the cccDNA, S, C and X gene of transfected cells with siRNA expression plasmids where as the calibrators were the cccDNA, S, C and X gene of transfected cells with negative control plasmids.

Table 9 PCR program for real-time PCR (HBV cccDNA and mRNAs).

Steps	Remarks	Temperatures	Times
Initiation	Denaturation	95°C	10 minutes
Cycle (40×)	Denaturation	94°C	20 seconds
	Annealing	60°C (for cccDNA, C, X, S and beta-globin genes) 62°C (for beta-actin)	20 seconds
	Extension	72°C	20 seconds
	Detection	76°C (for cccDNA and beta-globin genes) 77°C (for S gene) 85°C (for X, C and beta-actin genes)	20 seconds
Final	Melt analysis	Default program of real-time PCR machine	50 minutes

9. Statistical analysis

Student's t-test was used for comparing data between means from two independent samples. A statistic t was calculated using the formula:

$$t = \frac{(A_1 - A_2)}{S_p \sqrt{\frac{1}{n1} + \frac{1}{n2}}}$$

When $$S_p^2 = \frac{(n_1 - 1)S_1^2 + (n_2 - 1)S_2^2}{(n_1 - 1) + (n_2 - 1)}$$

A_1 = Mean of samples 1

A_2 = Mean of samples 2

n_1 = Number of sample 1

n_2 = Number of sample 2

S_1 = Standard deviation of sample 1

S_2 = Standard deviation of sample 2

The t distribution was used with the degree of freedom (df) = $n_1 + n_2 - 2$. A *p*-value was determined from the probability table (Table 10).

Table 10 Table of t values (Student's t-test).

Degrees of freedom	Probability, p			
	0.10	0.05	0.01	0.001
1	6.31	12.71	63.66	636.62
2	2.92	4.30	9.93	31.60
3	2.35	3.18	5.84	12.92
4	2.13	2.78	4.60	8.61
5	2.02	2.57	4.03	6.87
6	1.94	2.45	3.71	5.96
7	1.89	2.37	3.50	5.41
8	1.86	2.31	3.36	5.04
9	1.83	2.26	3.25	4.78
10	1.81	2.23	3.17	4.59
11	1.80	2.20	3.11	4.44
12	1.78	2.18	3.06	4.32
13	1.77	2.16	3.01	4.22

Determination of the cell viability and the siRNA-induced interferon response

1. Cell viability assay (MTT assay)

The HepG2 cells were seeded on 24-well plates with the approximately 10^5 cells in each well. After 24 hours, the cells were transfected in quadruplet with the plasmids using the Lipofectamine^{TM}2000. In details, 300 ng of pHBV-48 was co-transfected with 100 ng of pCMV-RLuc and 600 ng of various siRNA expression plasmids. (pShPRE1317-1337, pShH1-1357, pShH1-1644 and pShH1-Neg). After incubation for 48 hours at 37°C, the old media in each well were replaced with 200 µL of new media and 10 µg of MTT (that was dissolved in PBS). Next, the cells were incubated for 3 hours at 37°C. After that, media were removed and DMSO was then added to dissolve the pure formazan of MTT. After incubation for 5 minutes at room temperature, the absorbance was then measured by a microplate reader at a wavelength of 570 nm.

2. Interferon response assay using real-time PCR

The HepG2 cells were seeded on 24-well plates with the approximately 10^5 cells in each well. After 24 hours, the cells were transfected in triplicate with the plasmids using the Lipofectamine^{TM}2000. For transfection, 300 ng of pHBV-48 was co-transfected with 100 ng of pCMV-RLuc and 600 ng of various siRNA expression plasmids. (pShPRE1317-1337, pShH1-1357, pShH1-1644 and pShH1-Neg). Moreover, the untransfected cells were untreated and treated with 100 and 1,000 ng of peginterferon alfa-2a. After incubation for 48 hours at 37°C, the cells in each well were lysed with TRIzol to extract the RNA using above protocol. Next, the RNA solution was treated with DNase I and then reversed to the first-strand cDNA. The total cDNAs were adjusted a final concentration to 300 ng/µL. The cDNAs were analyzed the levels of STAT1 and OAS1 using real-time PCR, followed by program in Table 11. In addition, the amount of plasmid was optimized for this experiment.

In this study, the reference gene was beta-actin gene. The collection data were calculated by delta-delta Ct method and analyzed using t-test as described previously in section 8 and 9 respectively.

Table 11 PCR program for real-time PCR (STAT1, OAS1, beta-actin and beta-globin).

Steps	Remarks	Temperatures	Times
Initiation	Denaturation	95°C	10 minutes
Cycle (40×)	Denaturation	94°C	20 seconds
	Annealing	60°C (for beta-globin genes) 62°C (for STAT1, OAS1 and beta-actin genes)	20 seconds
	Extension	72°C	20 seconds
	Detection	76°C (for beta-globin genes) 81°C (for STAT1 and OAS1 genes) 85°C (for beta-actin gene)	20 seconds
Final	Melt analysis	Default program of real-time PCR machine	50 minutes

RESULTS AND DISCUSSIONS

Results

Prediction of the effective siRNA target sites

1. Prediction of siRNA target sites

To predict siRNA target sites, HBV PRE sequence was input into eleven programs. The sequencher program was then applied to group siRNA target sites (Method section: identification of RNAi target sites No. 1). The results of predicted siRNA target sites were clearly observed into nine clusters (Fig. 14A). The highly specific identified siRNA target site that contains position-base preferences by Reynolds *et al.* (2004) was then selected to represent each cluster (Fig. 14A: shown as red lines). The representative siRNA target sites for clusters 1-9 were: 1176-1196, 1260-1280, 1317-1337, 1357-1377, 1409-1429, 1548-1568, 1575-1595, 1606-1626 and 1644-1664, respectively (Fig. 14A). Within the nine representative siRNA target sites, the reported siRNA target site position 1317-1337 (Fig. 14A: Group III, Fig. 14B) (Panjaworayan *et al.*, 2010) was highly detected by all programs and found to be located within the previously reported regulatory element known as a splicing regulatory element-1 (SRE-1) (Panjaworayan *et al.*, 2007) (Fig. 14C). In addition, the predicted siRNA target site 1644-1664 (Group IX) was also highly detected and appeared to be a very conserved region (Fig. 14D). Subsequently, the representative siRNA target sites from clusters III (1317-1337), IV (1357-1377) and IX (1644-1664) were selected for further analysis.

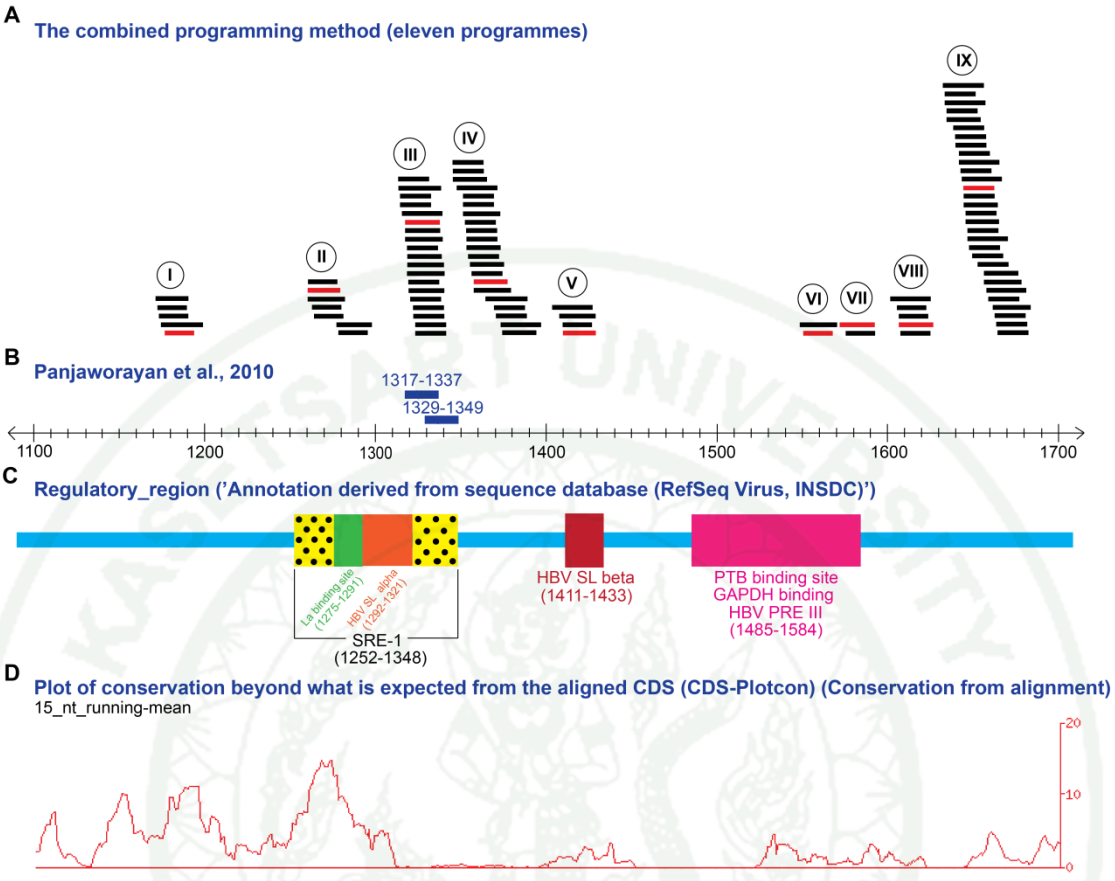

Figure 14 Nine clusters of the predicted siRNA target sites. (A) Clusters of predicted siRNA target sites. A represent sequence is shown using red line. (B) Previous reported siRNA target sites within HBV PRE. (C) Reported regulatory elements within HBV PRE. (D) Conserved regions of HBV PRE predicted by CDS-Plotcon.

Source: Thongthae and Panjaworayan (2011)

2. Prediction of secondary structures of selected siRNA target sites in the contents of luciferase and HBV mRNAs

According to the results from mfold of luciferase conjugated with HBV PRE, pgRNA, preS1, preS2/S and X transcripts, selected siRNA target sites position 1357-1377 and 1644-1664 could potentially form hairpin structures. On the other hand, the predicted siRNA target sites position 1317-1337 was found to be located

between two stem-loop structures (Figure 15-18). Interestingly, all siRNA target sites were shown as part of secondary structures in HBV X transcript (Figure 19).

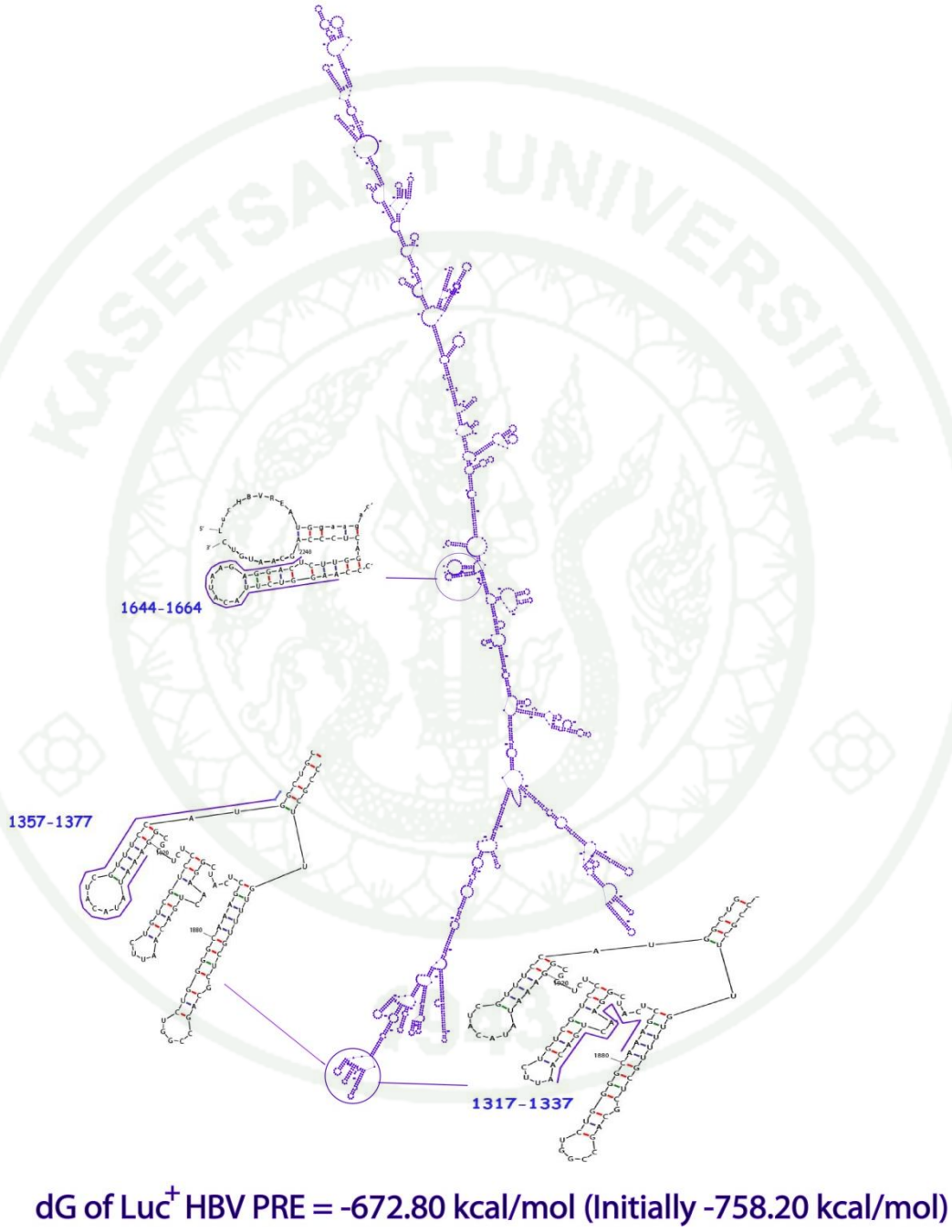

Figure 15 The secondary structure of Luc^{+} conjugating with HBV PRE (ΔG = -672.80 kcal/mol). The magnified structures of selected siRNA target sites position 1317-1337, 1357-1377 and 1644-1664 are identified.

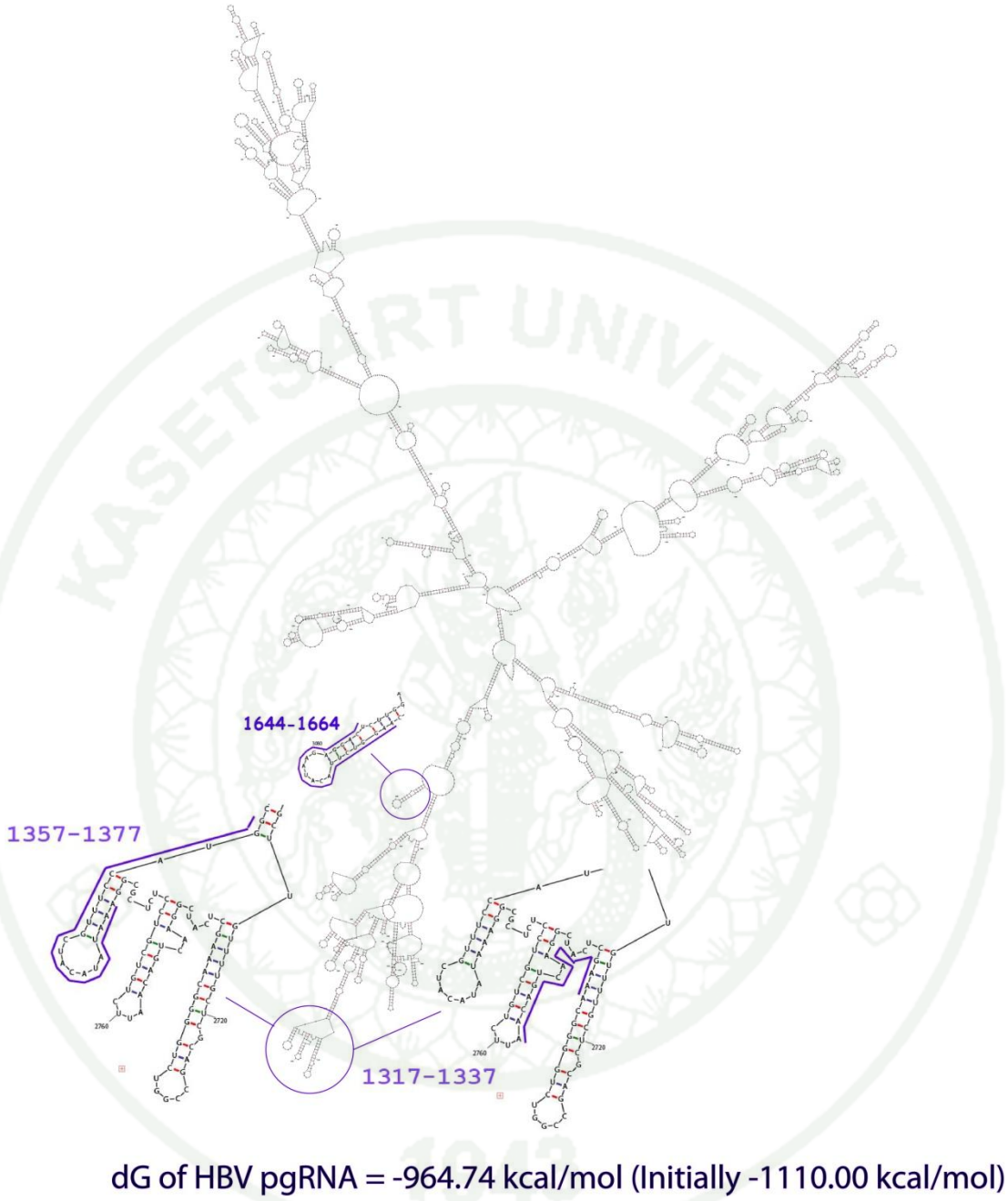

Figure 16 The secondary structure of HBV pgRNA (ΔG = -964.74 kcal/mol). The magnified structures of selected siRNA target sites position 1317-1337, 1357-1377 and 1644-1664 are identified.

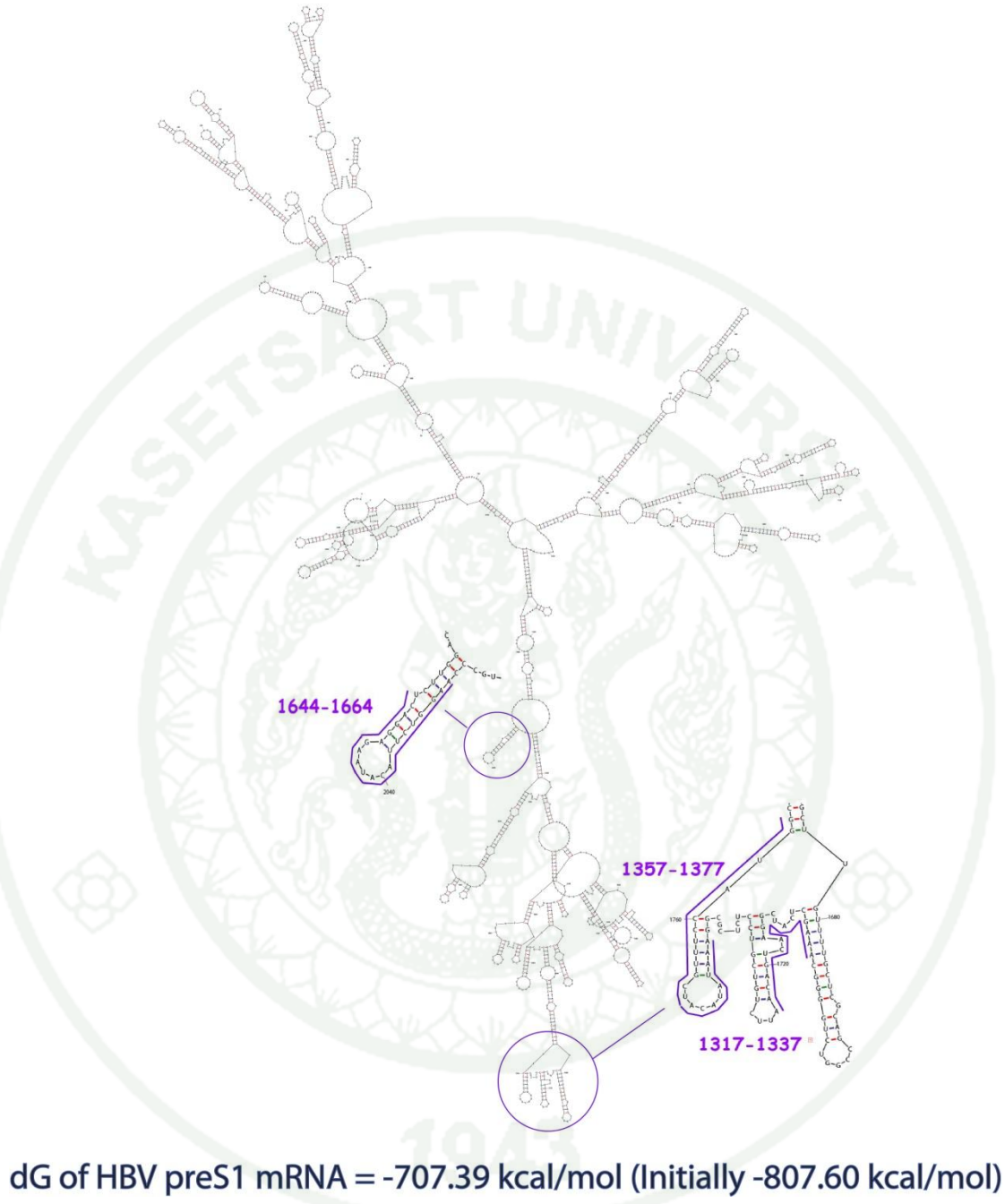

Figure 17 The secondary structure of HBV preS1 mRNA (ΔG = -707.39 kcal/mol). The magnified structures of selected siRNA target sites position 1317-1337, 1357-1377 and 1644-1664 are identified.

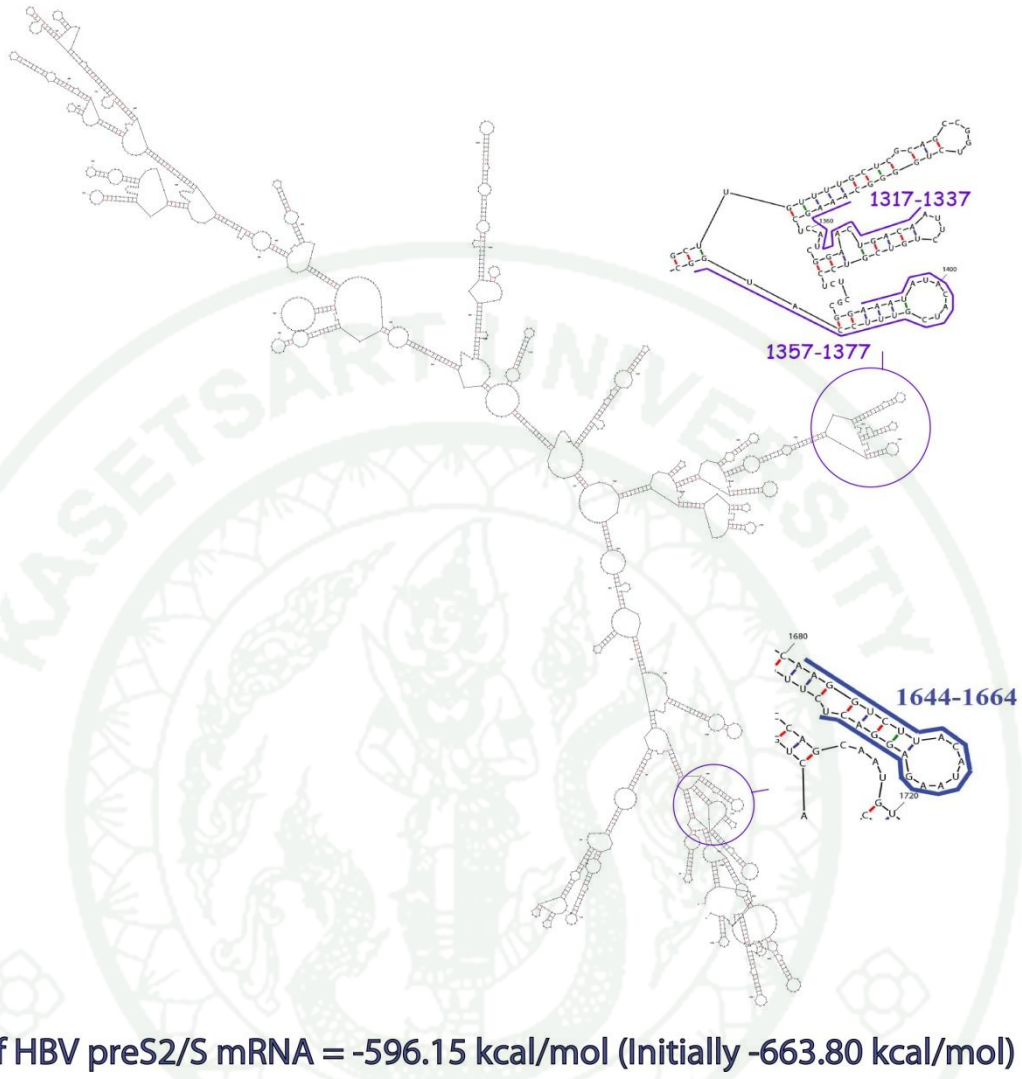

Figure 18 The secondary structure of HBV preS2/S mRNA (ΔG = -596.15 kcal/mol). The magnified structures of selected siRNA target sites position 1317-1337, 1357-1377 and 1644-1664 are identified.

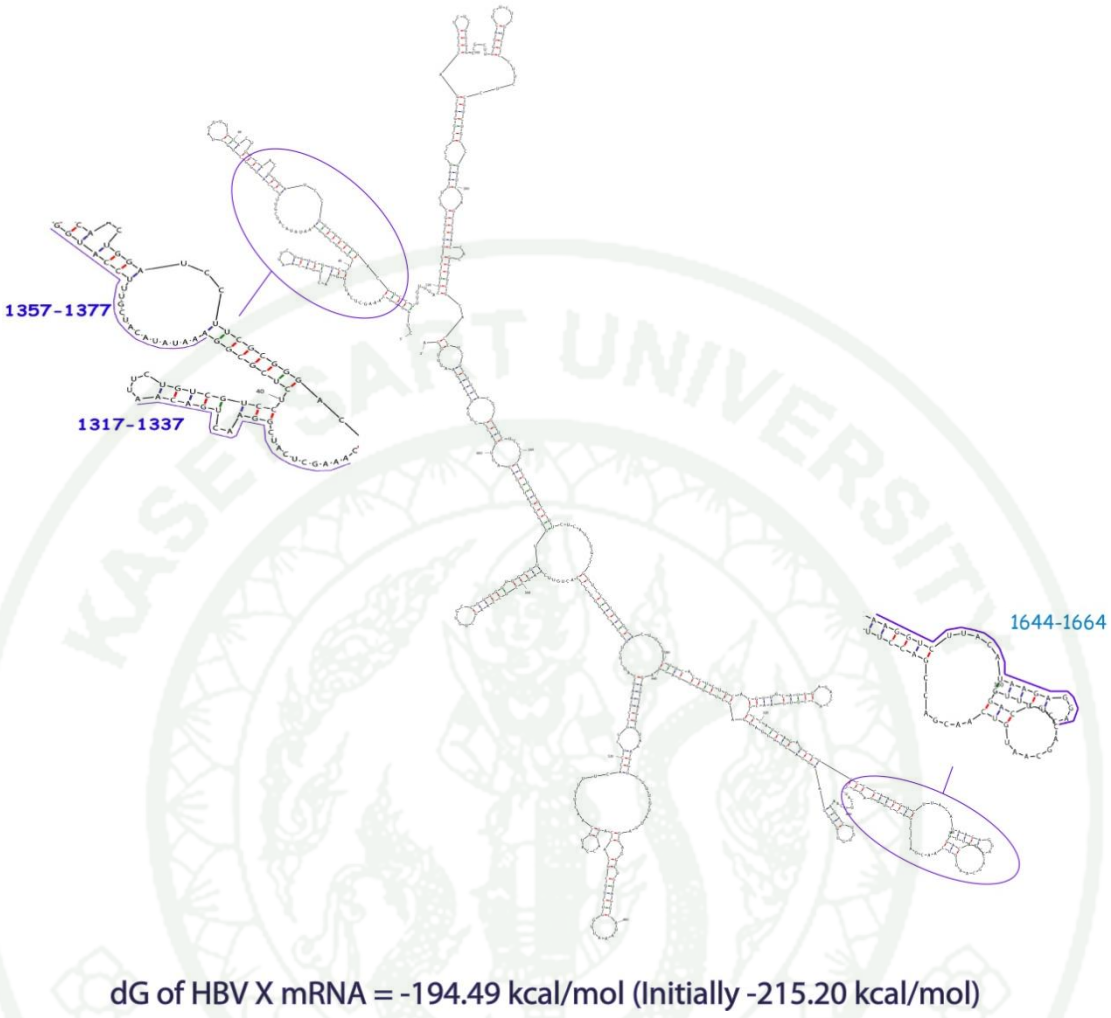

Figure 19 The secondary structure of HBV X mRNA (ΔG = -194.49 kcal/mol). The magnified structures of selected siRNA target sites position 1317-1337, 1357-1377 and 1644-1664 are identified.

3. Sequence characterization of selected siRNA target sites.

For identification of key features of siRNA target sites, some important properties such as thermodynamics, base preferences, conservation and secondary structure were analyzed. Based on base preferences reported by Reynolds *et al*. (2004), all selected siRNA target sites contained about 50% of preferential bases. However, each selected siRNA target site contained different Reynolds-base preferences (Table 12). Moreover, all selected siRNA target sites were highly specific as they were less than 81% match to human genome (Table 12). Furthermore, all siRNA target sites had an optimal GC content (%). The siRNA target site position 1317-1337 had the highest ΔG of 5'-end of AS whereas the position 1357-1377 had the lowest value. On the other hand, the siRNA target site position 1357-1377 had the highest ΔG of 3'-end of AS whereas the position 1644-1664 had the lowest value (Table 12). In addition, only siRNA target site position 1317-1337 was not form actual hairpin structure of any tested genes (Table 12).

Table 12 Characterization of selected siRNA target sites. "*" indicates base preferences from Reynolds *et al.* (2004).

Properties	Positions of HBV genome (accession no.: AM282986)		
	1317-1337	1357-1377	1644-1664
mRNA target sequence (5'->3')	AAAGCUCAUCGGAACUGACAA	AAAUAUACAUCGUUUCCAUGG	AAGGUCUUACAUAAGAGGACU
Sense strand sequence of siRNA (5'->3')	AGCUCAUCGGAACUGACAAUU	AUAUACAUCGUUUCCAUGGUU	GGUCUUACAUAAGAGGACUUU
Antisense strand sequence of siRNA (5'->3')	UUGUCAGUUCCGAUGAGCUUU	CCAUGGAAACGAUGUAUAUUU	AGUCCUCUUAUGUAAGACCUU
Percentage of GC content	42.9	33.3	38.1
Predicted by	9 programs	7 programs	9 programs
An "A" base at position 3 of sense strand*	No	Yes	No
An "U" base at position 10 of sense strand*	No	No	Yes

Table 12 (Continued)

Properties	Positions of HBV genome (accession no.: AM282986)		
	1317-1337	1357-1377	1644-1664
Not "G" base at position 13 of sense strand*	Yes	Yes	No
An "A" base at position 19 of sense strand*	Yes	No	No
ΔG of 5'-end of antisense strand (kcal/mol)	-0.9	-3.3	-2.1
ΔG of 3'-end of antisense strand (kcal/mol)	-2.1	-1.1	-3.3
Secondary of mRNA target site	Between two stem-loops	On stem-loop	On stem loops
Percentage of similarity to a host genome	67% match TSPAN7 and 81% match chr. 2 genomic contig	67% match BPIL2 and 76% match chr.11 genomic contig	76% match chr. 20 genomic contig

4. Oligonucleotide designed for construction of siRNA expression plasmids

To construct the siRNA expression plasmid, double-stranded hairpin siRNA (shRNA) sequence was required. Therefore, the selected siRNA target sites were designed to have a hairpin structure by adding sense, loop, antisense, H1 terminator sequence and enzyme restriction sites (*Bam*HI and *Hind*III). Subsequently, top and bottom strands of synthetic shRNAs were annealed together (Figure 20). These complementary oligonucleotides were then subjected to ligate (Method section: plasmid cloning No. 1) with vector p*Silencer* 3.0-H1 Negative (Appendix Figure A4) and p*Silencer* 4.1-CMV neo Negative (Appendix Figure A5).

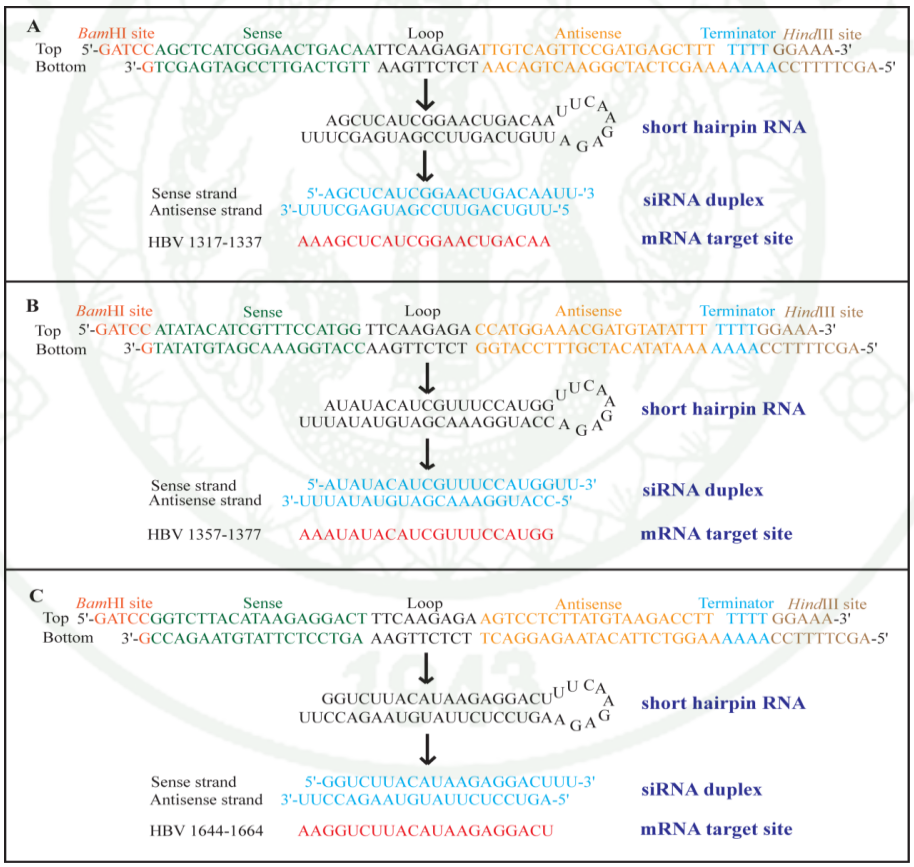

Figure 20 Oligonucleotides of selected siRNA target sites and their image pictures of shRNAs and duplexes. (A) The siRNA target site at nucleotide position 1317-1337. (B) The siRNA target site at nucleotide position 1357-1377. (C) The siRNA target site at nucleotide position 1644-1664.

Construction of siRNA expression plasmids

1. Vector digestion and ligation

To construct siRNA expression plasmids, p*Silencer* 3.0-H1 (Appendix Figure A4) and *pSilencer* 4.1-CMV neo (Appendix Figure A5) were used as the parent vectors for expressing siRNAs under RNA polymerase III (H1 promoter) and RNA polymerase II (CMV promoter) respectively. Prior to ligation, the vectors were digested with *Bam*HI and *Hind*III and then gel purified (Figure 21).

Figure 21 The 1% agarose gel shows sizes of cut vectors and shRNA fragments.
M: GeneRuler^{TM} DNA Ladder Mix (Fermentas)
1: A cut p*Silencer* 3.0-H1 Negative (2734 bp)
2: A cut p*Silencer* 4.1-CMV neo Negative (4891 bp)
3: A shRNA 1317-1337 fragment (63 bp)
4: A shRNA 1357-1377 fragment (63 bp)
5: A shRNA 1644-1664 fragment (63 bp)

2. Plasmid verification

After ligation and transformation, plasmid constructs were prepared and named pShH1-1357, pShH1-1644, pShCMV-1317, pShCMV-1357 and pShCMV-1644. To check whether the plasmids were constructed correctly, the plasmids were cut with *Bam*HI and *Hind*III. Their DNA sizes were checked using 1% agarose gel (Figure 22) and their sequences were verified by DNA sequencing. As a result, the DNA sequencing confirmed that all plasmids were constructed correctly.

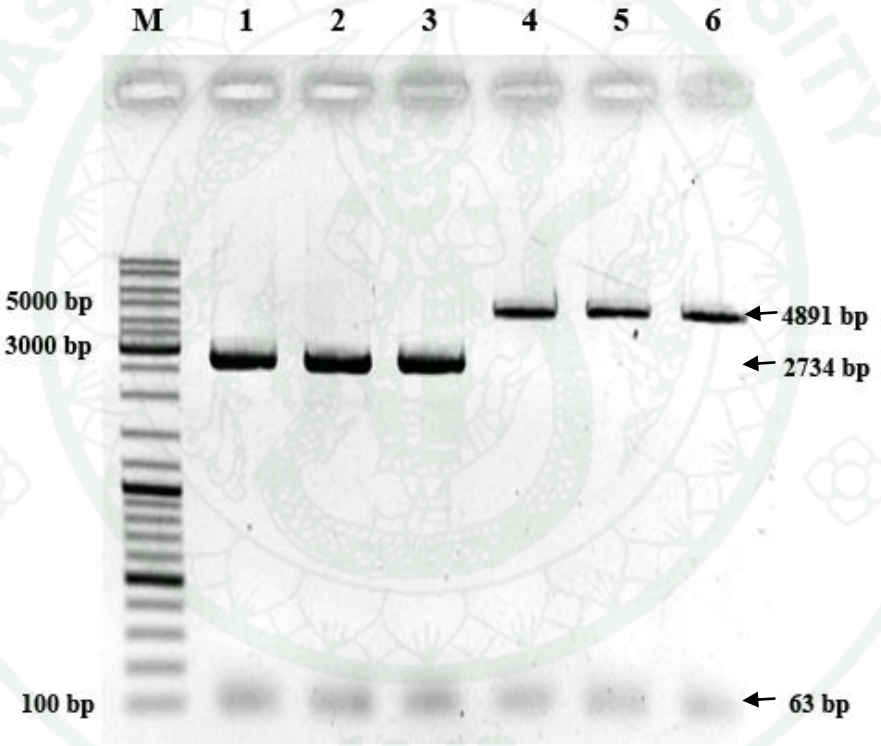

Figure 22 The 1% agarose gel shows digestion of siRNA expression plasmids.
M: GeneRuler^{TM} DNA Ladder Mix (Fermentas)
1: pShPRE 1317-1337 was cut with *Bam*HI and *Hind*III (2734 and 63 bp)
2: pShH1-1357 was cut with *Bam*HI and *Hind*III (2734 and 63 bp)
3: pShH1-1644 was cut with *Bam*HI and *Hind*III (2734 and 63 bp)
4: pShCMV-1317 was cut with *Bam*HI and *Hind*III (4891 and 63 bp)
5: pShCMV-1357 was cut with *Bam*HI and *Hind*III (4891 and 63 bp)
6: pShCMV-1644 was cut with *Bam*HI and *Hind*III (4891 and 63 bp)

Inhibitory effects of siRNA expression plasmids

1. Luciferase assay

Firstly, the inhibitory effect of siRNA expression plasmids were tested on firefly luciferase (Luc^{+}) reporter protein (Figure 23). The experiment included *Renilla* luciferase as an internal control and it was used to normalize the luciferase activity (Figure 23).

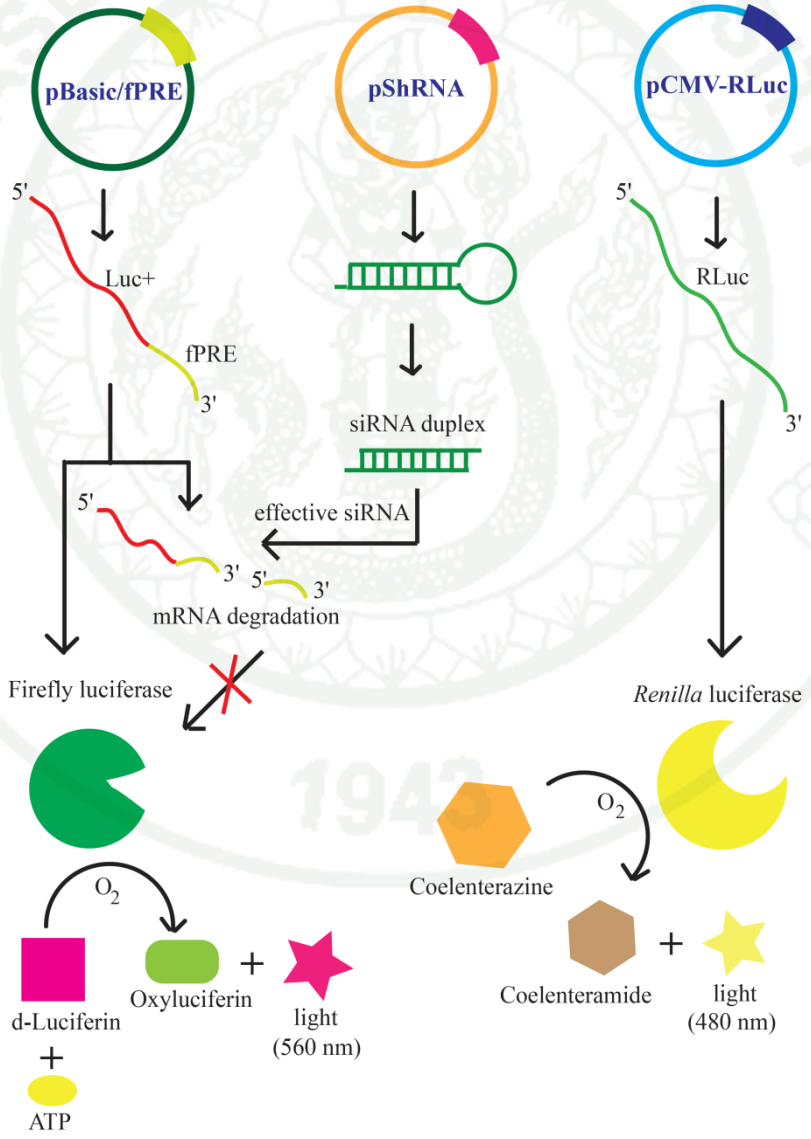

Figure 23 Diagram shows the outline of luciferase assay in this study.

For the siRNAs driven by H1 promoters, siRNA 1317-1337 had the most inhibitory effect with 90.47% reduction ($p < 0.001$). In addition, siRNA 1644-1664 significantly reduced the Luc^+ activity with 70.64% reduction ($p < 0.001$), but siRNA 1357-1377 only slightly reduced the Luc^+ activity with 13.48% reduction ($p < 0.05$). However, all siRNAs driven by CMV promoter showed no effects on the luciferase activity in HepG2 cell line (Figure 24).

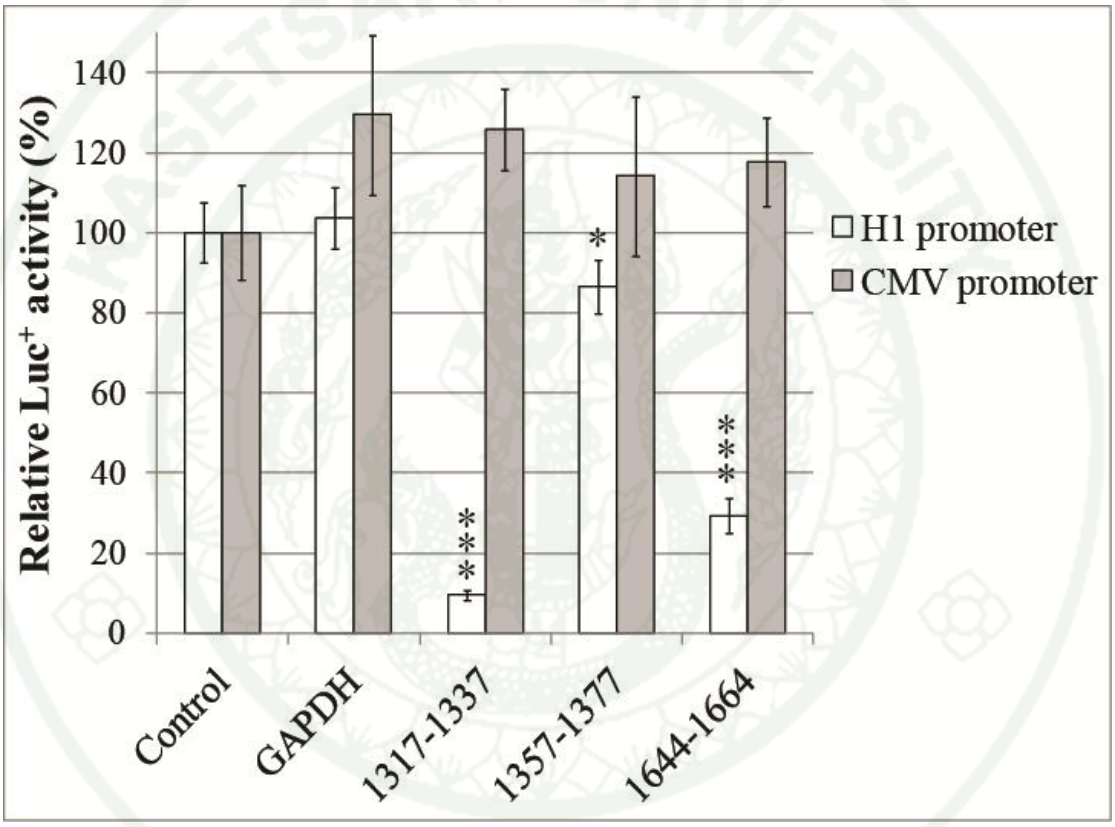

Figure 24 This graph shows the relative Luc^+ activity (%) of various siRNA expression plasmids. Opened box indicates siRNA expression plasmids driven by H1 promoter and closed box for CMV promoter. "*" and "***" indicate significant inhibitory effect when compared with the control at $p < 0.05$ and $p < 0.001$ (by t-test) respectively. This result was plotted from two-independent experiments.

2. Optimization of real-time PCR

Prior to real-time PCR experiment, an optimal temperature for amplifying each amplicon was tested using a gradient PCR. After optimization (Table 9 and 11), correct and specific size of each amplicon was detected using the 2.5% agarose gel. The sizes of cccDNA, core, surface, X, beta-globin, beta-actin, STAT1 and OAS1 amplicons were 261, 181, 98, 93 102, 351, 107 and 142 bp, respectively (Figure 25).

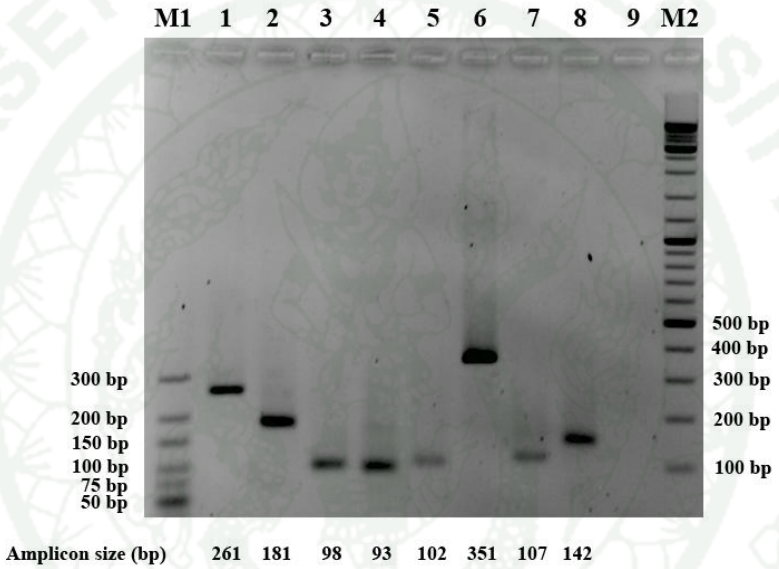

Figure 25 The 2.5% agarose gel shows the sizes of real-time PCR amplicons.
M1: GeneRuler^{TM} Ultra Low Range DNA Ladder (Fermentas)
1: A cccDNA amplicon (261 bp)
2: A core amplicon (181 bp)
3: A surface amplicon (98 bp)
4: An X amplicon (93 bp)
5: A beta-globin amplicon (102 bp)
6: A beta-actin amplicon (351 bp)
7: A STAT1 amplicon (107 bp)
8: An OAS1 amplicon (142 bp)
9: No template control
M2: GeneRuler^{TM} DNA Ladder Mix (Fermentas)

In real-time PCR analysis, SYBR green was employed and its fluorescent signal is generated after it integrates into any dsDNAs or dsRNAs. Therefore, the signal from primer-dimer must be identified and it could be denatured by addition of detection step. Notably, the optimal temperature for denaturating dimers of cccDNA, core, surface, X, beta-globin, beta-actin, STAT1 and OAS1 amplicons were 76, 81, 77, 81, 76, 85, 81 and 81^{o}C, respectively (Appendix Figure B1-B8).

3. The effects of siRNA expression plasmids on HBV cccDNA

As cccDNA is a template for HBV transcription, inhibition of cccDNA by siRNAs might reduce the expression of all HBV transcripts. Therefore, this experiment was tested to investigate whether the constructed siRNA expression plasmids could inhibit cccDNA expression or not. Surprisingly, only the siRNA 1357-1377 driven by H1 promoter could significantly reduce cccDNA with 36.21% reduction ($p < 0.05$) in HepG2 cell line. Notably, beta-globin gene was used as the reference gene (Figure 26).

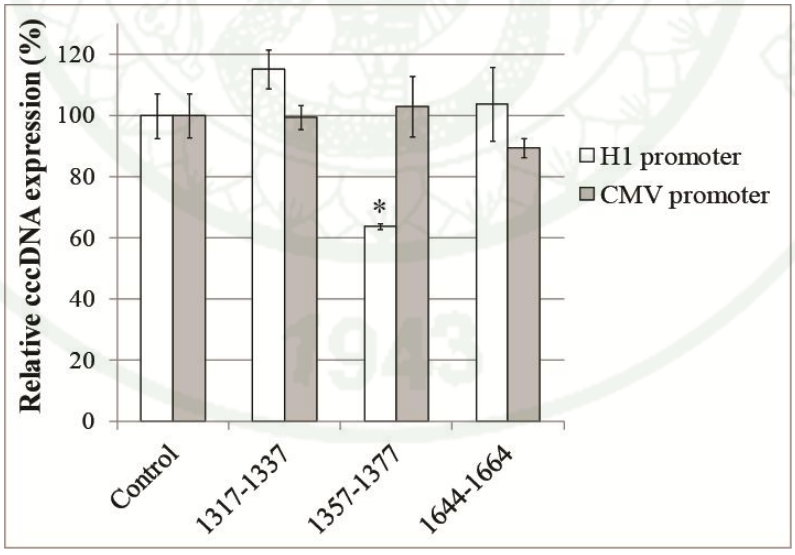

Figure 26 The graph shows the cccDNA reduction (%). Opened box and closed box indicate siRNA expression plasmids driven by H1 and CMV promoter respectively. "*" indicates significant inhibitory effect when compared with the control at $p < 0.05$ (by t-test).

4. The effects of siRNA expression plasmids on HBV transcripts

From the bioinformatic analysis, siRNA target sites at nucleotides position 1317-1337, 1357-1377 and 1644-1664 are found on all HBV transcripts. In addition, position 1317-1337 and 1357-1377 are also located on the polymerase ORF whereas position 1644-1664 is located on the X ORF (Figure 27). This experiment was aimed to investigate whether the selected siRNA target sites could potentially inhibit expression of core, surface and X transcripts or not.

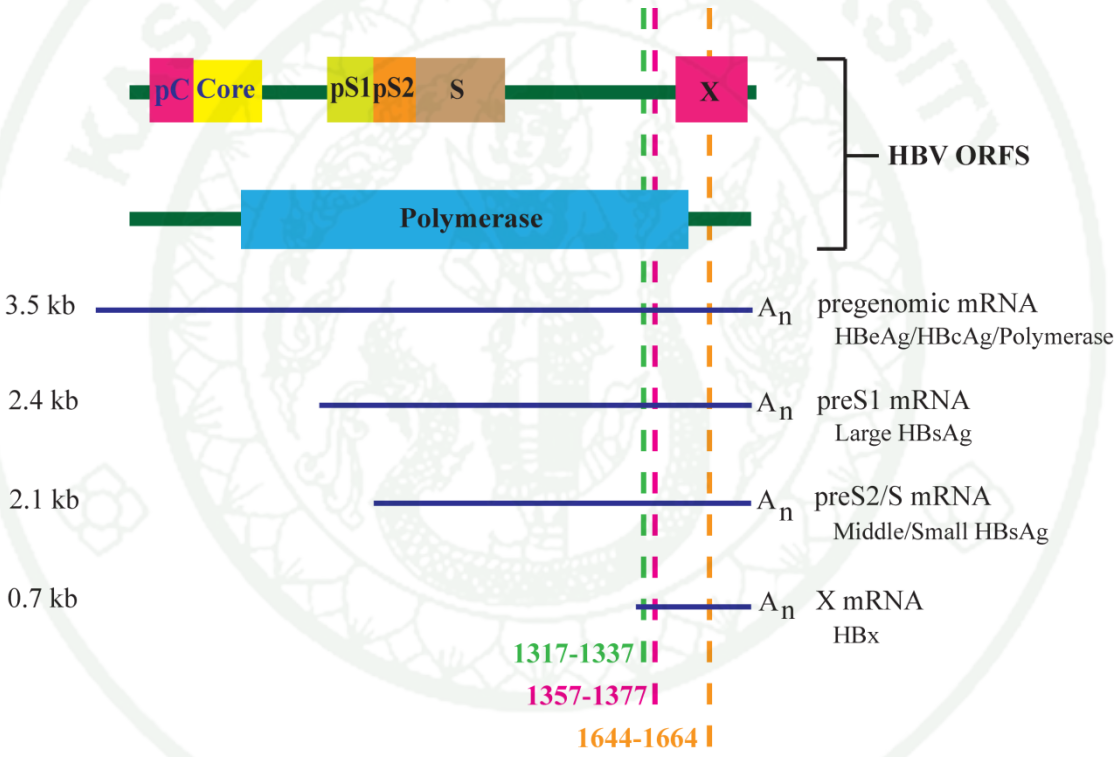

Figure 27 Diagram shows three siRNA target sites which are found on all HBV transcripts and their ORFs.

As siRNA expression plasmids driven by the H1 promoter had a greater effect than those with CMV promoter, they were then chosen to be continued in this experiment. In HepG2 cell line, siRNA target site at nucleotide position 1317-1337 had the best inhibitory effect by reducing the expression of core, surface and X mRNAs with 94.10%, 94.76% and 97.48% of reduction, respectively. Similarly, siRNA target sites at nucleotide position 1357-1377 and 1644-1664 drastically decreased core, surface and X mRNAs. The core, surface and X mRNAs were suppressed by 70.32%, 74.36% and 88.74% of reduction with siRNA 1357-1377 and about 75.70%, 80.96% and 97.84% of reduction with siRNA 1644-1664, respectively. In addition, the greatest inhibitory effect of all tested siRNAs was observed on the X mRNA. Notably, beta-actin gene was used as the reference gene (Figure 28).

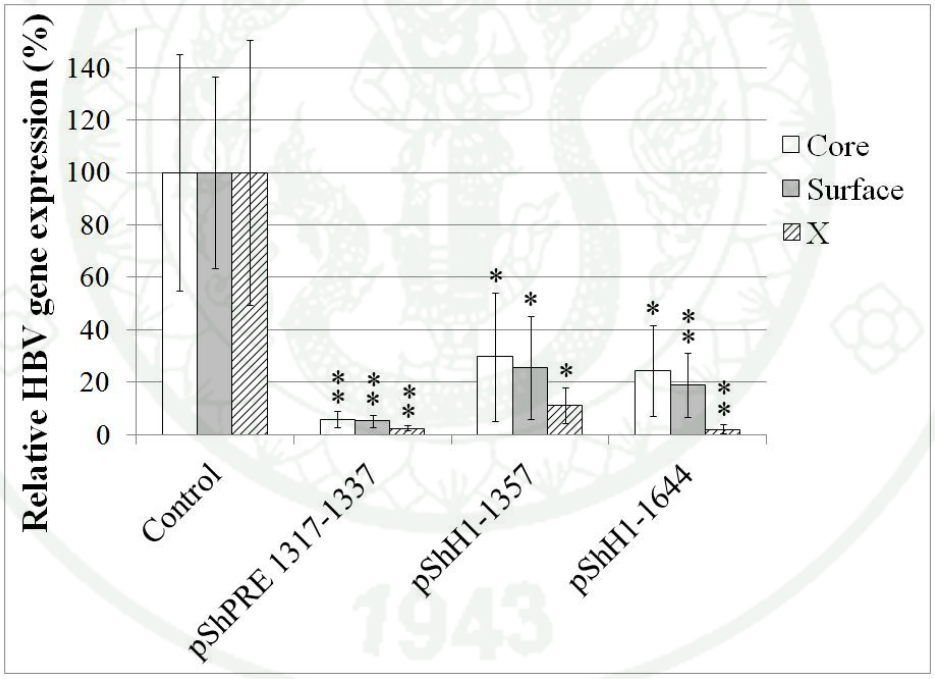

Figure 28 The graph shows the relative HBV gene expression (%) that was affected from various siRNA expression plasmids driven by H1 promoter. Opened box, closed box and lined box indicate the core, surface and X mRNAs, respectively. "*" and "**" indicate significant inhibitory effect when compared with the control at $p < 0.05$ and $p < 0.01$ (by t-test) respectively. This result was plotted from two-independent experiments.

Determination of the cell viability and the siRNA-induced interferon response

1. Cell viability assay (MTT assay)

This study was aimed to test whether the condition used to perform RNAi assay could cause cytotoxicity on cells or not. Therefore, HepG2 cells were transfected with the same condition as indicated in the RNAi assay. Then, cell viability was determined by using MTT assay. As a result, none of the siRNA expression plasmids caused cytotoxicity of the cells in the given condition (Figure 29).

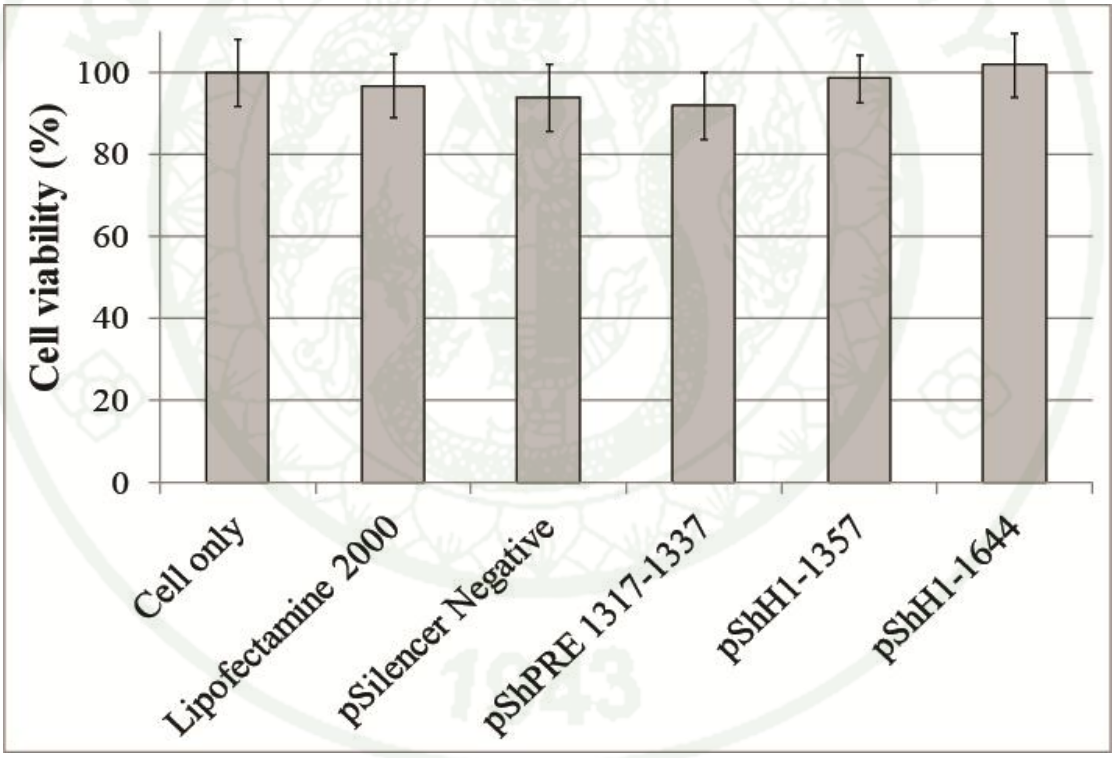

Figure 29 The graph shows the cell viability (%) of this experiment. The result was plotted from three-independent experiments.

2. Interferon response assay

Lastly, the experiment was conducted to check whether the selected siRNA expression plasmids could cause induction of interferon response or not. This experiment included cell treated with 100 ng of IFN as a positive control. As a result, all siRNA expression plasmids did not induce the expression of STAT1 or OAS1 (the down-stream genes of IFN). Moreover, transfection of siRNA expression plasmids did not affect expression of the house-keeping gene, beta-globin (Figure 30).

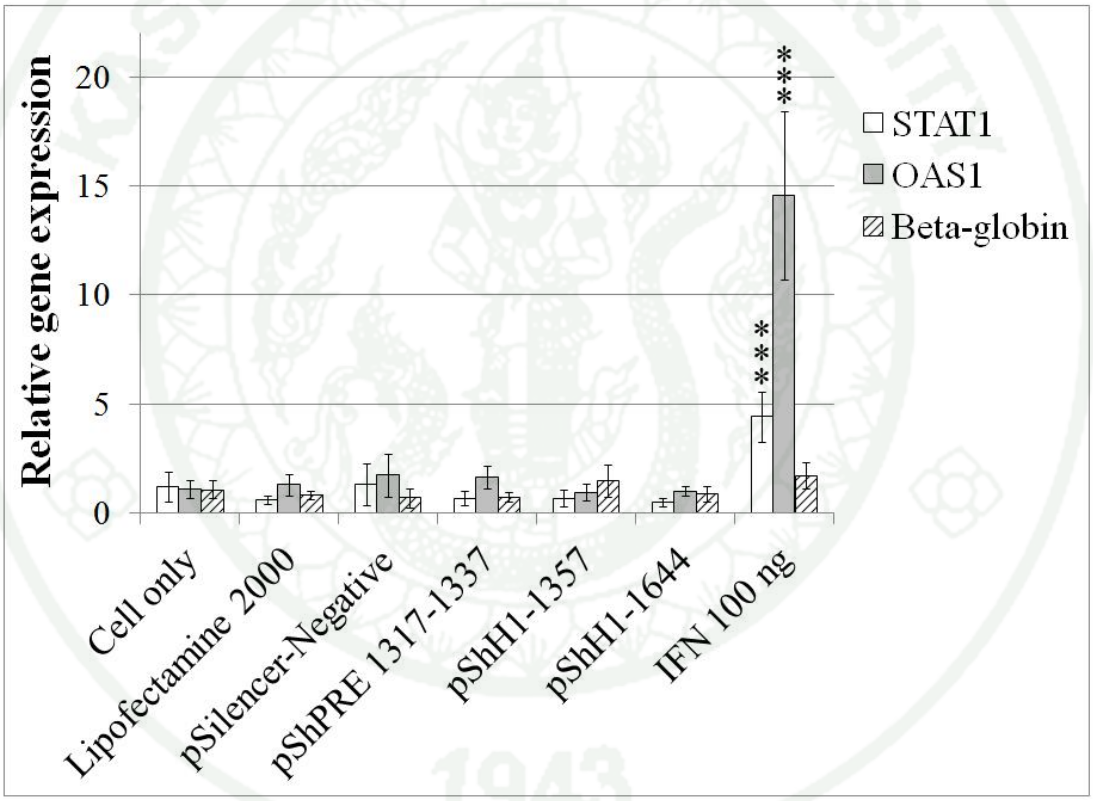

Figure 30 The graph shows the relative human gene expression. Opened box, closed box and lined box indicate the STAT1, OAS1 and beta-globin mRNAs, respectively. "***" indicates statistical difference of gene expression when compared with the cell only at $p < 0.001$ (by t-test). This result was plotted from two-independent experiments.

Discussions

1. From this study, siRNA expression plasmids driven by the H1 (RNA polymerase III) promoter had greater inhibitory effects than the plasmids driven by the CMV (RNA polymerase II) promoter (Figure 24 and 26). Since the RNA polymerase III promoters are naturally ubiquitously and constitutively expressed (Wu *et al.*, 2007); thus, they can produce high levels of shRNAs that may cause cytotoxicity of tissue culture cells (McBride *et al.*, 2008). High levels of shRNAs generated from RNA polymerase III has been reported to saturate and compete components of the endogenous miRNA processing pathway (Castanotto *et al.*, 2007) and cause off-target effects due to the accumulation of excessive AS (McBride *et al.*, 2008). Therefore, optimal conditions used in RNAi assay must be strictly determined when using siRNA expression plasmids driven by RNA polymerase III. Alternatively, plasmids containing RNA polymerase II such as CMV promoter are used to resolve the problems mentioned above (Snyder *et al.*, 2008). In addition, this study successfully performed RNAi assay with siRNAs expressing from RNA polymerase III without the induction of cell cytotoxicity and IFN response (Figure 29 and 30).

2. Results from the Luc^+ activity confirmed that the siRNA target site at nucleotide position 1317-1337 could significantly inhibit the Luc^+ activity as previously reported by Panjaworayan *et al.* (2010). However, the siRNA target site at nucleotide position 1317-1337 had no effect on the expression of cccDNA in HepG2 cell line in this study. This result contrasts with Panjaworayan *et al.* (2010) as they demonstrated that siRNA 1317-1337 had a strong inhibitory effect on the level of cccDNA in Huh-7 cell line. The likely explanation is that a genotype of the cells can cause result's variation as reported by Snyder *et al.* (2009). Furthermore, this is the first report indicating that siRNA 1317-1337 effectively inhibits core, surface and X of HBV transcripts and it had the best effect among three selected siRNAs (Figure 24 and 28).

3. The siRNA target site at nucleotide position 1644-1664 also showed a great inhibitory effect on the Luc^+ activity, HBV core, surface, and X expression (Figure 24 and 28). The result correlated very well with Sun *et al.* (2007), who reported that this site could reduce the total of HBV RNA by 90%.

4. The siRNA target site at nucleotide position 1357-1377 had a mild inhibitory effect on the Luc^+ activity, but it had a potent effect on the expression of HBV core, surface, and X transcripts as similar as the effect observed from the siRNA 1644-1664 (Figure 24 and 28). Moreover, only the siRNA 1357-1377 could significantly inhibit the level of cccDNA in HepG2 cells (Figure 26). Therefore, the siRNA 1357-1377 may also be involved in RNAi-like mechanism (Verdel *et al*., 2004). However, further investigation must be performed to confirm roles of siRNA 1357-1377 in the RNAi-like mechanism.

5. According to results of the RNAi assay, the selected target sites at nucleotide position 1317-1337 served as the best siRNA because it had the greatest inhibitory effect on the Luc^+/fPRE and HBV mRNAs (Figure 24 and 28). This great potent of the siRNA 1317-1337 is likely due to the presence of essential features within its sequence. For example, siRNA 1317-1337 contains the highest numbers of base preferences when compared to the other two selected siRNA target sites (Table 13). The siRNA 1317-1337 has the most suitable GC content and it also has the best score of the thermodynamic stability. Moreover, the sequence of siRNA 1317-1337 on the Luc^+/fPRE, pgRNA, preS1, and preS2/S mRNAs were not predicted to form actual stem-loops. Interestingly, none of the selected siRNA target sites were predicted to form proper hairpin structure of the X transcript (Figure 19). Furthermore, all of the sites could strongly inhibit the expression of the X gene in the similar extent. Therefore, the result suggested that the secondary structure is the very important feature that affects the activity of siRNAs. This finding supports the previous report from Yoshinari *et al.* (2004) and Schubert *et al.* (2005).

Table 13 The summarized scores of essential properties of siRNA target sites. "-" indicates unwanted features.

Properties	Score of positions			References
	1317-1337	1357-1377	1644-1664	
Average GC content (32-79%) (full scores = 4)	4	3	3	Elbashir *et al.* (2002), Amarzguioui and Prydz (2004), Reynolds *et al.* (2004) and Takasaki *et al.* (2004)
Base preferences (full scores = 15)	5	-2	2	Amarzguioui and Prydz (2004), Hsieh *et al.* (2004), Reynolds *et al.* (2004), Takasaki *et al.* (2004), Ui-Tei *et al.* (2004) and Katoh and Suzuki (2007)
Thermodynamic stability (full scores = 6)	3	0	2	Amarzguioui and Prydz (2004) Reynolds *et al.* (2004) and Ui-Tei *et al.* (2004)
Consensus sequence (AAN_{19}) (full score = 1)	1	1	1	Elbashir *et al.* (2002)
Not form secondary structure (full scores = 5)	5	1	1	Zuker (2003)

6. This study will potentially be developed as an antiviral therapeutics for HBV chronic patients. However, the stably expression of siRNA expression plasmids and the delivery system have to be further investigated.

CONCLUSION

1. By using combined-predicting siRNA programs, predicting siRNA target sites were grouped into 9 clusters. Three potential siRNA target sites were chosen from the three major clusters (III, IV and IX). The selected siRNA target sites were named siRNA 1317-1337, 1357-1377 and 1644-1664.

2. The siRNA expression plasmids namely pShH1-1357, pShH1-1644, pShCMV-1317, pShCMV-1357 and pShCMV-1644 were successfully constructed.

3. The siRNAs that were expressed under RNA polymerase III (H1) promoter had a strong inhibitory effect whereas the siRNAs expressed under RNA polymerase II (CMV) promoter had no effect on the Luc^+ and HBV transcripts. The inhibitory effects of siRNAs driven by H1 promoter were summarized in Table 14.

Table 14 The summarized table of inhibitory effect from H1 promoter.

Inhibitory effects	Positions					
	1317-1337		1357-1377		1644-1664	
	%	*p*-value	%	*p*-value	%	*p*-value
Luc^+	90.47	0.001	13.48	0.05	70.64	0.001
cccDNA	No effect	-	36.21	0.05	No effect	-
Core	94.10	0.01	70.32	0.05	75.70	0.05
Surface	94.76	0.01	74.36	0.05	80.96	0.01
X	97.48	0.01	88.74	0.05	97.84	0.01

4. All effective siRNA expression plasmids such as pShPRE 1317-1337, pShH1-1357 and pShH1-1644 did not induce IFN response and had no cytotoxicity effect.

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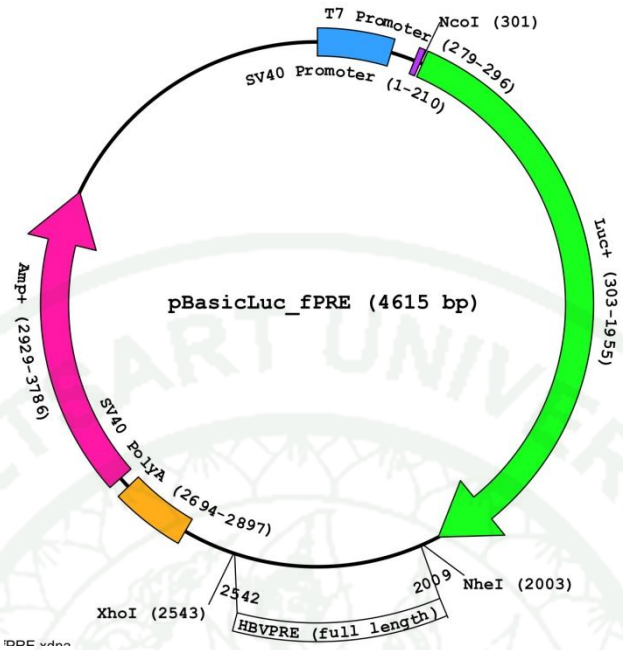
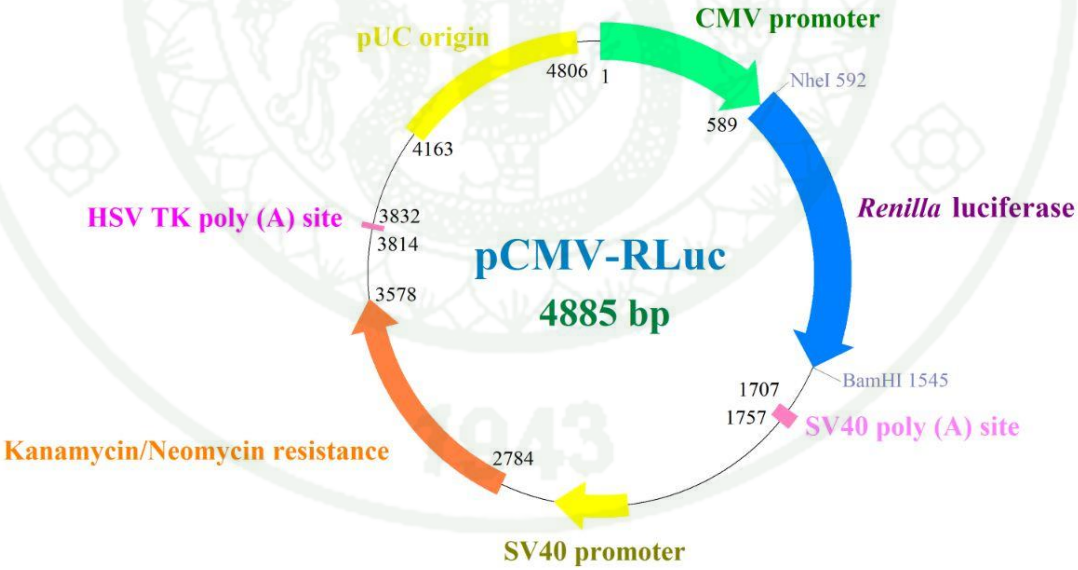
APPENDICES

Appendix A

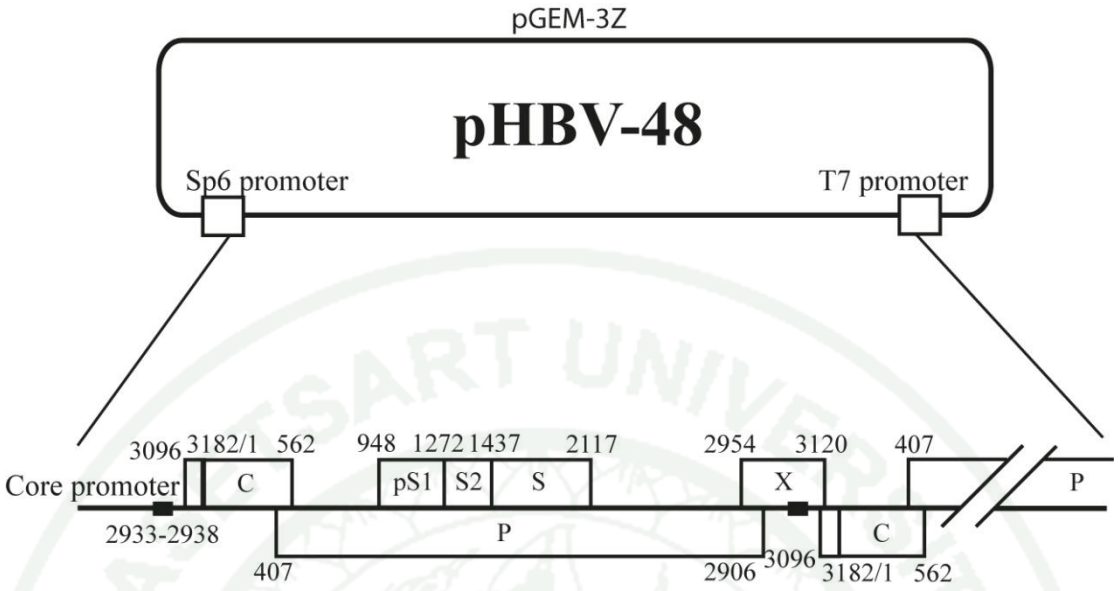
Vector maps



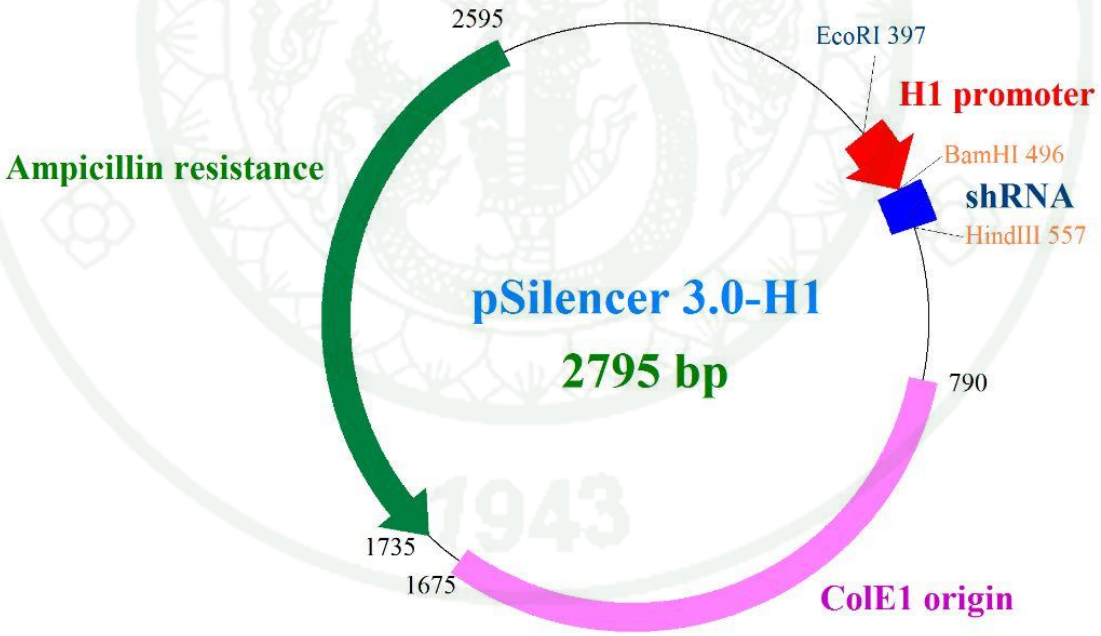
Appendix Figure A1 Vector map of pBasic/fPRE.



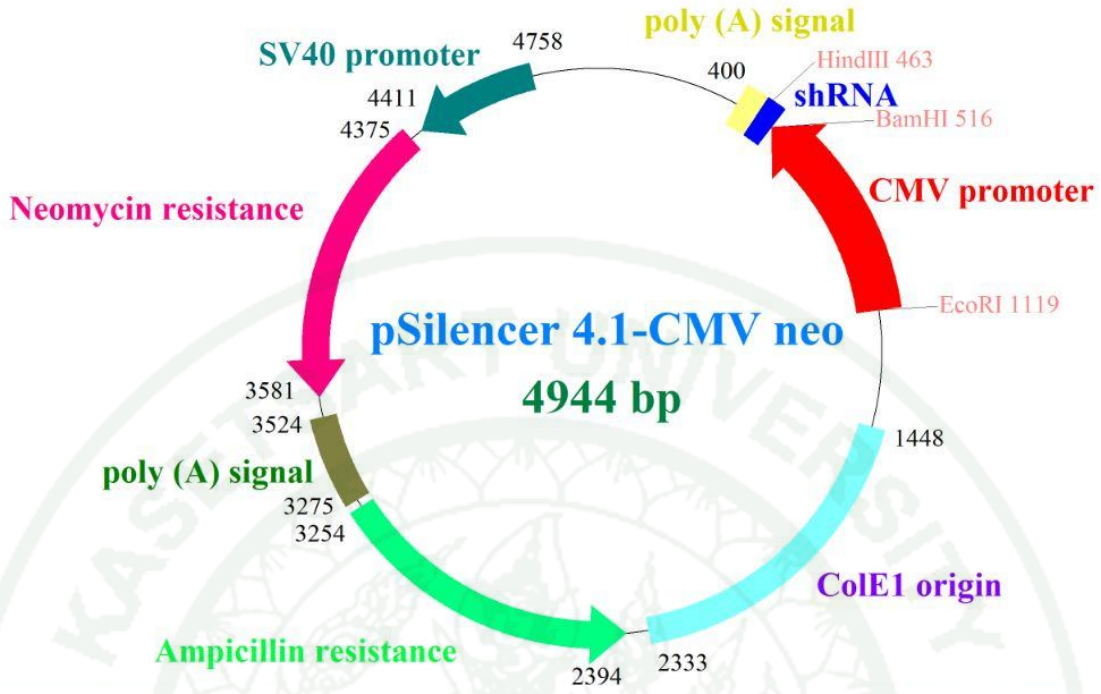
Appendix Figure A2 Vector map of pCMV-RLuc.



Appendix Figure A3 Vector map of pHBV-48.



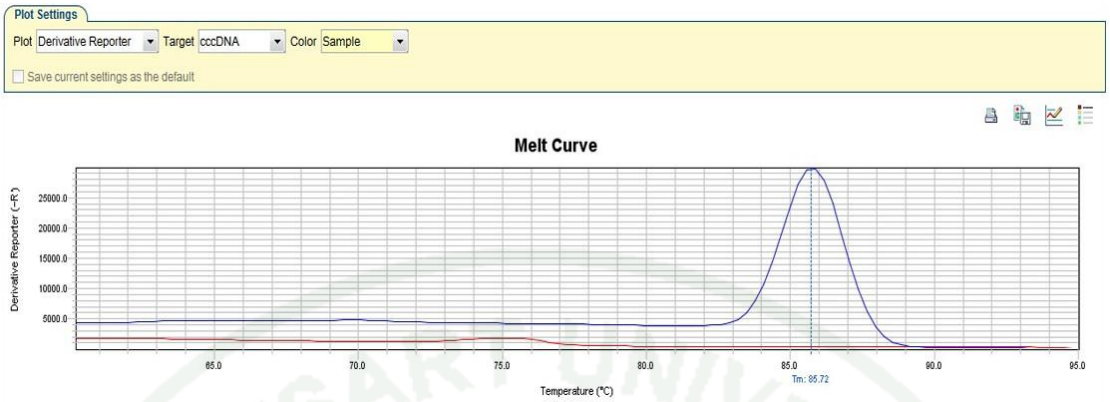
Appendix Figure A4 Vector map of p*Silencer* 3.0-H1.



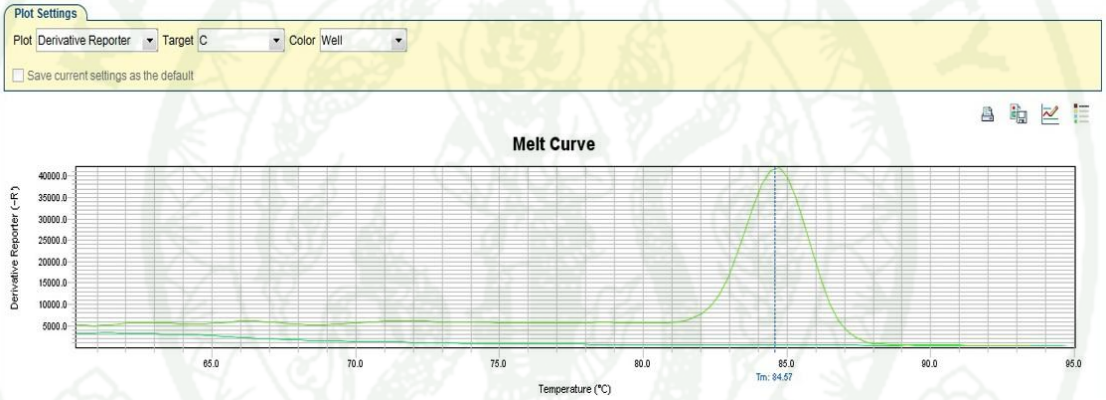
Appendix Figure A5 Vector map of p*Silencer* 4.1-CMV neo.

Appendix B

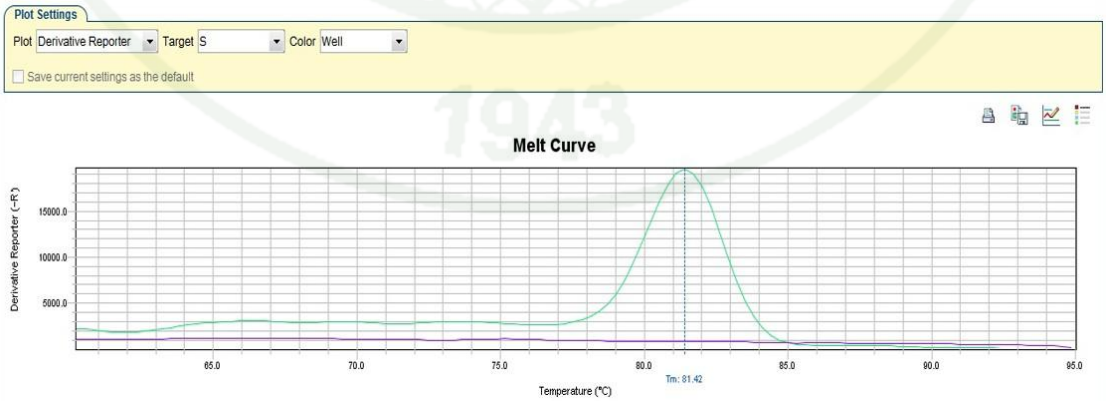
Melt curve analysis



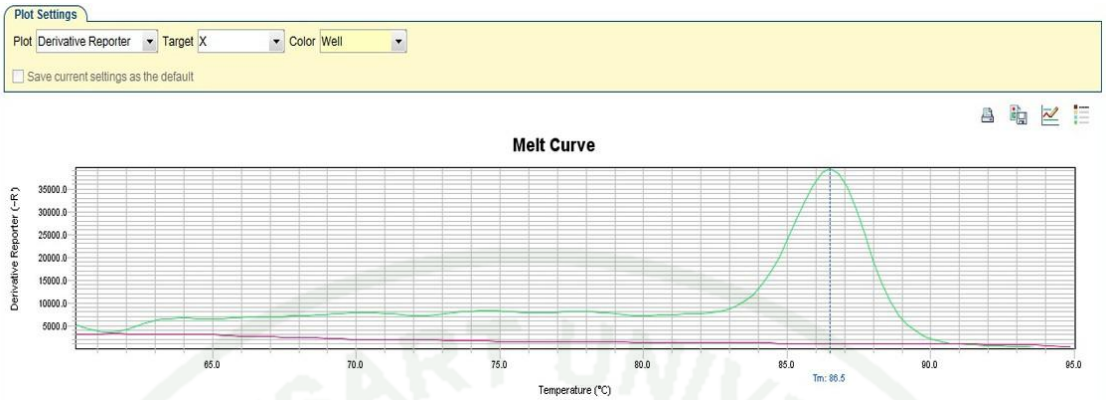
Appendix Figure B1 The melt curve analysis of cccDNA amplicon.



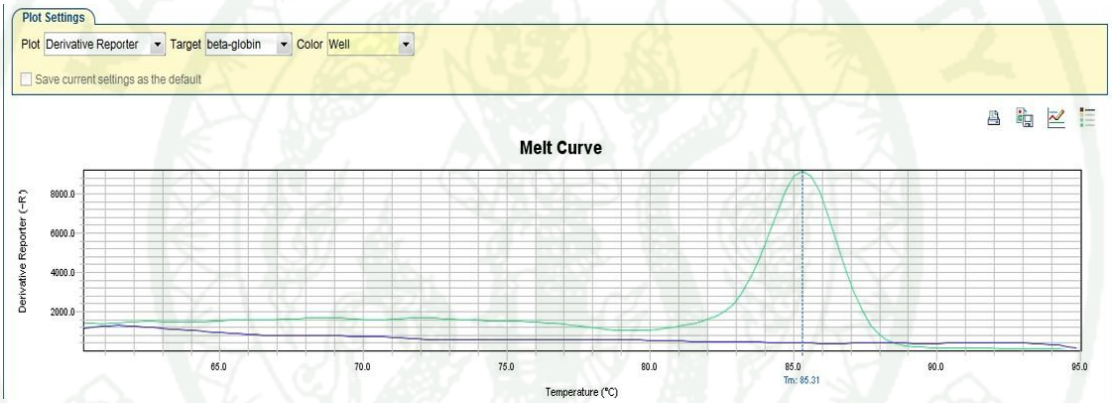
Appendix Figure B2 The melt curve analysis of core amplicon.



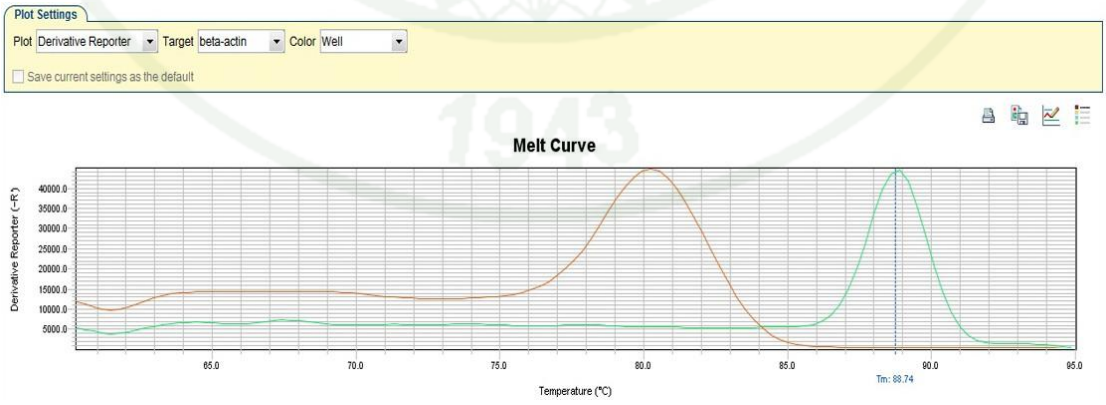
Appendix Figure B3 The melt curve analysis of surface amplicon.



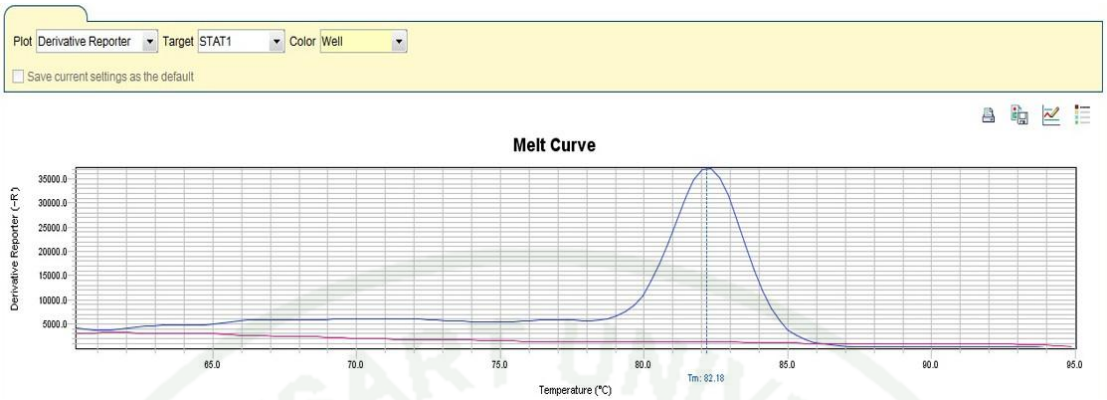
Appendix Figure B4 The melt curve analysis of X amplicon.



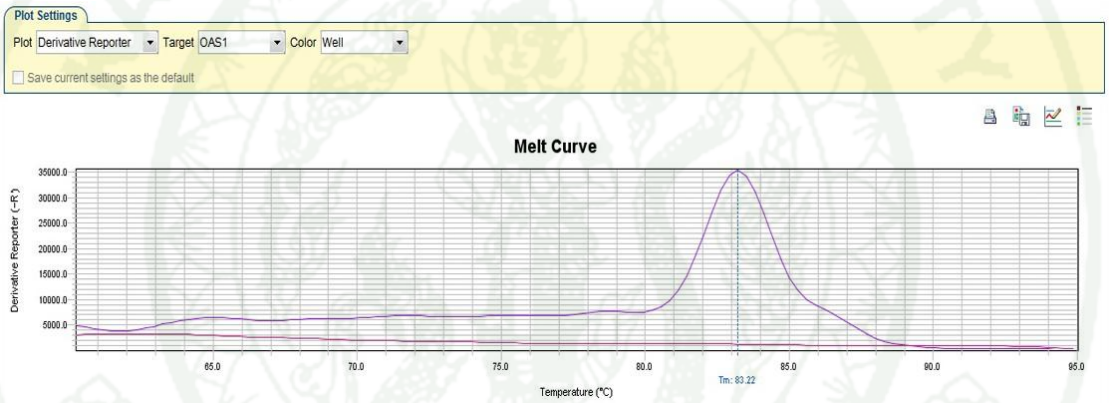
Appendix Figure B5 The melt curve analysis of beta-globin amplicon.



Appendix Figure B6 The melt curve analysis of beta-actin amplicon.



Appendix Figure B7 The melt curve analysis of STAT1 amplicon.



Appendix Figure B8 The melt curve analysis of OAS1 amplicon.

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