DEVELOPMENT OF RNA-BASED MOLECULES FOR THE INHIBITION OF INFLUENZA A VIRUS

by

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A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Animal Science

Spring 2014

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ACKNOWLEDGMENTS

First I want to give my sincere thanks to my advisor Dr. Serguei Golovan for his guidance for my research, his thoughtful and enlightening ideas that contributed to my project for the past-four-year's work. Without his advise, I would not be able to finish my Ph.D. project. I also want to give my thanks to Dr. Calvin L. Keeler, Jr. Although only being as my advisor for one year, he has provided thoughtful ideas as well as possible experimental equipment in order for me to finish my project. Also I greatly appreciate the enlightening discussions with him, which have encouraged me to be not just a experimentally skillful Ph.D. but most importantly, an independent researcher.

I want to give my thanks to my committee member Dr. Carl Schmidt for his helpful advise on my project and also being as my advisor guiding my bioinformatics project. I could not obtain my bioinformatics certificate without his help. I also want to give my thanks to Dr. Jack Gelb, Jr., for his intellectual contributions to my project and also providing equipment and space for me to finish my experimental work on influenza virus. My sincere thanks also go to Dr. Daniel R. Perez for providing thoughtful ideas on my project and kindly providing training opportunities and experimental skills. I also want to thank to Dr. Mark Parcells for his help and thought discussions on my experiments.

My thanks also go to my labmates Nick Siano and Sabari nath Neerukonda for their helpful discussions and suggestions for some of my experiments. They became

not only labmates but also important friends in my life through the past few years' working together.

I also want to thank Brian Ladman, Lauren Preskenis, Dr. Jose Miguel Ruano and Marcella Murphy for their help in my first-year project. Also, I want to thank Dr. Hongjun Chen and other people in Dr. Daniel Perez's research group for their training for my experimental skills. I also want to thank Cynthia Boettger in Dr. Keeler's group for her kind help with laboratory arrangement.

I want to thank all my friends, Qiaoyu Yang, Dr. Qian Gou, Wenqing Xu, Huimin Dong, Lixing Huang and others who have accompanied me in UD. I have spent the best time with them for the past five years.

Finally I hope to give my greatest thanks to my parents and grandparents in China. Without their unconditional support and constant care for my study and life, I could not finish all these in past five years.

This dissertation is contributed to my beloved parents Yanmin Mi and Qisheng Wang and my grandparents Xiulan Shang and Yuanqing Wang.

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ABSTRACT

Avian influenza has always been a serious threat to the poultry industry and public health. Limitations in the effectiveness and possible adverse effects of vaccines and antiviral drugs demonstrate the need to develop new prophylactic and therapeutic approaches. RNA silencing, an evolutionarily conserved pathway in many eukaryotic cells, has been utilized as a powerful tool to reduce gene expression levels as an approach for potential therapeutic uses. Previous studies have shown that small interfering RNAs (siRNAs), synthetic 19-21nt double-stranded RNAs, can significantly inhibit influenza virus replication both *in vitro* and *in vivo* by targeting viral mRNAs for degradation.

The studies herein focus on developing RNAi molecules and constructs as potential alternative methods to control avian influenza. With the restrictions of sequence complementarity for efficient siRNA targeting and yet the existence of high sequence variation within a dynamically changing influenza population, viral RNAi targets has to be selected within highly conserved regions of the influenza virus genome. However, not all the conserved regions were found to be optimal for siRNA design.

To address these challenges, I first developed a system for the accurate and rapid quantitation of influenza infectious titer and for monitoring the antiviral activity of siRNAs. Influenza vRNA/cRNA promoter-controlled GFP/luciferase expression plasmids were developed and compared for their sensitivity and accuracy in

determining influenza virus infectious titers. The vRNA-promoter driven luciferase expression reporter was selected for its high sensitivity and lower background.

Secondly, siRNAs targeting highly conserved regions across different influenza A virus strains were designed and optimized with sequence, structural or size modifications. Several modifications were found to improve the selected siRNAs' antisense-strand targeting efficiency and antiviral activity. Furthermore, to extend the practical application of anti-influenza RNAi, an influenza infection-inducible microRNA expression cassette was developed and demonstrated to have strong inhibitory activity on influenza virus replication.

Chapter 1

INTRODUCTION

Influenza virus belongs to the family *Orthomyxoviridae*. It has three major types, A, B and C. Among these, influenza A viruses are the most important zoonotic pathogens (1). Wild birds are the natural reservoir for influenza A viruses, the virus population can also infect a wide range of hosts including human, birds, pigs and horses and are responsible for severe epidemics as well as pandemics. Influenza A viruses are lipid-enveloped viruses containing eight single-stranded, negative-sense and segmented RNAs. According to differences of two surface antigens, hemagglutinin (HA) and neuraminidase (NA), influenza A viruses are classified into sixteen known HA subtypes and nine known NA subtypes (2).

Avian influenza (AI) is caused by influenza A viruses that infect various domestic as well as wild bird species. According to the clinical signs of AI, avian influenza viruses are classified into highly pathogenic avian influenza (HPAI), which was known as 'fowl plague' in the 1980s (3) or low pathogenic avian influenza (LPAI). The World Organization for Animal Health (OIE) specified HPAI as a notifiable avian influenza virus (NAI). According to OIE, notifiable HPAI (HPNAI) avian influenza viruses are defined as meeting any one of the following criteria (4): a) Avian influenza strains that can kill more than three-fourths of at least eight 4- to 8-week-old susceptible chickens inoculated intravenously in 10 days; b) Avian influenza strains that have intravenous pathogenicity index (IVPI) greater than 1.2 when inoculated intravenously into ten susceptible 4- to 8-week-old susceptible chickens; c)

H5 and H7 subtype avian influenza viruses that have a multiple basic amino acid cleavage sequence at the hemagglutinin coding region similar to those that are found in other HPNAI. Furthermore, any H5 or H7 low pathogenicity avian influenza viruses that are neither pathogenic to chickens nor have multiple basic amino acid sequences (4) were also considered notifiable and defined as LPNAI for the concerns that H5 and H7 LPAI would become virulent strains by mutations when infecting chickens (5).

HPAI leads to high mortality among domestic poultry. Both historical and ongoing outbreaks bring huge economic losses to the poultry industry. LPAI viruses although only cause mild to subclinical signs in domestic poultry, can potentially mutate into HPAI viruses. Current prevention and control measures for AI depend on adequate biosecurity and therefore more effective methods should be implemented.

1.1 General Information on Avian Influenza Virus

1.1.1 Clinical signs in chickens and the economic significance of AI

LPNAI infection in poultry is usually asymptomatic. A temporary 5-30% drop in egg production among layers can be observed, which returns to normal after recovery. Infection of turkeys by LPNAI is usually more severe than in chickens. Inappetence and decreased water consumption are usually observed (6). The common clinical signs in domestic ducks and geese are usually respiratory symptoms, such as nasal discharge and swollen sinuses (6).

HPAI causes high mortality among domestic poultry. With an incubation time of 3-7 days in birds, the most frequent sign of infection for chickens is sudden death, usually in large numbers (6). After the first sign of illness, mortality rates can almost reach ~100% after 2-12 days. The canonical clinical signs of HPAI infection include

marked depression, termination of egg production, and watery diarrhea 24-48hr before death. Neurologic signs such as torticollis and ataxia and respiratory signs such as coughing, sneezing and mucus accumulation are less frequently observed (6).

The most important biological difference, which results in the differential pathogenicity between HPAI and LPAI, is that HPAI is able to replicate systemically while LPAI can only replicate locally in the enteric or respiratory tract. This phenomenon is due to the presence of the multiple basic amino acid sequence on the HPAI hemagglutinin protein, which can be recognized by ubiquitous intracellular proteases and be activated for virion release. Instead, the hemagglutinin protein of LPAI can be recognized by an extracellular trypsin-like protease, which only exists in enteric and respiratory tracts (7).

The greatest economic losses brought by HPAI outbreaks happen in commercial farms with dense populations of domestic poultry. Economic costs directly involved with HPAI outbreaks include high morbidity and mortality. The indirect costs include the exercise of subsequent surveillance and prevention measures such as depopulation, disposal and the impact on live bird market (LBM). The two large outbreaks of HPAI that occurred in the 19th century in the U.S. cost over $64 million dollars ($116 million adjusted to 2001) in direct loss as to the poultry industry (8,9).

There is an increasing trend in the number of avian influenza outbreaks in recent years. Since avian influenza was identified as the agent causing “fowl plaque” in 1955, there were 17 outbreaks of AI reported between 1955 and 1998 (10,11) and HPAI outbreaks was considered rare events. However, from 1998, there have been a significant increase in the number of HPAI avian influenza outbreaks that have

occurred in both developing and developed areas including Mexico (12), Pakistan (13), Hong Kong (14,15), the Netherlands, Asia(16), and Africa (17). Those outbreaks have resulted in huge economic losses and clearly require more effective control and prevention measures.

1.1.2 Public health concerns

Avian influenza virus can also cause direct interspecies infection to human. Before 1997, sporadic cases of human infection with A/H7N7 avian influenza virus *via* direct animal-to-human transmission were reported (18–20). Most of them resulted in conjunctivitis and no death was reported. The outbreak of H5N1 avian influenza in Hong Kong in 1997 (21) involved several instances of human infection with clinical respiratory illnesses and deaths. This has raised public's attention. Since then, more cases of human infection by avian influenza have been reported in more than 12 countries including Asia, Europe and Africa (22). The subtypes of avian influenza virus involved in those infection cases include the H5 subtype (H5N1), H7 subtypes (H7N2, H7N3, H7N7 and H7N9) and the H9 subtype (H9N2) (22,23). Before 2013, most of the cases were caused by highly pathogenic H5N1. According to the WHO (24), since 2003 till 2011, more than 600 cases of human infection by H5N1 with ~60% fatality were reported (24). However, a recent outbreak of low pathogenic avian influenza H7N9 infection in human in China on March 2013 has caused more than 100 cases of infections with acute respiratory symptoms and a 22% fatality within a year (23,25). Besides, the H7N9 infection appears to be asymptomatic in poultry, which makes it harder to control.

The clinical symptoms caused by avian influenza virus in humans also varies depending on the type of avian influenza virus and ranges from conjunctivitis to

respiratory symptom to death (26). Most of the cases occurred after closely handling sick or dead poultry. Cases of direct human-to-human transmission of avian influenza infected disease are rare, inefficiently supported, and have been limited (27–29). However, several recent reports showing that artificially mutated or re-assorted highly pathogenic avian influenza H5N1 strains confer the ability to transmit among guinea pigs (30) and ferrets (31,32) through respiratory droplets has drastically increased public concerns for the potential to widely spread HPAI infections among human.

Two antiviral drugs, oseltamivir or zanamivir are currently the recommended treatments for avian influenza virus by the CDC and the WHO (26). However, because of the high risk of antiviral resistance development, their use is limited to large pandemic events. Several H5N1vaccines have also been developed and licensed for use in the U.S. (33,34) and Australia (35). However, they are reserved for use in the event of large H5N1 pandemics and are not commercially available.

1.1.3 Current prevention and control measures for avian influenza virus

According to FAO (Food And Agricultural Organization)/OIE/WHO (World Health Organization)'s suggestions on global strategies for control and prevention of avian influenza virus, generally accepted strategies for the prevention of avian influenza virus infections include enhanced biosecurity, strict regulations of live bird markets and the vaccination of poultry (36). High standards of biosecurity for poultry growth on farms are considered the cornerstone for prevention. This primarily requires preventing contact between domestic poultry and wild birds, including shared path for water consumption and appropriate cleaning and disinfection procedures when handling poultry (37). Live bird markets where transmission of avian influenza among poultry as well as to humans could happen, also need to be strictly controlled.

Vaccination is also recommended by OIE and FAO (36). However, it needs to be applied in combination with the other control measures mentioned above and is only applied in high-risk areas (36).

Effective control measures during an outbreak suggested by FAO (38) include the controlled movement of birds in the infected "zones", "Stamping-out" policies (depopulation of the infected and at-risk poultry), proper disposal of infected poultry and contaminated animal products, and vaccination. Although these measures have reduced the prevalence of AI in several countries (36), large economic costs put burdens on the effective control and prevention of avian influenza viruses, especially in some developing countries. More cost-effective measures still need to be developed.

1.2 Molecular Virology of Influenza A Virus

1.2.1 Structure and protein information of influenza A virus

The influenza A virus genome consists of eight negative single-stranded RNA segments, which encode up to 17 proteins (1,39). The three subunits of influenza viral RNA polymerase (PB2, PB1 and PA) are encoded by segments 1 to 3. Recent studies showed that segment 2 and segment 3 are capable of encoding extra proteins via ribosomal frameshifting or leaky ribosomal scanning. Segment 2 encodes three proteins, PB1, PB1-F2 and PB1-N40. PB1-F2 is transcribed by segment 2 in a +1 reading frame, while PB1-N40 is a N-terminally deleted form of PB1. Both of them are as a result of leaky ribosomal scanning. Some strains encode a functional PB1-F2 protein with 87 or 90aa in length and some strains encode a truncated version of PB1-F2 (39,40). Segment 3 encode encodes two proteins, PA and PA-X (41,42). PA-X is transcribed from a +1 ribosomal frameshifting and studies suggested that PA-X is

universally expressed across different strains (41). The surface glycoprotein hemagglutinin (HA) by segment 4, nucleoprotein by segment 5, and another surface glycoprotein neuraminidase (NA) by segment 6. The segments 7 and 8 each could encode two distinct proteins through messenger RNA (mRNAs) splicing. Segment 7 encodes an internal matrix protein (M1) and a third surface protein (M2) while segment 8 encodes nonstructural protein 1 (NS1) and nonstructural protein 2 (NS2). NS1 is not packaged into virions but it is produced in a large quantities during infection (43). NS2 exists in small amounts inside of the virion (44). The specific functions of each protein are listed in Table **1.1**.

The influenza A virion is spherical or filamentous shaped with an average diameter of 100 nm (45). A schematic of the influenza A virus structure is shown in Figure **1.1**. The two major antigenic glycoproteins HA and NA are embedded on the surface of the virus lipid envelope in a ratio of about 4 to 1 (1). The HA protein binds to the sialic acid on the host cell surface to facilitate viral attachment. The NA protein destroys sialic acid binding to prevent aggregation of progeny virions. It is the target of antiviral drug Oseltamivir and Zanamivir, which inhibits neuraminidase activity and cause aggregation of viral particles (46), thus inhibit influenza reproduction. The M2 protein is a transmembrane protein and functions as an ion channel. It is activated by low PH and facilitates the uncoating of virions (47). M2 is the target of antiviral drugs Amantadine and Rimantidine. Due to the relative frequent emergence of resistant-strains to these two drugs by mutations, CDC suggested discontinuing use of them (48). Underneath the envelop lies a layer of M1 proteins (49).within the virion, RNA segments are encapsidated by nucleoprotein and exist in the form of ribonucleoprotein

Table 1.1 Influenza A virus gene and protein

RNA segment*	Length**	Protein encoded	Protein size	Protein function
1	2341	PB2	759	Recognizes and binds to 5'capped of host pre-mRNAs; subunit of viral RNA polymerase
2	2341	PB1	757	RNA-dependent RNA polymerase activity; subunit of viral RNA polymerase
		PB1-N40	718	Maintenance of PB1-F2 and PB1's expression level
		PB1-F2	Truncated or 87-90	Exists in certain strains of influenza A virus and enhance cell apoptosis through mitochondria regulation
3	2233	PA	716	Endonucleolytic cleaves pre-mRNA and generate primers for viral transcription; subunit of viral RNA polymerase
		PA-X	252	Virulence factor and host response
4	1778	HA	566	Viral attachment to sialic acid on the surface of host cells; major antigenic determinant; membrane fusion; proteolytic cleavage for activation; hemagglutinin.
5	1565	NP	498	Binds to viral RNA to form viral ribonucleoprotein (vRNP); Transports vRNA from cytoplasm to nuclear; involved in viral RNA synthesis.
6	1413	NA	454	Major antigenic determinant; neuraminidase activity; cleaves silalic acid to

				destruct receptor binding; facilitate release of newly synthesized virions
7	1027	M1	252	Major component protein underneath lipid envelope; Facilitates viral replication; Facilitates packaging/budding of newly synthesized virions; Export of progeny vRNP into cytoplasm; most abundant proteins
		M2	97	Transmembrane protein; ion channel activity important for virus uncoating and virion formation
8	890	NS1	230	Inhibit host mRNA processing; interferon antagonism; binds to double-stranded RNA (dsRNA); interact with proteins involved in cell signaling and reduce cell apoptosis
		NS2	121	Facilitates nuclear export of vRNP; interacts with M1

* The table is adapted from Briedis (2011), Swayne and Halvorson (1997) and Dias et al. (2009).

** Lengths are based on the A/PR/8/34 strain.

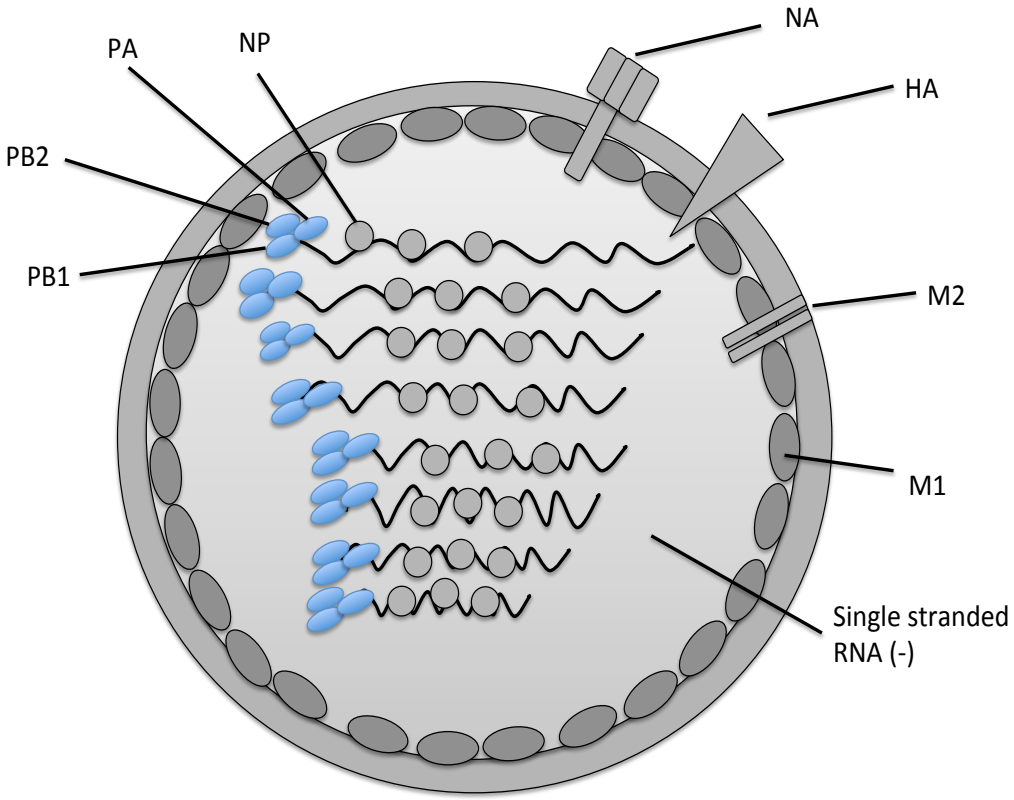


Figure 1.1 **Schematic diagram of the influenza A virus structure.** Picture adapted from Horimoto and Kawaoka, 2005 (51). Influenza A virus has a spherical shape with a lipid envelop. On the surface are embedded two major antigens HA and NA. An M2 ion channel is incorporated within the surface membrane. Underneath lies a layer of M1 protein. Inside the virion, eight RNA segments are wrapped with nucleoproteins and a single copy of RNA polymerase.

(RNP). Each vRNP is attached to a viral RNA polymerase, composed of three subunits PA, PB2 and PB1, needed for viral replication and transcription (45). The RNA polymerase has RNA-dependent RNA polymerase activity. The 5' and 3' termini of each segment are complementary to each other and can form panhandle or "corkscrew" structures (52,53). This replication model will be discussed in more details in Chapter 1.2.2.

1.2.2 Influenza A virus life cycle

Influenza A virus attaches to host cells through sialic acid binding by hemagglutinin (HA). Variations in the receptor-binding affinity of influenza A virus is determined by the different linkage type of terminal sialic acid to the galactose. In general, avian and equine influenza virus contains HA that preferentially recognize sialic acid having α- (2, 3) linkage while human influenza virus contains HA that prefers an α- (2, 6) linkage (54,55). The influenza virus replication cycle is demonstrated in Figure **1.2**. Binding of silaic acid to HA protein initiates receptor-mediated endocytosis. This features a pH drop, which triggers the conformational change of HA. This allows HA to be inserted into the endosomal membrane and induces the fusion of the viral and cellular membranes (56). The M2 ion channel protein on the surface of virions then conducts protons and acidifies the interior of the virions, which triggers the uncoating of virions and the release of the vRNPs into the cytoplasm (57,58). vRNPs are then transported into the cell nucleus for replication and transcription (59).

The viral RNPs serve as templates for the synthesis of both mRNAs and complementary RNA (cRNA) molecules under the control of influenza RNA-dependent RNA polymerase (RdRp) (1,60). The 13 nucleotides at 5'-end and 12

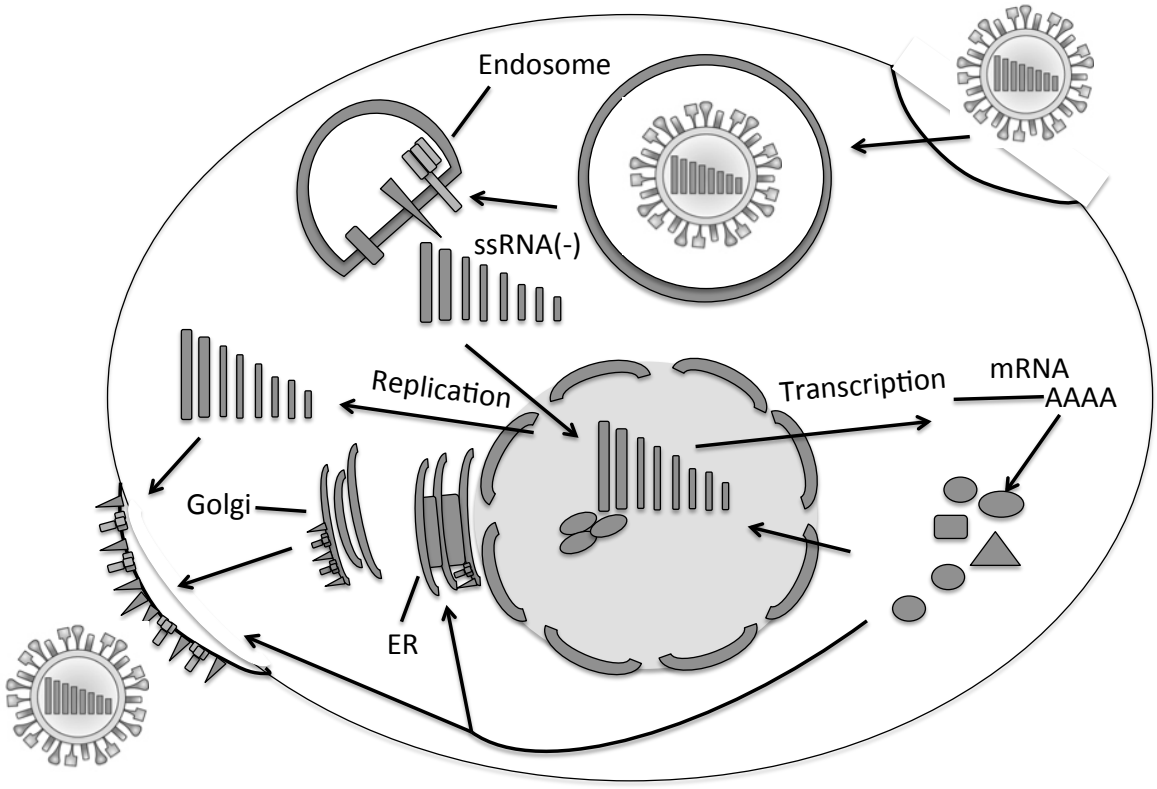


Figure 1.2 **Influenza A virus replication cycle**. This picture was adapted from Neumann et al. 2009 (61). Steps of influenza A virus life cycle include attachment, virus entry, viral replication and transcription, protein translation, packaging, assembly, budding and release.

nucleotides at 3'-end of non-coding regions of vRNA are highly conserved among the different segments and across different IAV strains. Recent studies suggested that these conserved nucleotides, along with several segment-specific nucleotides at 5' and 3'ends, can form a "corkscrew" structure (62) recognized by RdRp to initiate the primer-dependent mRNA transcription or *de novo* cRNA synthesis. Luytjes et al. (1989) have demonstrated that 22 nucleotides from 5'end and 26 nucleotides from 3'end of vRNA were sufficient to support RNA transcription and replications. The complementary cRNA can also form similar structures and interact with RdRp in *trans* (63) for the production of progeny vRNAs. Virion RNA and its complementary strand cRNA form similar structures yet with unique features, which enables the differential recognition of the viral polymerase complex. Viral replication is error-prone. Lacking proofreading properties, the RdRp has a mutation rate in the range of 10^{-3}~10^{-5} substitutions per nucleotide (64). Mutations of HA and/or NA genes can cause amino acid substitutions of HA/NA epitopes, which is called "antigenic drift" (1). The antigenic variants of mammalian influenza viruses are usually selected by immune pressure (1). For avian influenza viruses, the antigenic diversity of HPAI is more frequently observed among vaccinated poultry (65). On the other hand, transcription of messenger RNAs is initiated by a "cap-snatching" mechanism. Recent studies have found that the PA subunit has the endonuclease activity. It cleaves near the 5' cap of host pre-mRNAs and generates 10-13 nucleotides to prime virus mRNA transcription (66). The mRNA produced are polyadenylated by transcribing the poly-(A) signal existing at the 5'end of the vRNA (67).

Recently, via manipulating the influenza vRNA promoter to express reporter proteins such as Enhanced Green Fluorescent Protein (EGFP) and luciferase (Luc),

researchers successfully developed constructs and cell lines for the detection of influenza virus replication/transcription and the expression of foreign proteins (68–71). The inducible expression of reporter proteins only in presence of viral polymerase makes these constructs and cell lines suitable for the detection of viral infectious titer in a relatively short period of time and they are used for conducting large screening of antiviral reagents (72,73).

Once polyadenylated, viral mRNAs are exported into the cytoplasm for translation. In the cytoplasm, mRNAs are recognized by ribosomes for translation (Figure **1.2**). Three RNA polymerase subunits and NP proteins are transported back into nucleus after production to facilitate transcription and replication (74). Three surface proteins HA, NA and M2 are translocated into the endoplasmic reticulum for post-translational modifications such as glycosylation and oligomerization, after they are synthesized by membrane-bound ribosome. They are then transported to Golgi apparatus (75) followed by trafficking to cell membranes for subsequent viral assembly. Export of vRNP is mediated via the assistance of NS2 and M1 proteins (76). M1 is found to be associated with vRNA and NP, which could further stabilize the vRNP structure. It has also been found to interact with NS2 protein and together they facilitate the export the vRNP through nucleoporins (77). The assembly of newly synthesized virions is facilitated by M1 protein which directs the vRNP to be associated with the membrane-bound protein HA, NA and M2 (1). The packaging of segmented vRNPs was suggested as a selective procedure (78,79). Recent studies suggest that packaging signals that exist at the 5’ and 3’ end of the segmented RNA are necessary for incorporation. Moreover, there is one more bundling signal which exists in the encoding regions and which ensures the full sets of genomes are packaged

(80). Budding of newly synthesized virions is also facilitated by M1 (49). The HA protein still binds to sialic acid after budding, so the release of virus particles is through the destruction of the interaction by NA protein cleavage. The processing of HA is very important to the virulence of the influenza virus. An HA protein that contains a multibasic cleavage site can be processed by intracellular protease such as furin or furin-like protease into the active forms HA1 and HA2, which are bound by disulfide bonds (81), while the one that does not contain the cleavage site still remains the inactive precursor form HA0, until recognized by extracellular protease such as trypsin-like protease for activation. The HA0 form does not respond to the lowering PH and undergo conformational change during the receptor-mediated endocytosis without cleavage (81,82). This would lead to unsuccessful viral entry and the influenza virus is thus non-infectious.

1.3 Background of RNAi

RNA interference (RNAi), first identified in nematode worms *Caenorhabditis elegans* (83), is a highly conserved pathway found in many eukaryotic cells that could inhibit gene expression in a sequence-specific manner. The phenomenon was also observed in plants (84) and fungi (85,86) before, but this mechanism of RNAi was only revealed until 1998 by Fire et al. (83). Afterwards, it was found to be also conserved in other species including insects (87), protozoa (88) and mammalian cells (89), which suggested the ancient origin of it from a same ancestor. In higher plants, RNA interference functions as a naturally occurring antiviral response, which could be transmitted systemically to combat virus infection (90). Similarly, Li et al. observed that in *Drosophila* cells, the RNA virus infection could induce strong RNA silencing, suggesting that RNAi serves as an adaptive antiviral defense (91). In mammalian cells,

the sequence-specific RNAi pathway was originally thought to be unlikely present because the long dsRNAs (>30bp) are able to induce sequence-independent gene silencing through activation of protein kinase (PKR) (92) and 2'-5' oligoadenylate synthetase (2'- 5' OAS) . The former could result in phosphorylation of translation factor eIF2α and subsequent translational inhibition (93) while the latter could promote the dimerization of RNase L and result in global degradation of mRNAs (94). Elbashir et al. (2001) found that the 21-nucleotide siRNA with a 2-nt 3' overhang could evade these cellular sensors and be capable of inducing efficient sequence-specific gene silencing and demonstrated the existence of the RNA interference pathway in mammals. It was long argued whether RNA interference should also be considered as a defense system for virus infection in mammalian cells (95) since some animal viruses carry viral proteins that could suppress RNAi in insects and plants (96–99). Until recently, two studies on encephalomyocarditis virus and Nodamura virus infection of mammals revealed that both *in vitro* and *in vivo* , RNAi played an important role in the innate antiviral function in mammals (100,101).

RNAi could induce gene silencing through two different pathways (as demonstrated in Figure **1.3**) post-transcriptional gene silencing, which include mRNA degradation and translational inhibition, and transcriptional gene silencing. The post-transcriptional silencing induced by dsRNAs/siRNAs, which usually have sequences fully complementary to their target mRNAs, is the most common pathway for gene silencing. The model has been established well in *Drosophila*, which includes two steps, an initiation step and an effector step (102). In the initiation step, the dsRNA is recognized by Dicer, an ATP-dependent enzyme that belongs to the RNase III ribonuclease family (103–105) and cleaved into ~22-nt small interfering RNAs

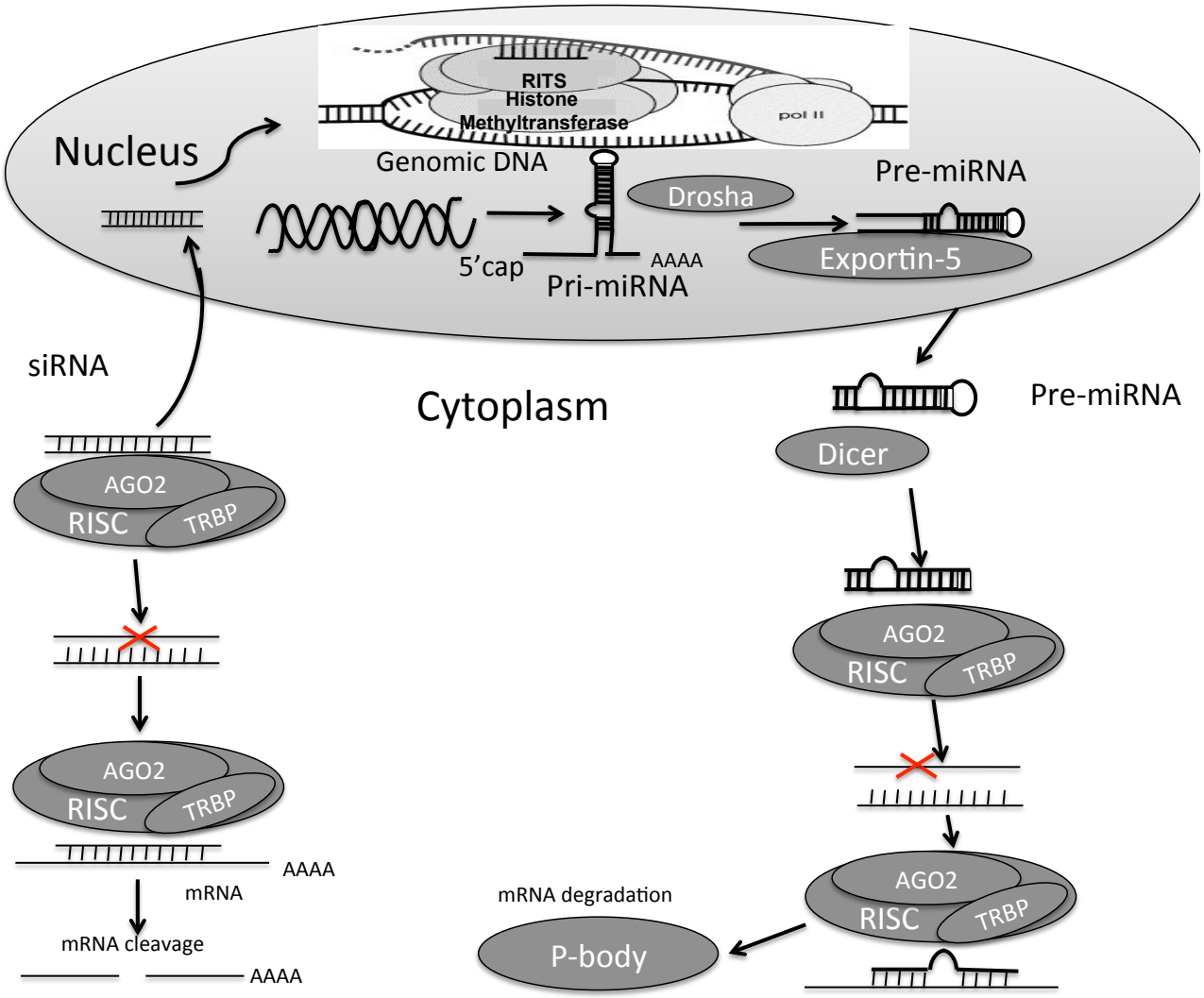


Figure 1.3 **Schematic graph of RNAi mechanism in eukaryotic cells.** Adapted from Antonin et al. 2007 and Bayne and Allshire 2005 (106,107). The graph demonstrates three major pathways for RNAi induced gene silencing that happen at the transcriptional and post-transcriptional level. The transcriptional level RNAi happens inside of the nucleus and the post-transcriptional level RNAi happens inside of cytoplasm including exogenous siRNA pathway and endogenous microRNA pathway

(siRNA). After processing, the siRNAs are ~22 nucleotide with 5'-bi-phosphate and 2-nt 3' overhangs (108). During the effector step, the siRNAs is recognized by a protein complex, RNA-induced silencing complex (RISC) and is unwound (109). One of the strands from the siRNA is incorporated into RISC while the other strand, called the passenger strand or sense strand is cleaved and degraded (110). This selection procedure is not random. Studies in *Drosophila* showed that 5' terminal thermodynamic difference could be sensed by Dicer R2D2 (Dcr-2 associated protein), and this determines which strand to be loaded (111). The strand with lower 5' termini thermostability is more likely than the other strand to be functional for mRNA targeting. The selected strand, called guide strand/antisense strand, associated with RISC and finds the mRNA with sequence complementarity, which is then cleaved by the Argonaute 2 protein (Ago2) in the middle of the complementary sequence (112). The cleaved mRNA will be destroyed by the cellular system and the corresponding gene will not be expressed.

The post-transcriptional silencing induced by endogenous microRNA (miRNA), due to its imperfect homology to its mRNA target, usually results in translational inhibition. The biogenesis of miRNA happens in the nucleus as shown in Figure **1.3**. It is first transcribed as a long primary transcript, called primary miRNA (pri-miRNA). It is first processed by Drosha, a class II RNase-III-like enzyme, inside of the nucleus, into ~70nt long hairpin-shaped precursor miRNA (pre-miRNA) (113,114). The pre-miRNAs are exported out of the nucleus by a nuclear export receptor protein, exportin-5 (Figure **1.3**) (115). In the cytoplasm, the pre-miRNA is recognized by another RNAse III enzyme, Dicer, and further processed into bulged dsRNA with 5' phosphate and 2-nt 3' overhangs (104,116). The processed miRNA

then unwinds and one strand, the mature miRNA, is incorporated into RISC. Studies showed that strand selection of miRNA also likely follows the asymmetry rule. The strand with high free energy/low thermodynamic stability at 5' termini will be incorporated into RISC (110). However, the passenger strand of miRNA, miRNA*, is unlikely to be destroyed for elimination during the selection procedure. Several studies showed that some miRNA/miRNA* ratio have different patterns in a tissue/developmental-specific manner(117,118), suggesting that miRNA* might also play a role in gene regulation. The RISC associated mature miRNA (miRNP) then finds its target usually located on the 5' or 3' untranslated region (UTR) of the mRNA (119). Partial complementarity results in repression of translation. It is suggested that miRNP directs the target transcript to P-bodies, where cap-dependent RNA degradation happens (120,121).

Several studies suggest that RNAi can also induce transcriptional gene silencing (TGS) in the nucleus. It has been shown in plants, fission yeast and in humans that dsRNAs/siRNAs targeting the promoter regions of the genome can induce DNA methylation or formation of heterochromatin, and thus shut down the expression of downstream genes (122–126). Basically, the dsRNAs are processed by Dicer/R2D2 into ~22-nt siRNAs, which are then incorporated into an RNAi-induced Transcriptional Silencing complex (RITS), which contains protein subunits that catalyze histone H3 lysine 9 methylation (126). This leads to the establishment of heterochromatin in eukaryotic cells and thus gene silencing at the transcriptional level.

1.4 Applications of RNAi

1.4.1 Triggers of RNAi

The discovery of RNAi-induced gene silencing made it a powerful approach to knock down gene expression. It has been explored in different fields for application especially in genome function screening (127) and RNAi-based therapeutics against human diseases, like cancers (128), eye diseases (129), and also viral infectious disease (130–132).

For application in mammalian cells, there are two popular types of triggers currently used to conduct RNAi research: i) Chemically synthesized short interfering RNAs (siRNAs) 19 bp dsRNAs with 2nt overhangs at each end or recent endoribonuclease-prepared siRNAs (esiRNAs) (133,134) ii) RNA polymerase III-driven expression of short hairpin RNAs (shRNAs) or artificial microRNAs, which are designed to act as miRNA precursors (pre-miRNA).

The canonical siRNA is a 21-nt long dsRNA with 2-nt overhang at each terminus. Elbashir et al. (2001) showed that the synthesized 21-nt siRNA duplexes with 2-nt symmetric overhang at 3'end sufficiently reduced expression of two reporter genes, *Renilla* luciferase and firefly luciferase on a plasmid in *Drosophila* S2 and mammalian cells. They also found that the silencing effect is still relative potent at a concentration as low as 1.5nM. Besides, unlike the dsRNA (>30nt), siRNA could evade the recognition of PKR, 2', 5'-OAS and avoid the interferon induction (89). Recently, the endoribonuclease-prepared siRNAs (esiRNAs) based on the RNase III processing of dsRNAs, become an alternative approach for generations of siRNA pools (133–135). In the light of cellular processing of dsRNAs by RNase-III enzyme Dicer, this approach was first developed by Yang and his colleagues (134). They

found that limited digestion of long dsRNAs by the *Escherichia coli* RNase III, could generate 20- to 25-nt siRNAs pool, named esiRNAs. Their study showed that these esiRNAs were not only capable of inducing sequence-specific silencing with no non-specific interferon (IFN) response in mammalian cells, but also were more potent compared to chemically synthesized siRNAs and shRNAs. Then Myers et al. (135) also successfully showed that siRNA pools with 20- to 21-bp could be prepared by a recombinant human Dicer (r-Dicer) from dsRNAs with a yield of ~70%. These methods made possible the development of siRNA libraries for given targets in a fast and easy fashion.

Vectors expressing short-hairpin RNAs (shRNA) are another popular trigger of RNAi. The design of shRNA is based on naturally occurred precursor miRNAs (136), with a hairpin-like structure, an antisense and sense strand (stem) connected by a single strand loop sequence. It is usually transcribed from a plasmid under the control of RNase III promoter, which gives transcripts well-defined initiation and termination sequences. Human origin H1 and mouse origin U6 promoters are the most widely used promoters (137). After transcription, the shRNAs can be exported out of nucleus by exportin-5 (138) and processed by Dicer into siRNAs in the cytoplasm for subsequent gene silencing. Unlike the transient silencing effect provided by siRNA, vector-expressed shRNAs could have a more prolonged gene-silencing effect. Plasmids with selective genes have been used to develop stable transformed cell lines constitutively expressing shRNAs that have been shown to down-regulate target gene expression for several months (139). shRNAs have also been utilized for the development of transgenic animals to study the phenotypic effect of genes of interest. Through electroporation (140) or lentiviral delivery (141), an shRNA expression cassette can be

in introduced into animal embryonic stem cells to develop transgenic animals. This approach to develop transgenic animal usually takes much less time compared to the conventional knockout approaches (137).

Recently, as the structure and maturation procedures of endogenous microRNAs become well characterized, shRNAs designed based on the structure of naturally occurred pirmary microRNAs (pri-miRNAs) have been developed. These are called artificial microRNA or shRNA-mir (142). Zeng and her colleagues first showed that an artificially synthesized mir-30 precursor expressed by RNA polymerase II (Pol II) could block mRNA expression in human cells (143). Silva et al. (2005) successfully developed the second-generation shRNA-mir libraries based on the primary mir-30 backbone against human and mouse genes. The structure of shRNA-mir is usually a small interfering RNA in the sequence context of a precursor miRNA (shown in Figure **1.4**). Unlike shRNAs, shRNA-mir expression is usually driven by RNA polymerase II, which results in a transcript with a 5' cap and a 3' polyadenylation. The primary transcript of shRNA-mir can be recognized by Drosha in the nucleus and processed into pre-miRNAs. Once in the cytoplasm, pre-miRNAs are processed by Dicer into siRNA to complete subsequent gene silencing pathway described above. It is reported that compared to conventional shRNAs, shRNA-mirs appear to be ~12-fold more potent in gene silencing (142). Also, studies haven shown that artificial microRNAs can circumvent the interferon off-target effect that was strongly triggered by the shRNAs in the primary cell line (144). In addition, the Pol-II-driven expression of shRNA-mir makes it possible to apply genetic engineering for the expression of the aritificial microRNAs, such as expressing a reporter protein upstream of the miRNA sequence at the 3' UTR to monitor miRNA processing (145)

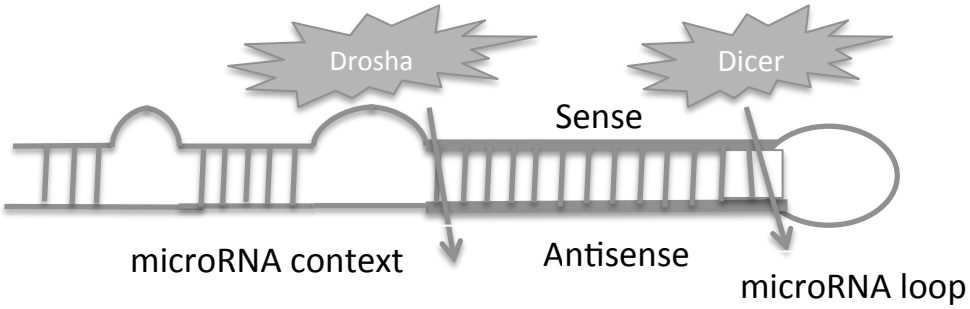


Figure 1.4 Schematic diagram showing the structure of artificial microRNA (shRNA-mir). The shRNA-mir, also called artificial microRNA, is a structure of shRNA in a microRNA sequence context. The artificial microRNA is first processed by Drosha in the nucleus at the 5' and 3' termini. In the cytoplasm, the processed shRNAs-mir is processed by Dicer into siRNAs. The graph marked the approximate position where Drosha and Dicer cut.

or to make a poly-cistronic expression of multiple artificial microRNAs in a single transcript (146).

1.4.2 Design of siRNAs

It was originally assumed that siRNA could target mRNAs with any sequence context for gene silencing (147). As siRNAs were employed as a common tool, it was found that different siRNAs targeting even the same mRNAs conferred drastically varied activities (148). Then researchers found that during RISC incorporation, the strand selection of siRNAs for subsequent mRNA targeting is not random but biased (110,149). These studies showed that the strand with a higher free energy, or the lower thermodynamic stability (A/T rich) at the 5' termini, is preferred to be loaded into RISC. The molecular mechanism for strands selection bias was further investigated in *Drosophila*. It was found that Dicer-2/R2D2 could interact with duplex siRNA and assist its loading into RISC. R2D2 acts as the protein sensor for the thermodynamic asymmetry of siRNAs (111). Based on the asymmetric selection of the siRNA strand, Reynolds et al. systemically examined 180 siRNAs targeting two genes and summarized eight criterias for rational design (Figure **1.5**): low G/C content, low thermodynamic stability at the 5' end of the antisense strand (A/U rich), high thermodynamic stability at the 3' end of the sense strand (G/C rich), base preference at position 3, 10, 13 and 19, absence of inverted repeats.

Recently, studies showed that thermodynamic stability alone is not enough to determine the efficiency of siRNAs (Reviewed in Kurreck, 2009). Overhoff et al. found that the accessibility for an siRNA to hybridize to a mRNA might be another

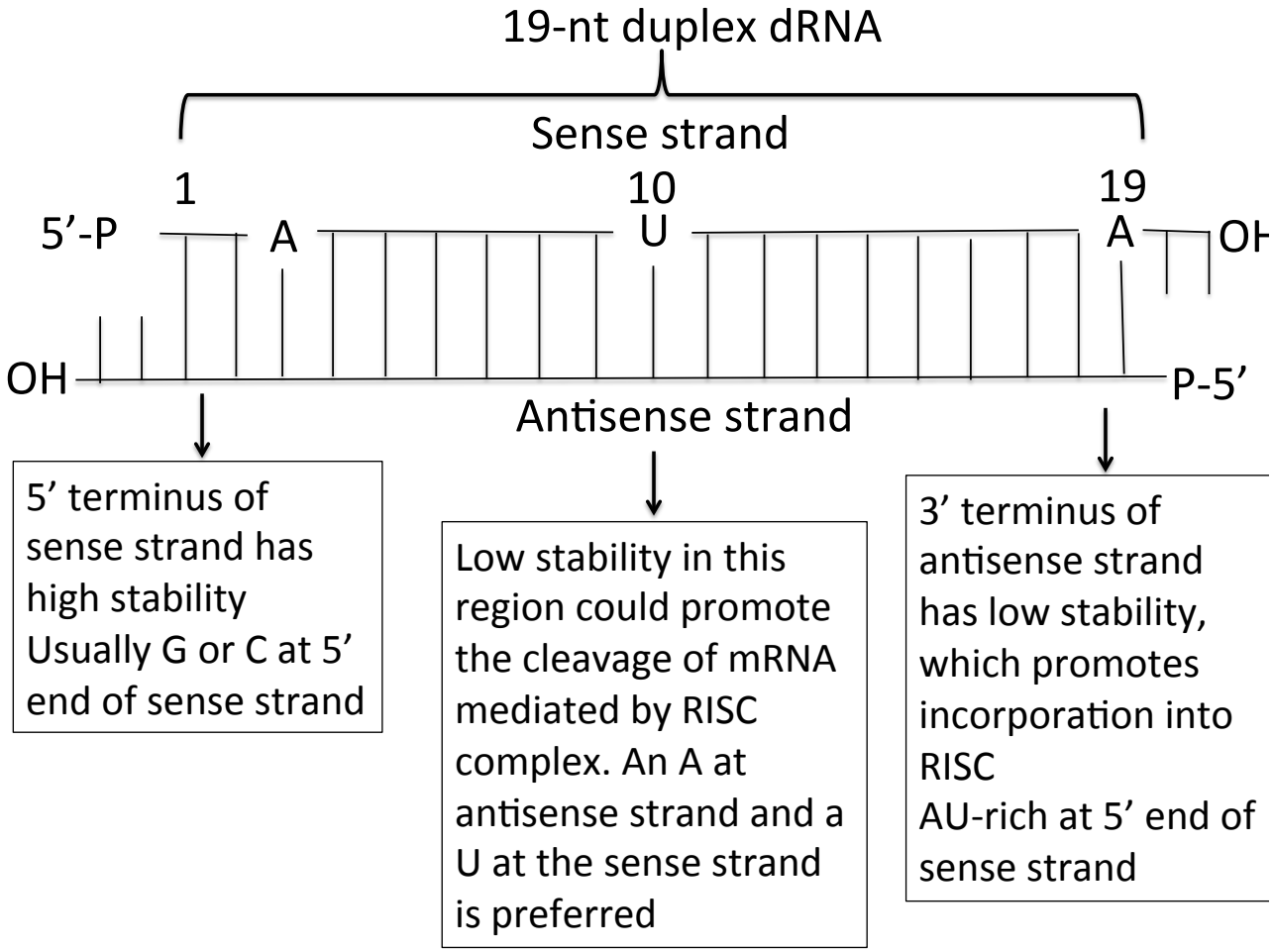


Figure 1.5 Schematic graph showing the criteria for the small interfering RNAs design. This picture was adapted from Mittal, 2004 (150)**.** siRNA structure is a 19-nt duplex with two 3' overhangs. The top strand is the sense strand and the bottom strand is the antisense strand. The positions of the nucleotide. According to Reynolds et al. (148), the 5' termini of antisense strand have a lower stability compared to the 5' termini of the sense strand as well as the cleavage site in the middle. Also, the base preference for sense strand at position 3, 10, 19 was also marked in the graph.

important factor for efficient silencing (151–154). Accessibility to the target mRNA is determined by both the secondary structure of target region on the mRNA as well as the secondary structure of the antisense strand. Overhoff et al. systemically analyzed a large set of siRNAs against two genes in cell culture (154). Accessibility was determined for the target region of each siRNA. They found that the half maximal inhibitory concentration (IC_{50}) of the accessible siRNAs was 10-fold lower compared to the inaccessible ones, suggesting that the accessibility of local target structure might play an important role in silencing efficiency (154). Then two other studies systemically examining large set of siRNAs/shRNA efficiency along with their local target accessibility as well as their thermodynamic asymmetry indicated that the thermodynamic asymmetry is the first step to determine the efficacy of siRNAs while the local mRNA target accessibility could further affect the silencing efficiency (155,156). Some siRNA designing softwares such as Sfold (157), have incorporated both factors above into the algorithm to achieve better designs.

1.5 Antiviral RNAi

RNA interference is an ancient conserved defense system against viral infection in plants, insects and has recently been found in mammals (90,91,100,101). As a powerful tool for gene knockdown, it has also emerged as a promising approach for antiviral therapeutics. Bitko and Barik (2001) first applied RNA interference as an antiviral approach in cell culture against respiratory syncytial virus (RSV), a virus that can cause respiratory or lung infection in humans. They showed that a 21-nt dsRNA that targets the phosphoprotein mRNA could inhibit RSV growth in cell culture, reflected by the complete loss of syncytia in infected cells. Further studies on the RNAi effect indicated that the dsRNA inhibited viral growth via the induction of gene-

specific mRNA degradation, with no interferon induction (158). Also, viral genomic and antigenomic RNA remained intact due to encapsulation (158). Later, they showed that siRNA could effectively protect mice from RSV and parainfluenza virus infection by intranasal administration,but without activating the innate immune response (132). Zhang et al. also showed that another siRNA targeting the NS1 protein could provide protection against RSV in a mouse model through intranasal delivery by nanoparticles (159). Currently, an anti-RSV siRNA (ALN-RSV01) is being developed by Alnylam (www.alnylam.com), a major RNAi pharmaceutical company has finished Phase II clinical trials (160). For ease of delivery and effortless administration, the RNAi approach have also been employed to combat against other acute respiratory viruses including severe acute respiratory syndrome coronavirus (SARS-CoV) (161,162), influenza virus (163–166), and metapneumovirus (167,168) in cell culture, mouse and the rhesus macaque models. The approach also holds promise for antiviral therapeutics against some chronic infectious disease including human immunodeficiency virus-1 (HIV-1) (169–171), hepatitis B virus (HBV) (172–174) and hepatitis C virus (HCV) (175–178). Different strategies have been utilized against these viruses, including siRNAs, vector-expressed shRNAs or artificial miRNAs.

The major target for RNAi on virus is the mRNA transcript that encodes important viral proteins during viral life cycle. Other targets include important viral genome RNA motifs as well as host co-factors that assist viral replication. Theses targets varied depending on different virus type. The siRNAs against some paramyxovirus family viruses and influenza viruses have been designed to target the highly conserved protein-coding regions of the RNA polymerase components (132,158,179) that are important to viral replication/transcription, showed efficient

inhibition of viral growth. Further study indicated that the RNA interference could induce the gene-specific mRNA degradation (158,179). To treat SARS-CoV, siRNAs were designed to target the spike protein, which is important to viral entry (161,162). Designs showed efficacy and specifity in protection against SARS-CoV. For HIV-1, people have developed effective RNA interference molecules targeting the coding region of some regulatory proteins, such as Rev/Tat (180), *gag* (181) and *nef* (182), the 5'-untranslated leader sequence (ldr) (170,182), and also the promoter region, which could induce TGS (183). HBV virus is a DNA virus, whose its pregenomic RNA and mRNA transcripts overlap with each other. RNAi molecules can have more than one target (173,174,184). For HCV virus, siRNAs/shRNAs that target the core protein, polymerase protein and non-structural protein genes all exhibited an inhibitory effect on viral growth (175,176). Several experiments using shRNAs targeting single host genes (176) or screened sets of siRNA/shRNA targeting human genes from RNAi libraries (177,178,185) also showed significant reduction of HCV replication in both cell culture and on animal models. These host genes were usually important co-factors for viral replication (176,177).

Compared to traditional vaccine and antiviral drugs, there are several advantages of applying the RNA interference in treating viral infectious diseases. First, according to Bitko et al. (2005), to treat respiratory diseases, delivery of RNAi molecules through intranasal pathway is easy to administrator. Secondly, the preparation of RNAi molecules is straightforward while vaccine preparation usually takes time, not to mention that some viruses do not grow well in cell culture (186) and the vaccine is hard to purify (186). Thirdly, the side effects of siRNAs can also be minimal, especially when applied through intranasal delivery. Transient effects should be

sufficient to inhibit viral replication and reduce viral load (171). Finally, studies on treating HIV-1 infectious disease suggested that treatment by combinations of RNAi molecules targeting different regions on virus might delay or prevent viral escape (169,187).

1.6 Challenges of Antiviral RNAi

RNAi indeed showed promise in combating viral infectious disease with several RNAi drugs undergoing development. However, there still exist challenges in the utilization of RNAi as potential anti-viral measures. Practical problems such as viral escape, potent siRNA, off-target effect and stability of siRNAs all need corresponding solutions in order to develop an effective and efficient antiviral RNAi treatment.

Targeted viruses usually replicate rapidly and accumulate mutations easily, especially RNA viruses, which often existed as viral quasispecies (64) due to the error-prone properties of RdRp. On the other hand, RNA interference requires full sequence complementarity or at least seed region complementarity (188) between the antisense strand and the mRNA, so a single mismatch might result in the viral escape (189). Current strategies to solve this problem include designing targets to conserved regions of the virus genome, developing combinatorial RNAi libraries against multiple sites or a combination of the two. Since conserved regions usually have important functions during viral life cycle, mutations on those regions are usually deleterious to virus fitness, making them robust candidates for RNAi targets. Nevertheless, those conserved regions are not usually ideal for siRNA designs. As discussed in Chapter 0, siRNA designs need to follow the thermodynamic asymmetry rule and have well target accessibility. With sequence limitations on those conserved regions, the siRNA

designs might not achieve optimal potency. Expression of multiple copies of RNAi molecules targeting different sites can be another alternative. Several experiments have demonstrated that combinations of siRNAs/shRNAs targeting different sites could delay or prevent viral escape (169,190). A combination of the two methods above might have synergistic effect and enhance the overall inhibition effect. For example, Chen et al. has shown that the tandem expressed artificial microRNA targeting PA, PB1 and NP was significantly more potent in inhibition of influenza virus compared to microRNA targeting NP alone (146). Targeting host cofactors important in virus life cycle is another way to avoid viral escape. Several experiments have been explored in this direction. Some host proteins involved with viral entry (191,192), intracellular transport (193), virus replication (194,195) and transcription (196) all showed potentials as efficient targets for inhibition of virus growth. Currently, several large experiments that use RNAi libraries to screen host factors important to influenza virus growth in cell culture were conducted (197–199), making this approach as potential treatment also for influenza virus. Despite the advantages it might have, long-term target of host genes important in normal cell and tissue functions could have deleterious and toxic effects. As a result, host cofactor targets need to be carefully examined to avoid that.

Delivery is another issue for efficient RNAi targeting. To treat the acute infectious diseases, a transient delivery mediated by transfection regents, such as polymers or surfactant, is sufficient to reduce the viral load and avoid symptoms. This method has been shown effectively in inhibiting RSV, influenza virus and SARS-CoV in animal models (132,162,164). To achieve durable inhibitory effect on viruses causing chronic infectious disease, there's more focus on gene therapy approach by

developing transduced cell line expressing shRNAs /miRNAs. It was proposed that engrafting patients with their isolated CD4 + hematopoietic stem cells (HSCs) transduced with anti-HIV-1 shRNAs could possibly extended their life span (200) .These shRNAs/miRNAs were usually delivered by viral vectors and tissue-specific.

Off-target effect is a big concern for choosing proper dosage of RNAi (189). It has been shown that high dosage of synthetic siRNAs could induce severe innate immune response including interferon response and production of inflammatory cytokines (201). Besides, off-target on host genes caused by partial sequence complementarity inducing unintended microRNA-like targeting or sense-strand targeting (202,203) could result in non-specific silencing and toxicity. Moreover, the exogenous delivered RNAi molecules share the same pathway of processing with the endogenous microRNA in mammalian cells, so a high dosage could result in saturation of enzymes (204) and energies (109) in the RNAi pathway. One recent study has shown that the shRNAs designed against hepatitis delivered through adeno-associated virus (AAV) could induce liver toxicity by inhibiting cellular miRNA pathway (205). To overcome these problems, first is to develop highly potent siRNAs. The thermodynamic asymmetric design, as described in Chapter 0, is the basis for biased strand selection into RISC. siRNAs design with less stable 5' end of antisense could help to reduce the sense strand targeting and improve the potency of siRNAs. Second is to develop inducible RNAi expression cassettes. Strayer et al. has successfully developed a HCV-infection inducible vector. In this vector, the anti-HCV siRNA expression is under the control of the LTR promoter from HIV, which could respond to the HCV infection-induced NF-κB expression (206).

1.7 RNA Nucleotides-based Strategies for Inhibition of Influenza A Virus

Beside the RNAi approaches mentioned above, other molecular strategies are being developed as anti-influenza measures. Several strategies including utilization of 5'capped RNA oligonucleotides (207), RNA decoys (208), morpholino oligomers (209,210) and also ribozymes (211) have been investigated for inhibition of influenza virus. Some of them exhibit potent inhibition of influenza virus both in cell culture, chicken embryos (163) or mouse (164,209,211,212).

According to Luo et al. and Chung et al.'s studies (207,208), 5' capped RNA oligonucleotides and RNA decoys can interfere with the replication or transcription cycle of influenza virus. The 5' capped RNA oligonucleotides could mimic the cellular mRNAs, binding to the viral RNA polymerase but failing to prime the transcription due to its short length so that it could serve as a potent inhibitor for influenza virus transcription (213). Developed by Luo et al., a mini-RNA decoy, consisting of the 5' and 3' terminal sequences from influenza A virus, binds to and sequesters viral RNA polymerase, inhibiting both steps of viral replication and transcription (208). On the other hand, the morpholino oligomer, ribozymes and small interfering RNA molecules usually inhibit influenza virus by binding to viral mRNAs and inhibit translational or by targeting them for cleavage, although it was shown by Gabriel et al. and Ge et al. (209,214) that morpholino oligomers designed to be complementary to the 3' untranslated regions (UTR) of vRNAs could also efficiently inhibit viral replication, which means that morpholino oligomers also potentially interfer with influenza replication or transcription. The inhibition effect on influenza virus by morpholino oligomers and ribozymes, similar to RNA interference molecules, were both highly sequence-specific. Due to the high genetic instability of influenza virus, they were designed targeting conserved regions of the influenza virus genome to

prevent viral escape. According to Julie et al, the ribozyme was designed to target conserved regions present in five segments (except HA and NA segment) of influenza A virus strains (211), while the oligomers were designed to target conserved regions identified across H1N1, H5N1 and H7N7 influenza A virus strains in both coding and non-coding regions (209,210).

Among these methods, ribozyme RNAi and RNA decoy were also considered for potential development of an influenza-resistant transgenic chicken. In 2011, Lyall et al. tried to develop an influenza-resistant chicken by introducing RNA decoy constructs through a lentiviral vector (215). In their studies, although the transgenic chickens were still susceptible to initial infection of highly pathogenic avian influenza virus, the onward transmission frequency to both transgenic and non-transgenic chickens was significantly reduced. Development of more flu resistant chickens could be promising in the future for both the poultry industry and more important to protect public health.

1.8 Rationale and Objectives

Major prevention and control measures for avian influenza virus in poultry industry include application of robust monitoring systems, strict biosecurity and depopulation programs (216). Vaccines though proposed as possible solutions, only have limited applications for the concerns that they might induce antigenic change of circulated influenza virus and the emergence of resistant strains (4). Besides, no universal vaccines can prevent all different subtypes and even all variations of different strains within the same subtypes. So there is need for the development of new methods to prevent and control avian influenza virus in the poultry industry.

RNAi tools appear to be an ideal complement for their fast development, universal targeting, and possible development of influenza-resistant chickens when considering the limitations of current methods although they also have problems in large amount production and viral escape for target.

In this project, the overall objective is to develop escape-free and virus-inducible RNAi tools to inhibit influenza A virus replication. To accomplish this goal, several aims were achieved:

(I) Develop reporter constructs that can detect influenza A virus replication for fast screening of RNAi molecules (Chapter 3.1);

(II) Develop escape-free siRNA construct (Chapter 3.2)

(i) Identify the conserved regions of influenza A virus genome for the design of siRNAs

(ii) Enhance potency of siRNAs that target the conserved regions through increasing their asymmetry by structure, size and sequence modulations and reduce their off-target effect from the sense strand *in vitro*.

(iii) Evaluate siRNA-targeting efficiency on fusion transcripts and influenza virus replication using reporter plasmids as indicators.

(III) Develop amplifiable anti-influenza artificial microRNA constructs using influenza virus promoter (Chapter 3.3).

Chapter 2

MATERIALS AND METHODS

2.1 Cell Lines and Virus

2.1.1 Cell lines and their maintenance

Three cell lines used in this project were summarized in Table **2.1**. MDCK and HEK293 cells were maintained following ATCC's suggested protocol as seen in their website (217,218). Cells were maintained in complete medium DMEM (Dulbecco's Modified Eagle medium; Corning Inc.) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific), 100 IU/mL of penicillin, 100 μg/mL of streptomycin and 250ng/mL of amphotericin B (Corning Inc.) in 25 cm^2 or 75 cm^2 flasks (Corning Inc.). Cells were incubated at 37^oC and 5% CO_2 and split 1:4 regularly for maintenance. To split MDCK and HEK293 cells growth media was removed and the monolayer was rinsed with 1ml (25 cm^2) or 3ml (75 cm^2) PBS followed by the addition of 1 mL (25 cm^2) or 3 mL (75 cm^2) 0.25% Trypsin-EDTA (Corning Inc.) with an incubation time of 20 min (MDCK) or 5 min (HEK293) at 37^oC. Cell detachment was ended by adding 2 ml (25 cm^2) or 6 ml (75 cm^2) complete growth medium. Cells were centrifuged at 1000xg for 5min to remove Trypsin-EDTA and re-suspended in fresh complete medium before transferring into a new flask. If it was for cell stock, cells were resuspended into cryoprotective freezing medium, 95% fetal bovine serum supplemented with 5% DMSO, and then 0.1 mL aliquots with $1x10^7$ cells per vial were preserved in the liquid nitrogen.

Table 2.1 Cell lines used in the experiment

Name	Descriptions	Sources
MDCK	Madin-Darby Canine Kidney epithelial cell line	ATCC CCL-34
HEK293	Human Embryonic Kidney 293 cell line	ATCC CRL-1573
HD-11	Chicken macrophage cell line	Lab stock

HD-11 cells were grown in DMEM supplemented with 5% chicken serum, 5% fetal bovine serum and 100 IU/mL of penicillin, 100μg/mL of streptomycin and 250 ng/mL of amphotericin B in 25 cm^2 flask (Corning Inc.). HD-11 cells were incubated at 37^oC and 5% CO_2 and split 1:4 regularly for maintenance. To split HD-11 cells, growth medium were removed and the monolayer was washed with 1mL PBS, followed by the addition of 1 mL 0.025% Trypsin-EDTA. Trypsinization was complete after 2min and was ended by adding 3mL of complete medium before transfer into new flask. Cell stock was made similarly as described for MDCK and HEK293.

2.1.2 Virus stock preparation

Influenza A virus strain A/Puerto Rico/8/1934 (H1N1) was purchased from ATCC. The virus stock was diluted 1:1 into Dulbecco's Phosphate Buffered Saline (ATCC) supplemented with 100 IU/mL of penicillin and 100 μg/mL of streptomycin prior to the injection of 0.1 ml diluted virus into 11-day-old chicken embryos using the allantoic route of inoculation method. After 4 days, infected eggs were chilled overnight and allantoic fluid was collected from each egg. Allantoic fluid was tested by the hemagglutination assay for virus replication as described in section 2.1.3. Allantoic fluids from hemagglutination-positive eggs were pooled and 0.6ml aliquots were stored at -80^oC.

2.1.3 Virus titrations

Virus titer was determined by hemagglutination assay and by tissue culture. Hemagglutination assays were conducted as follows. Fifty μL of sample was added to the first well in each row of a U-bottom 96-well plate that contained 50 μL of

phosphate buffered saline (PBS) in each well. Two-fold serial dilutions of the sample were made by transferring 50 μL from the first well to the second well. This procedure was repeated across the entire row. Finally, 50 μL of a 0.5% chicken erythrocyte suspension in phosphate buffered saline (PBS) was added to each well and the plate was incubated for 20-30 min before checking for hemagglutination activity.

Infectious titers of virus stocks were determined by $TCID_{50}$. $TCID_{50}$ was carried out on MDCK cells in 96-well plates. MDCK cells were infected when they were about 70%~80% confluence. DMEM was removed from the monolayer, which was then washed with PBS. Virus stocks or collected supernatants were serially diluted 10-fold in serum-free DMEM complemented with 0.3% BSA (Life Technologies, Inc.), 10mM HEPES (Life Technologies, Inc.), 100 IU/mL penicillin and 100 μg/mL streptomycin and 100 μL of the virus dilutions were added into each well. Each diluted sample has 4 replicates of infection. Cells were incubated with virus for 1 hr with shaking every 20 min at 37^{o}C. The inoculum was removed and the wells were washed twice with PBS and replaced with virus infectious medium, the Eagle's Minimum Essential Medium (ATCC) supplemented with 2.5% Bovine Serum Albumin (7.5%) (Life Technologies), 2.5% 1M HEPES buffer (Life Technologies), 100 IU/mL of penicillin, 100 μg/mL of streptomycin, 250ng/mL of amphotericin B and along with TPCK-trypsin (Sigma, Inc.) at a final concentration of 2 μg/mL, for another 72-hours incubation. Each well was evaluated for hemagglutination activity by the addition of 0.5% chicken erythrocyte suspension. Reed and Muench method was used to determine $TCID_{50}$ based on the appearance of hemagglutination (219).

2.1.4 Cell transfection

Two transfection reagents were used for cell transfection. Lipofectamine 2000™ (Invitrogen Inc.) was used for plasmid transfection alone or cotransfection of siRNA and plasmid while X-tremeGENE (Hoffmann-La Roche Inc.) was used for siRNA transfections.

The standard transfection procedure of plasmid DNA/cotrasnfection plasmid DNA and siRNA were as followed. Lipofectamine™ 2000 transfection of plasmid DNA with HEK293 cells in a 96-well plate or 24-well plate was done following manufacture's protocol. Transfection efficiency was evaluated by co-transfecting pCAG-EGFP plasmid (Table **2.2**). Enhanced Green Fluorescence Protein was observed under fluorescence microscope every time after transfection following procedures described in Chapter 2.1.5. High transfection efficiency was achieved consistently. Lipofectamine™ 2000 (0.25μL) and 100 ng plasmid DNA were individually diluted in 25 μl serum-free DMEM, then mixed and allowed to react for 15 minutes. Reaction complex was plated on 96-well plate with 50 μL/well or 24-well plate with 100 μL/well. HEK293 cells (100 μL, $3.5x10^5$/mL for 96-well plate, or 500 μL, $7x10^5$/mL for 24-well plate) were then plated directly on the transfection reaction complex in a 96 well plate for incubation. Cotransfection of siRNA and plasmids were conducted similarly by mixing diluted Lipofectamine™ 2000 and diluted 100 ng of vLuc reporter plasmid along with 5 pmol/10 pmol (final concentration of 33 nM/66 nM) of siRNA into 25 μl serum-free for reaction as described above. Cells were incubated at 37^oC, 5%CO_2 for subsequent assay or RNA extraction.

Cell tansfection of siRNA with X-tremeGENE was optimized using Alexa Fluor 488 labeled siRNAs. The standard transfection procedure of siRNA transfection is as followed. Six to ten μL X-tremeGENE and 1 μL siRNA (10μM) were diluted into

15 μL serum-free DMEM separately, then combined and react for 20 minutes. The complex was added to 96-well plate and followed by adding $4x10^5$/mL HEK293 cells. Cells were incubated at 37°C, 5% CO_2 for subsequent assay or RNA extraction.

2.1.5 Fluorescence detection and luciferase assay

Cells transfected with reporter plasmids expressing Enhanced Green Fluorescence Protein (EGFP) were observed under a Nikon Eclipse TS100 fluorescence microscopy (Nikon Inc.). A SPOT Insight 2Mpixel Monochrome FireWire Digital Camera (SPOT Image Inc,) was used for fluorescence imaging. Fluorescence density images was analyzed by ImageJ (220)

To conduct luciferase assay, cells transfected with plasmid expressing firefly luciferase was removed of supernatant and were lysed by adding 100 μL the Glo Lysis buffer (Promega Inc.) for 5 minutes. Cell lysates were then analyzed using the Steady-Glo® Luciferase Assay System (Promega Inc.). Steady-Glo® reagent was prepared by mixing Steady-Glo® buffer with Steady-Glo® substrate. One Hundred μL of Steady-Glo reagent was the cells lysate and incubated for 5 minutes in the dark. Luminescence was measured in a Synergy TM 2 multi-mode microplate reader (Biotek, Winooski, VT) following the manufacturer's instruction.

2.2 Plasmid Cloning

Plasmid DNA used and constructed in this project was descried in Table **2.2**

Table 2.2 Plasmids used and constructed in this project

Name	Descriptions	Source
pHH21	Parental vector embedded with human polymerase I promoter and terminator for construct of virus replication reporter	Provided by Dr. Yoshihiro Kawaoka (University of Wisconsin-Madison)
pmirGLO	Parental vector expressing firefly luciferase protein for reporter luciferase assay;	Promega Inc.
pEGFP	Vector expressing EGFP protein for control of transfection efficiency	Addgene Plasmid 11150
pCAG-mir30	Vector that has human miR-30 5' and 3' stem sequence for construct of microRNA expression plasmid	Addgene Plasmid 14758
Luc-NP, Luc-PN	Plasmid expressing fusion transcript luciferase-NP: Segment NP from A/PR8/34 strain was cloned downstream of the luciferase gene.	Constructed based on pmirGLO
vGFP, vLuc	Based on pHH21, GFP or luciferase genes were cloned into pHH21 in anti-sense orientation and flanked by A/PR8/34 NP vRNA promoter sequence	Constructed based on pHH21
cGFP, cLuc	Based on pHH21, GFP or luciferase genes were cloned in sense orientation and flanked by A/PR8/34 NP cRNA promoter sequence	Constructed based on pHH21
pLB2-NP	plasmid expressing artificial microRNA targeting influenza A virus NP segment	Provided by Dr. JianZhu Chen (Massachusetts Institute of Technology, MA)

pLB2-PB1-NP-PA	plasmid expressing three artificial microRNAs targeting influenza A virus PB1, NP and PA segments	Provided by Dr. JianZhu Chen
pHH21-NPc	plasmid expressing artificial microRNA targeting influenza A virus NP segment	Constructed based on pHH21
pHH21-NPi(+)/pHH21-Npi(-)	plasmid expressing artificial microRNA targeting influenza A virus NP segment in sense(+)/antisense(-) orientation, flanked by vRNA promoter from A/PR8/34 NP segment	Constructed based on pHH21
pHH21-3c	plasmid expressing three artificial microRNAs targeting influenza A virus PB1, NP and PA segments	Constructed based on pHH21
pHH21-3i(+)/pHH21-3i(-)	plasmid expressing three artificial microRNAs targeting influenza A virus PB1, NP and PA segments in sense(+)/antisense(-) orientation, flanked by vRNA promoter from A/PR8/34 NP segment	Constructed based on pHH21

PCR primers for plasmid construct were all designed by Primer3 (221). Cloning PCR was conducted using Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific Inc.) following manufacturer's instructions. The reaction components include 25 μL 2x Phusion Master Mix, 1 μL (10 nmol/μL) forward primer, 1μL (10 ng/μL) reverse primer, 10 ng template DNA and deionized water which is added the reaction to 50 μL. The PCR reaction was conducted in the MJ Mini Thermo Cycler (Bio-rad, Inc.) with the following program described in Table **2.3**.

Colony PCR was conducted to determine the presence of DNA fragment of interest or correct insert orientation for engineered plasmid construct. Individual transformants were picked and dipped into 10 μL nuclease-free water in eppendorf tubes. The rest of it was streaked on corresponding positions in a LB agar plate supplemented with ampicillin (100 μg/mL) for preservation. The bacteria suspension was heated at 98^{o}C for 3 min and 1 μL was taken from the lysed bacteria as DNA template for subsequent PCR reaction. The PCR reaction was conducted following the manufacturer's suggested extension procedures for *Taq* DNA polymerase (Thermo Scientific). The components in a 50 μL reaction include 5 μL10X *Taq* Buffer, 5 μL dNTP Mix (2mM each), 1 μL (10 nmol/μL) forward primer and 1 μL (10 nmol/μL) reverse primer, 10 μL 25mM $MgCl_2$, 100 ng Template DNA, 1.25U *Taq* DNA polymerase and deionized water added up to 50 μL for the reaction. The reaction was conducted in a MJ Mini Thermo Cycler using the following the program as described in Table **2.3**.

Restriction endonuclease digestion of plasmid DNA or PCR product was conducted in 20 μL reaction. The restriction enzymes used in this study include

Table 2.3 PCR program for cloning PCR and regular PCR

a. Cloning PCR

Initial Denaturation	98°C	30s	
Denaturation	98°C	7s	Repeat 30 cycles
Annealing	Adjusted to primers	25s	
Extension	72° C	30s (30s/kb)	
Final extension	72°C	30s	

b. Regular PCR

Initial Denaturation	95°C	1 min	
Denaturation	95°C	30s	Repeat
Annealing	Adjusted to primers	30s	30 cycles
Extension	72° C	1 min (1min/kb)	
Final extension	72°C	10 min	

*Eco*RI, *Not*I, *Bam*HI, *Hin*dIII, *Xho*I and *Esp*3I (*Bsm*BI) (Thermo Scientific Fermentas Molecular Biology Solutions). Except restriction endonuclease digestion by *Esp*3I for pHH21-based plasmid cloning, standard restriction endonuclease digestion reactions include components of 2 μL 10X reaction buffer, 200 units of restriction enzyme, 0.5 μg of plasmid DNA or 10 μL of PCR reaction mixture and nuclease-free water added up to 20 μL. The reaction of restriction enzyme digestion by Esp3I needs to be included with 1 μL 20mM DTT in a 20 μL reaction. All reactions were incubated at 37^{o}C for 4 hours. The digested product was purified using QIAquick PCR Purification Kit (Qiagen Inc.) following manufacture's instructions (>100 bp) or was gel extracted using QIAquick Gel Extraction Kit (Qiagen Inc.) by running on an agarose gel as described below.

Separation of DNA or siRNAs was achieved by running on a 1% or 5% (w/v) agarose gel. 1% or 5% (w/v) agarose gel was made by followed procedures: 1g/5g of TopVision agarose (Thermo Scientific Fermentas Molecular Biology Solutions) was added in 100 mL 1X TAE electrophoresis buffer (Thermo Scientific Fermentas Molecular Biology Solutions) and the mixture was heated until agarose dissolved. The agarose solution was supplemented with 4 μg/mL of ethidium bromide (Thermo Fisher Scientific Inc.), solidified on a electrophoresis apparatus (Thermo Scientific Inc.). DNA samples were mixed with 6X Loading Dye (Thermo Scientific Fermentas Molecular Biology Solutions) and added to the wells with a DNA ladder included in each agarose gel: GeneRuler 100bp DNA Ladder (Thermo Scientific Fermentas Molecular Biology Solutions) for 100bp~1000bp DNA fragment, Lambda DNA/Hind III Marker, 2 (Thermo Scientific Fermentas Molecular Biology Solutions) for >2000bp DNA fragment and O'GeneRuler Ultra Low Range DNA Ladder (Thermo

Scientific Fermentas Molecular Biology Solutions) for siRNA fragment. The gel was run under 100 voltages for 30 ~ 60 minutes and then analyzed by Alpha Innotech FluorChem HD2 Imager. PCR products or endonuclease-digested reactions were run on the agarose gel for gel extraction purposes.

Ligation of digested plasmid DNA and PCR products was finished by T4 DNA Liagase (Thermo Scientific Fermentas Molecular Biology Solutions). Ligation was conducted in 10 μL reaction including 2 μL of 1:1 molar ratio of insert DNA and vector DNA, 10X T4 DNA ligase buffer, 1 μL of T4 ligase and nuclease-free water added up to 10 μL for 1 hour at RT.

Plasmid DNA was transformed into NEB 5-alpha Competent *E. coli* (New England BioLabs Inc.) following the manufacture's instructions: 100ng of plasmid DNA was added to 50 μL competent cells. Mixtures were heat shocked at 42 ^{o}C for 30 minutes and recover to grow at 37^{o}C for 60 minutes with vigorous shaking. The transformed bacteria were spread on the LB-agar (Thermo Fisher Scientific) plates containing ampicillin (100 μg/mL). After 24-hours incubation under 37^{o}C, colonies were used for subsequent small-scale or large-scale plasmid preparations.

QIAprep Spin Miniprep Kit (Invitrogen Inc.) was used for small-scale plasmid DNA preparations. Single colony transformed with plasmid DNA was picked to grow in 4 mL LB medium (1%Tryptone, 0.5% Yeast Extract and 0.5% Sodium Chloride) containing ampicillin (100 μg/mL) for 18 hours at 37^{o}C in a shaking incubator. The plasmid DNA was prepared following manufacturer's instructions. The plasmid DNA was eluted into 30 μL EB (10 mM Tris·Cl, pH 8.5) and preserved at -20 ^{o}C for further use. Large-scale plasmid DNA was prepared by QIAGEN Plasmid Midi Kit, bacteria were grown in 50 mL LB culture. The concentration of plasmid was determined by

NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc.). Quality of plasmid DNAs was estimated from Abs_{260}/Abs_{280}, ratio greater than 1.8 was considered as good quality.

Sequencing of plasmid DNA or PCR product was conducted at University of Delaware Sequencing and Genotyping Center using the ABI Prism 3130XL Genetic Analyzer (Life Technologies Inc.)

2.3 Preparation of Small Interfering RNA (siRNA)

RNA oligonucleotides were purchased from Dharmacon (Thermo Scientific). Corresponding oligos were annealed to generate double-stranded siRNAs. The annealing procedures were as followed. RNA oligonucleotides were dissolved in RNase-free water with a final concentration of 100 μM as stock. Each dissolved RNA oligonucleotides were then diluted in the annealing buffer (100 mM Potassium Acetate; 30 mM HEPES, pH 7.5) to a final concentration of 50 μM. Each strand of the diluted 20 μL RNA oligonucleotides were combined in equal volume. Additional 10 μL of annealing buffer was added to the mixture of two RNA oligos for annealing. They were incubated at 90 ^{o}C for 1 minute followed by slowly cool down until RT. The annealed siRNAs were stored at -80^{o}C for long-term use.

2.4 Real-Time RT-PCR

Viral RNAs were obtained by extraction of RNAs from MDCK cells infected with multiplicity of infection (MOI) = 1 A/PR/8/34 (H1N1) influenza viruses in a T-25 flask. vLuc/cLuc RNA transcripts were obtained from cells transfected by vLuc/cLuc followed by infection of MOI = 0.1 A/PR/8/34 (H1N1) influenza viruses in a 24-well plate. RNAs were extracted using RNeasy® Mini Kit (Qiagen Inc.)

following manufacturer's instructions. The total RNA concentration was quantified by Nanodrop 1000 spectrophotometer (Thermo Scientific Inc.). The quality of RNAs was checked using 2100 Bioanalyzer kits (Agilent Inc.) in a 2100 Bioanalyzer system (Agilent Inc.) following manufacturer's instructions. RNAs with RNA Integrity Number (RIN) higher than 5 were considered as good quality.

Viral 1st strand cDNAs from A/PR/8/34 (H1N1) for cloning were generated using AccuScript High Fidelity 1st strand cDNA synthesis kit (Agilent Inc.). Two microliter reaction includes 2 μL AccuScript RT Buffer, 1 μL NP segment specific primer (Appendix) (10 nmol/μL), 0.8 μL NP segment specific primer (Appendix) (10 nmol/μL), 1 μg Viral RNA and 0.8 μL dNTP mix (25mM each dNTP), 1 μg RNA and RNase-free water added up to 20 μL. The reaction mixture was incubated at 65^{o}C for 5 minutes in the MJ Mini Thermo Cycler and then cooled down to RT followed by adding with the following components: 2 μL DTT (100mM), 1 μL AccuScript RT enzyme, 0.5 μL RNase Block Ribonuclease inhibitor (40U/μL). The complete reaction mixture was incubated at 42^{o}C for 60 minutes and reaction was terminated at 70^{o}C for 15 minutes. The reaction mixture was either PCR amplified for cloning as described in Chapter 2.2 or for Real-Time RT-PCR as followed.

After reverse transcription, 1μl of each cDNA product were mixed with luciferase real time PCR detection primers and iQ™ SYBR® Green Supermix (Biorad, Hercules, CA). Human GAPDH gene was selected as the reference gene and Real Time PCR detection was conducted in the MyiQ2 two-color Real-time detection system (Biorad, Philadelphia, PA). The 2-step PCR thermo cycling conditions were 95°C for 3 min; 40 cycles of 95°C for 10 sec, 55°C for 30 sec. The specificity of amplification during real-time PCR was confirmed by checking the melting curve. All

PCR reactions were run in duplicates. The Ct values that varied by >0.5 unit (cycle) between duplicates were discarded and resultant Ct values were averaged. The amount of each strand (vRNA/cRNA/mRNA) in each sample was normalized to the GAPDH gene using the delta Ct method (222).

2.5 Statistical Analysis

Data was analyzed as a completely *randomized* block design using JMP statistical software (SAS Institute). The Student *t*-test was used to compare means of two groups. Tukey's test was used to perform multiple comparisons among several group means. Pearson's correlation analysis was done for luciferase expression by vLuc/cLuc reporter and their relative expression ratio of three strands (vRNAs/cRNA/mRNA). All experiments were repeated in triplicate or quadruplicates. The level of significance chosen to determine whether the data points obtained from several different samples were significantly different was $p<0.05$.

Chapter 3

RESULTS AND DISCUSSION

3.1 Developing and Comparing Two Plasmid-based Reporters for Detection of Influenza Viruses.

3.1.1 Introduction

During outbreaks of influenza, a multitude of tools and animal models must to be rapidly deployed to detect the infection and to characterize genomic sequence, antigenic characteristics, antiviral susceptibility, and pathogenic potential. Detection of influenza virus commonly requires amplification of the whole virus by replication in chickens eggs or cell culture, or amplification of specific genomic regions by nucleic acid amplification. Subsequently, influenza protein levels (hemagglutination, antibody based detection), viral nucleic acid levels (RT-PCR, Loop-mediated isothermal amplification (LAMP), next generation sequencing) or infectious particles (plaque assay, 50% Embryo Infectious Dose (EID_{50}) or 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay) are determined (223). Virus growth in canine MDCK cells, incubation in chicken eggs and terminal animal challenges are still the gold standard for diagnosis of influenza infection and pathogenic characterization of novel strains, but results from these tests can take weeks (224). Thus, there is a great need for fast high-throughput real-time methods of diagnosis and characterization of influenza infection and for the development of novel antiviral drugs.

Earlier studies have found that foreign genes flanked by influenza 5' and 3'RNA promoters could be expressed under the control of influenza RNA-dependent RNA polymerase (RdRp) (Figure. **3.2**) (225–227). Currently, two reporter approaches based on the influenza promoter have been tested. Reporter viruses were once produced through reverse genetics by replacing non-essential influenza genes, such as fusing or replacing NS1 or neuraminidase proteins with EGFP (228–230). However, this approach does not permit the detection of influenza infection in clinical samples. The characterization of novel strains requires construction of new reporter viruses, and selection pressures during passages *in vitro*/*in vivo* of the recombinant viruses can lead to loss of the reporter protein (229). Additionally, genes that might be non-essential *in vitro* are likely to be important *in vivo*, and introduction of reporter protein often seriously attenuates the viral pathogenicity *in vivo* (228,230). Alternatively, the plasmid DNA that transcribe a reporter-protein-encoding RNA flanked by influenza virus vRNA promoter sequence under the control of a RNA polymerase I (pol I) promoter could also function as reporter for infection. The transcripts produced could be amplified by viral RdRp and NP proteins either expressed from plasmid DNA or produced during influenza virus infection in suitable cell lines(69,231). Using influenza promoters, which are the highly conserved sequences found in all influenza strains, allows sensitive and broad based detection of even novel influenza strains. Furthermore the virus itself is not modified allowing the replication and pathogenicity of novel strains to be studied under more natural conditions, and real-time detection is also possible. This approach is also suitable for high-throughput screening of multiple clinical samples, and can be applied to the identification of novel anti-influenza drugs.

The objectives of the present study were to compare influenza reporters using vRNA or cRNA promoters. Two constructs were compared in detection of influenza A virus infection in cell culture by time-course and dose-response study. Two reporter proteins, enhanced EGFP (EGFP) and luciferase were also evaluated. Further, it was demonstrated that these constructs could be successfully applied to study the silencing kinetics of antiviral siRNA.

3.1.2 Construct of reporter plasmids

Reporter plasmids used as indicators for influenza replication activity were constructed based on pHH21 plasmid, kindly provided by Dr. Yoshihiro Kawaoka (University of Wisconsin-Madison, WI). The pHH21 plasmid carries a RNA polymerase I promoter and terminator. The plasmid map was shown in Figure **3.1**. Reporter plasmids expressing EGFP (vGFP/cGFP) or luciferase (vLuc/cLuc) were constructed separately. To construct vGFP/cGFP, the EGFP reporter gene was first PCR amplified from pCAG-GFP plasmid (Table **2.2**) by cloning primers vGFP-F/cGFP-F and vGFP-R/cGFP-R (Appendix). EGFP gene flanked by 23 nt sequence from 5' and 43 nt from 3'UTR of the NP vRNA of influenza A/WSN/33(H1N1) strain (69) with either directions were obtained and purified from agarose gel. They were digested with *Bsm*BI restriction enzyme (Figure **3.1**) and followed by ligation to the digested pHH21 plasmid. On the other hand, the firefly luciferase (*luc2*) reporter was amplified from the pmirGLO plasmid. vLuc and cLuc were constructed similarly as described for vGFP/cGFP. The finished constructs were confirmed by sequencing.

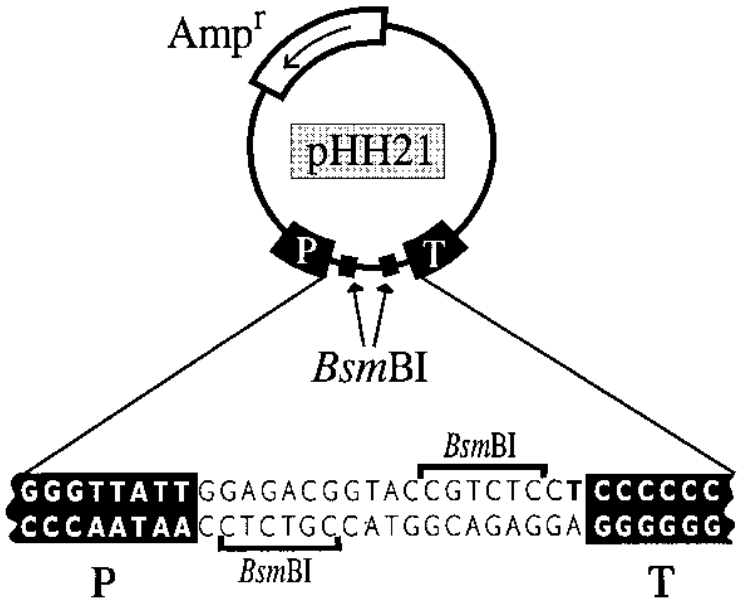


Figure 3.1 Schematic graph of pHH21 plasmid. The graph was adapted from Neumann et al. 1999 (232)**.** Plasmid pHH21 has a human polymerase I promoter (P) and terminator (T). The *Bsm*BI enzyme restriction site can be used for cloning purposes. vGFP/cGFP and vLuc/cLuc transcripts was inserted into this site for reporter plasmid construct.

The schematic diagram for the expression of reporter proteins by the four plasmid DNAs was shown in Figure **3.2**. The influenza virus vRNA-promoter-based reporters (vGFP/vLuc) produce vRNA-like transcript, which could be directly amplified by RdRp into mRNA for reporter protein translation (EGFP/luciferase), while cRNA-promoter-based reporters (cGFP/cLuc) produce cRNA-like transcript, which needs to be first replicated into vRNA-like transcript by RdRp and then transcribed into mRNA for reporter protein expression.

3.1.3 Detection of influenza A airus replication in HEK293 cells by using influenza-induced EGFP expression

The vGFP and cGFP reporter constructs were transfected into human HEK293 cells, which were subsequently infected with A/PR/8/34 (H1N1) as described in Figure **3.3**. Infection with influenza resulted in significant accumulation of EGFP protein inside of cells, reflecting the influenza RdRp replication (cRNA>vRNA) and transcription (vRNA>mRNA) activities (Figure.**3.2**). Influenza induced EGFP expression became detectable at 8 hpi. The EGFP signals became stronger as influenza replicated in the cells throughout the plate. Unexpectedly, the negative control transfected with pHH21-cEGFP but not infected with influenza showed sporadic EGFP expression indicating that low level translation of Pol I expressed cEGFP cRNA occurred. In contrast, the vEGFP reporter alone did not produce any visible EGFP expression. No significant differences were noticed between vEGFP and cEGFP based constructs at later time points.

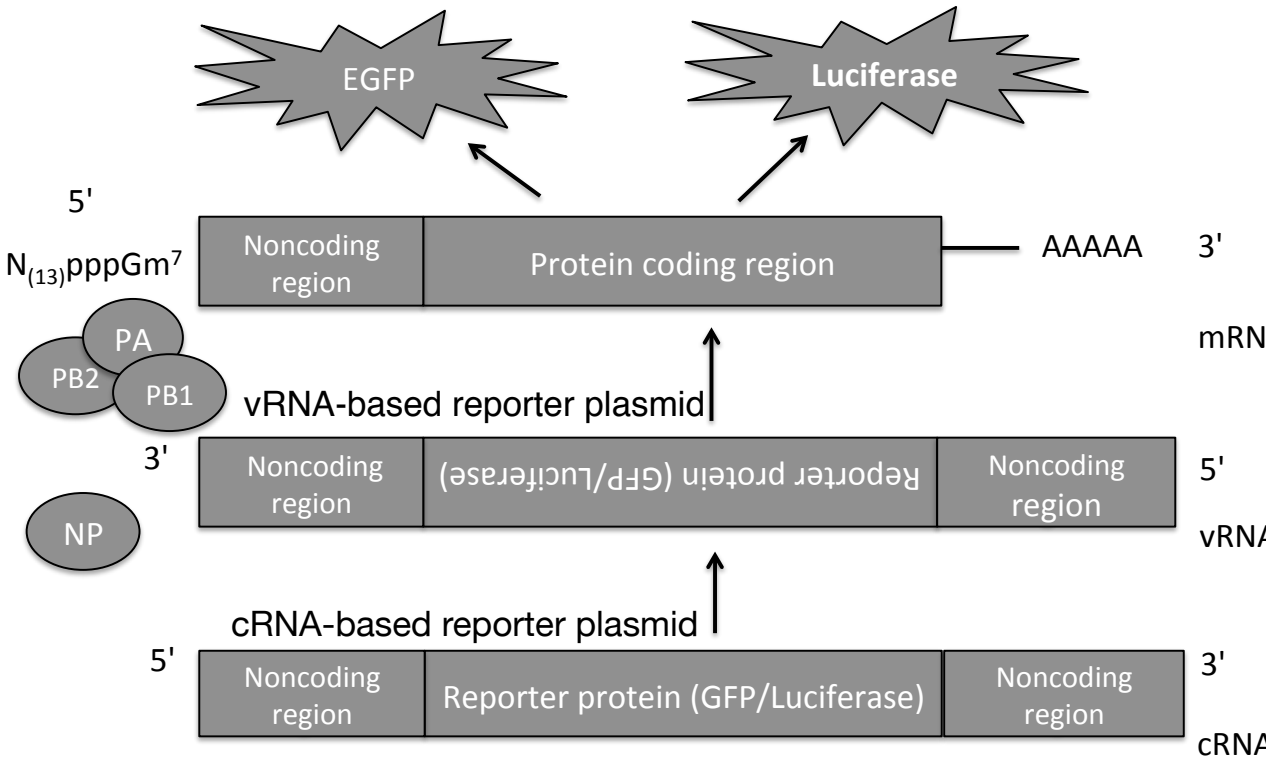


Figure 3.2 Schematic diagram for expression of foreign proteins by using influenza vRNA or cRNA promoters. Three RNA species (vRNA, cRNA and mRNA) were produced from the reporter plasmid vLuc/cLuc or vGFP/cGFP during influenza infection. EGFP, Enhanced Green Fluorescent Protein; Luc, luciferase; RdRp, influenza RNA-dependent RNA polymerase.

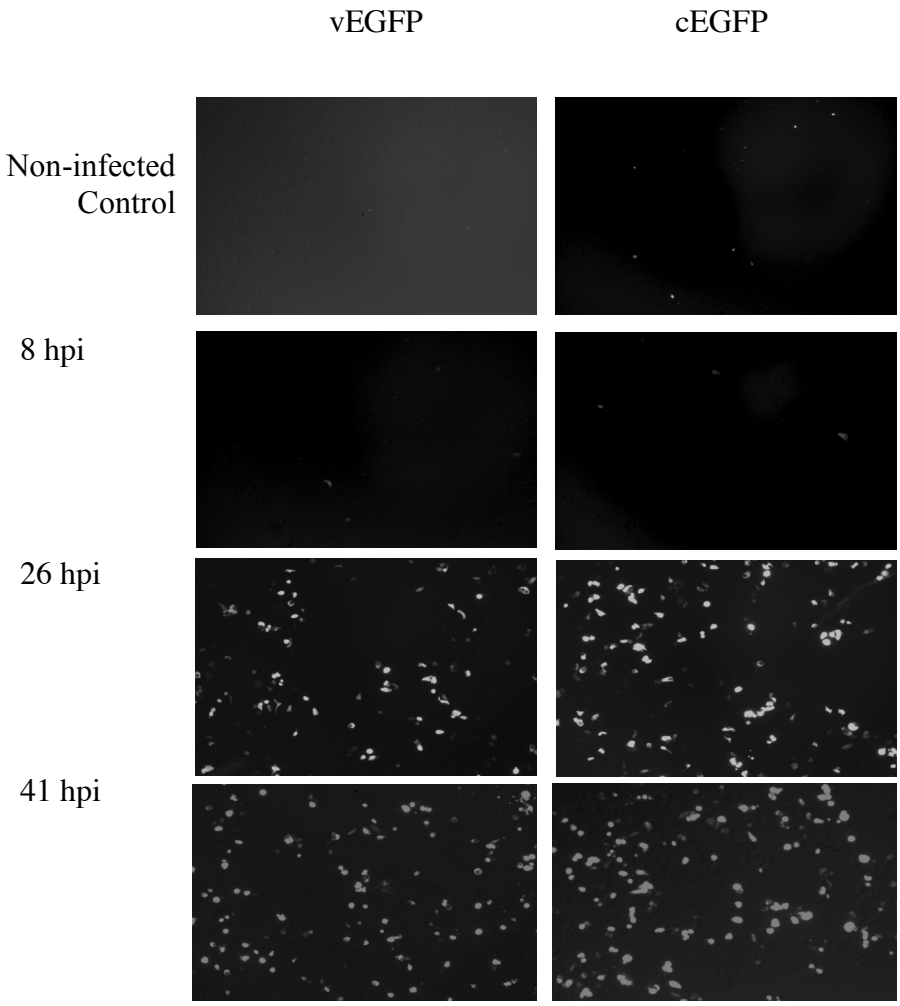


Figure 3.3 Time course study of influenza induced EGFP expression in HEK293 cells. HEK293 cells were reverse transfected with vEGFP or cEGFP constructs in 96 well plate and infected with A/Puerto Rico/8/1934 (MOI=1) for at 18h post transfection. Experiment was repeated twice. Non-infected control, negative control transfected with construct but not infected with influenza.

3.1.4 Time course study of influenza A virus replication in HEK293 cells using influenza-induced luciferase expression

The EGFP reporter was replaced with firefly luciferase gene (*luc2*) codon optimized for more efficient expression in mammalian cells. New vLuc and cLuc constructs were tested using the same transfection/infection protocol as described above in the time course study (Figure.**3.4**). The vLuc transfected, non-infected control remained at the same level as background signal from non-transfected cells. The cLuc non-infected control produced significantly higher signal ($p<0.05$) than the background, indicating low level translation of Pol I expressed cLuc cRNAs. This data corresponds to the previous observations with the cEGFP non-infected control.

The luciferase signal from vLuc or cLuc reporter transfected cells showed a similar overall trend during the course, with both constructs demonstrating significant increase in luciferase signal compared to the non-infected control. For vLuc, this difference was detected as early as 6 hpi ($p<0.05$), while for the luciferase signal became significantly different from the noninfected control ($p<0.05$) 17hpi. The signal for both constructs increased exponentially, peaked at 25 hpi and decreased gradually afterwards. At expression peak, the signal was 6767 fold higher than the non-infected control for vLuc ($p<0.05$) and 736 fold higher for cLuc ($p<0.05$). The vRNA promoter-driven luciferase expression started to increase at least 4 hr earlier resulting in 8 fold higher vLuc signal at 8 hpi ($p<0.05$). However, the cLuc signal increased faster and the differences between two was only 1.6 fold at 25 hpi ($p>0.05$). The latency for the production of reporter proteins from cLuc plasmid DNA could be due to the extra replication step (cRNA>vRNA) required for protein translation, as demonstrated in Figure **3.2**.

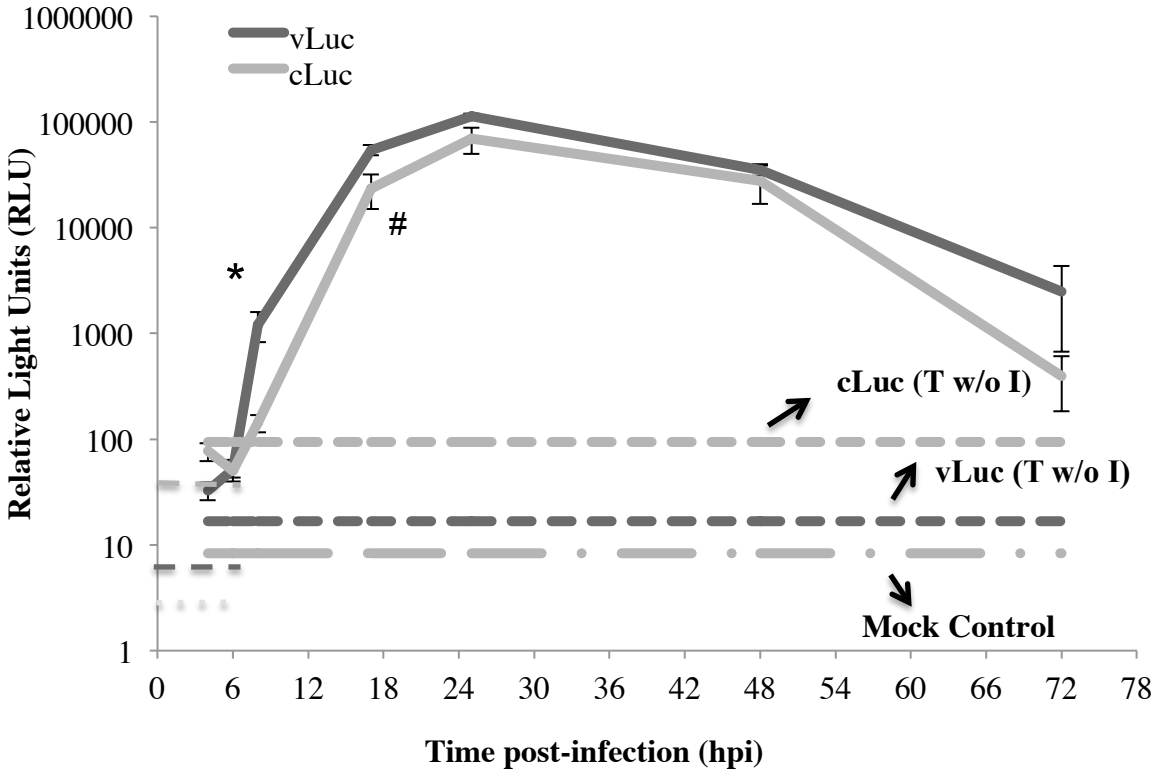


Figure 3.4 Time course study of influenza induced luciferase expression in HEK293 cells. HEK293 cells were reverse transfected with vLuc or cLuc constructs in 96-well plate and at 18h post transfection, infected with A/Puerto Rico/8/1934 (MOI=1). cLuc (T w/o I), cells transfected with cLuc but not infected with viruses; vLuc (T w/o I), cells transfected with vLuc but not infected with viruses; Mock Control, negative cell control transfected with construct but not infected with viruses. The error bars represent standard error (N = 3 biological replicates). Student *t*-test was used for statistical analysis. * , indicates significant differences between vLuc reporter and non infected control ($p<0.05$); # , indicate significant differences between cLuc and mock control ($p<0.05$).

3.1.5 Time course study of stability of reporter proteins

The luciferase signal was measured only in lysed cells in the previous study. To determine if luciferase proteins are lost into the cell culture supernatant during influenza induced cell apoptosis, the luciferase coming from both lysed cells and supernatant was quantified (Figure **3.5** a). Data indicated that only around 1% of luciferase was present in the supernatant even at later time points. Luciferase signal dropped gradually after 24 hpi around 4 fold at 48 hpi and 7 fold at 72 hpi for both lysed cells and the supernatant. This suggests that the decrease in luciferase signal was likely due to a decrease in expression and an increase in the degradation of luciferase at the later stages of influenza infection.

In contrast, for the EGFP reporter there seems to be a significant release of EGFP into the supernatant at all time points (Figure **3.5** b). About 24% of total EGFP signal was found in the supernatant at 24 and 48 hpi, while 47% of the total EGFP signal was located in the supernatant at 72 hpi; indicating a significant release of EGFP from cells into supernatant. Furthermore EGFP also accumulated slower than luciferase and achieved maximum signal in cells at 48 hpi compare to 25 hpi for luciferase. It is also more stable as no changes in total fluorescence intensity (cells plus supernatant) have occurred from 48 to 72 hpi.

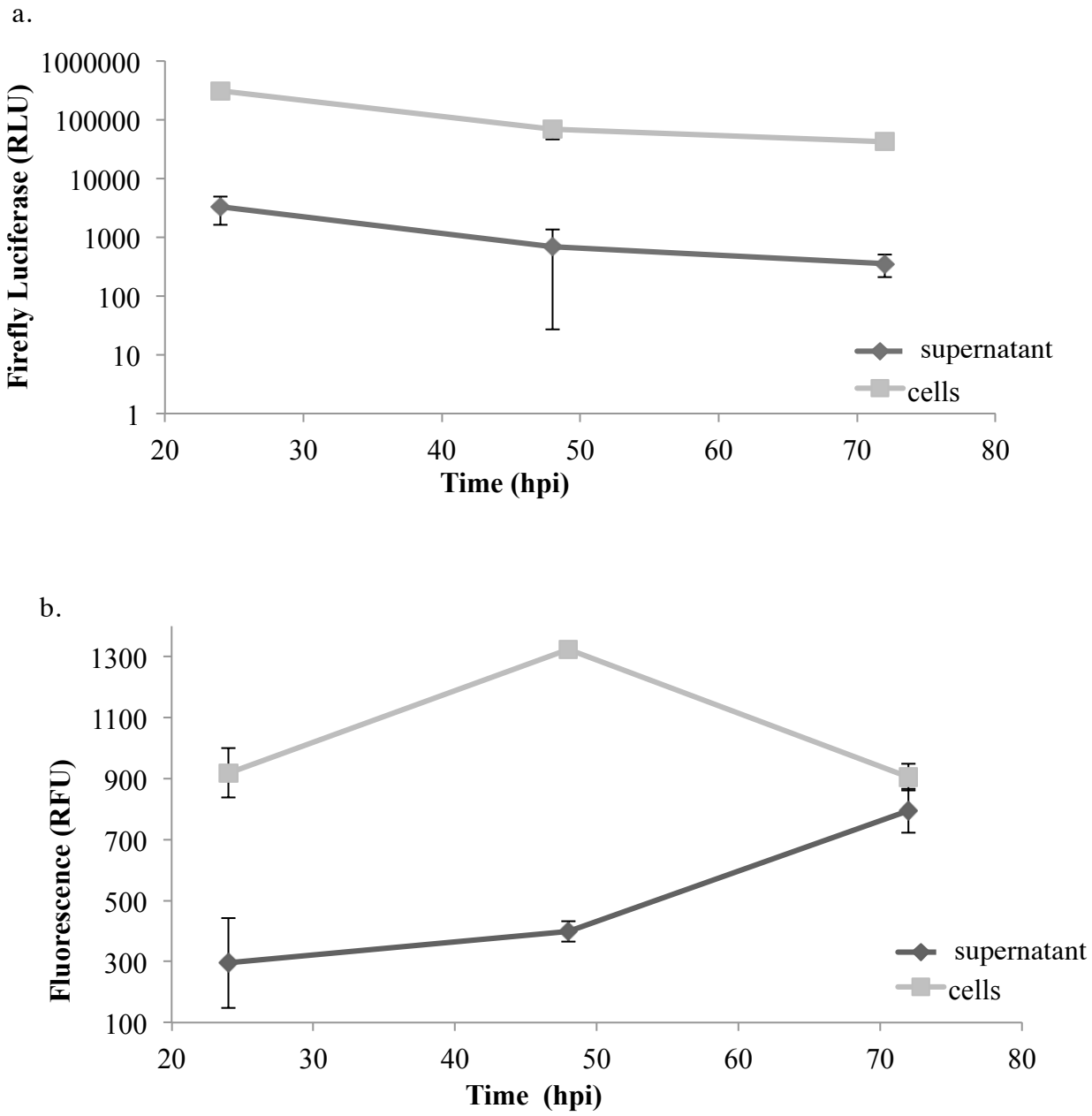


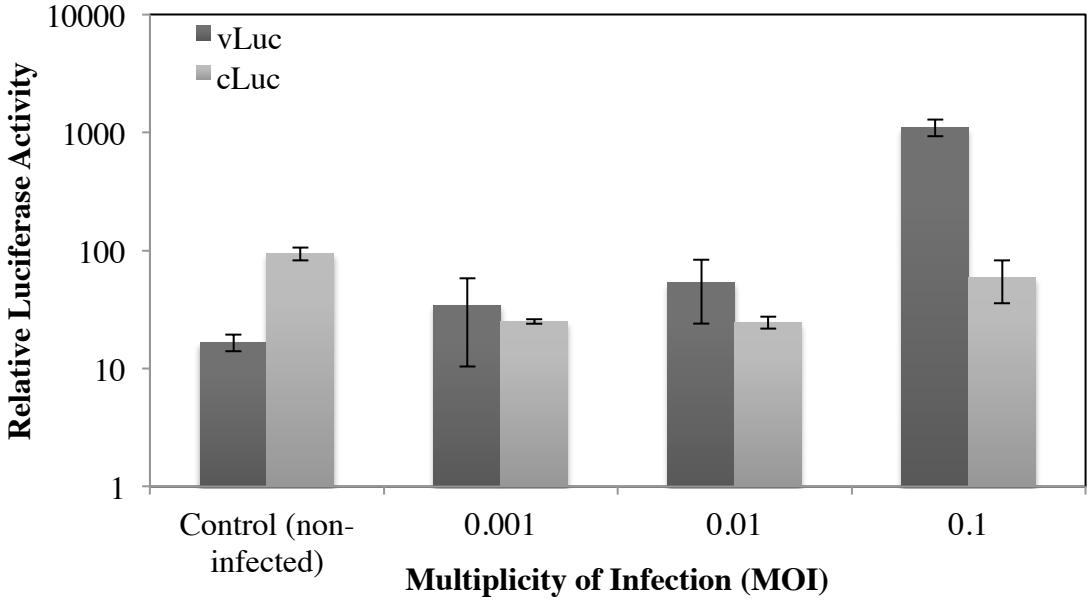
Figure 3.5 Time course study of stability of different reporter proteins. HEK293 cells were reverse transfected with vEGFP (a) or vLuc (b) construct in a 96-well plate and at 18h post transfection infected with A/Puerto Rico/8/1934. At selected time points the cells and supernatant were collected, the signal from each reporter protein was determined by deducting the signal from non-infected control.

3.1.6 Dose response study for vLuc and cLuc constructs

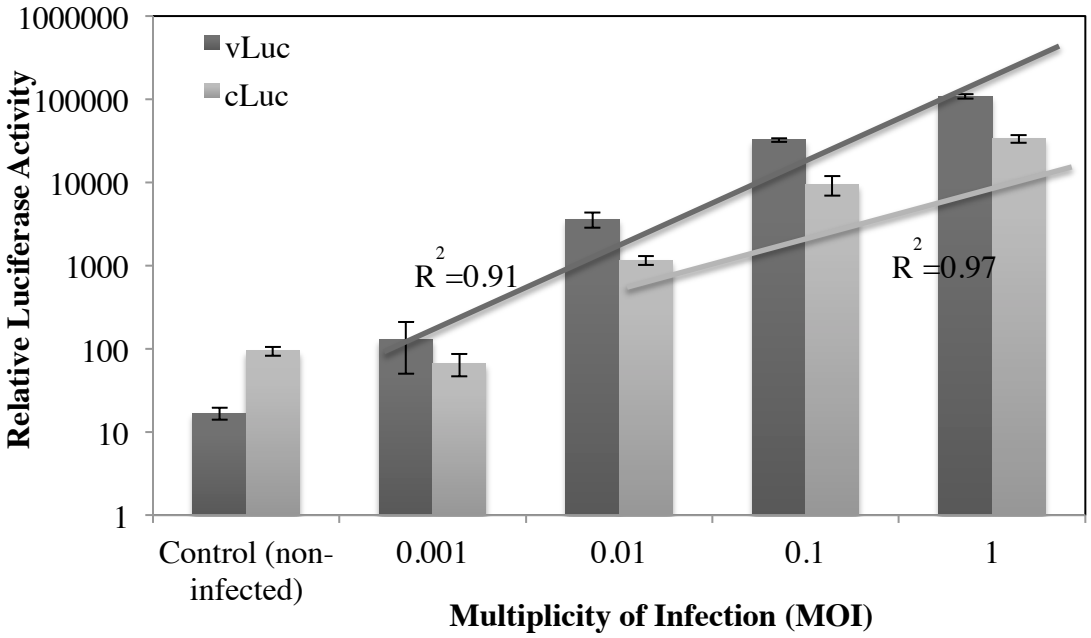
To compare the sensitivity of vRNA and cRNA constructs in detecting influenza A virus replication, HEK293 cells were transfected with vLuc or cLuc constructs and infected with A/PR/8/34 (H1N1) strain at different MOIs (MOI=0.001, 0.01, 0.1 and 1). Luciferase signal was evaluated at different time post infection (12, 24, 48 and 72 hpi) with MOI=1 only being evaluated at 24 hpi. The lowest detection limit for vLuc was MOI=0.001 at (12hpi and 24 hpi with $p<0.05$) and MOI=0.01 for cLuc reporter (24hpi, 48hpi and 72hpi with $p<0.05$) (Figure **3.6**). Both expression constructs demonstrated strong correlation between MOI and the logarithm of luciferase signal with R^2 of 0.91for vLuc ($p<0.05$), R^2 of 0.97 for cLuc at 24 hpi ($p<0.05$) (Figure **3.6** a) and 0.71 for vLuc at 72 hpi (Figure **3.6** c). It appears an upper detection limit was not reached in this experiment as no saturation plateau was detected even at high MOIs. Due to the lower sensitivity at early virus infection and the degradation of luciferase at later infection, luciferase signals and MOIs did not correlate well at 12 hpi (Figure **3.6** a) and 72 hpi, especially for cLuc construct (Figure **3.6** c).

Figure 3.6 Dose response study of influenza induced luciferase expression in HEK293 cells at different time post infection. HEK293 cells were reverse transfected with vLuc or cLuc constructs in 96 well plate and infected with A/Puerto Rico/8/1934 at MOI=0.001, 0.01, 0.1 or 1. Cells were lysed at 12 hpi (a), 24 hpi (b), 48 hpi (c) and 72 hpi (d) for luciferase assay. Cell control, untreated negative cell control; Non-infected control, cells controls transfected with construct but not infected with influenza. The error bars represent standard error (N = 3 biological replicates). R-Square of linear fit for vLuc between the log of relative luciferase activity and MOI is 0.91 at 24hpi and 0.71 at 72hpi ($p<0.05$). There is only significant correlation between the log of relative luciferase activity and MOI for cLuc at 48hpi and the R-square of linear fit for cLuc is 0.97 ($p<0.05$).

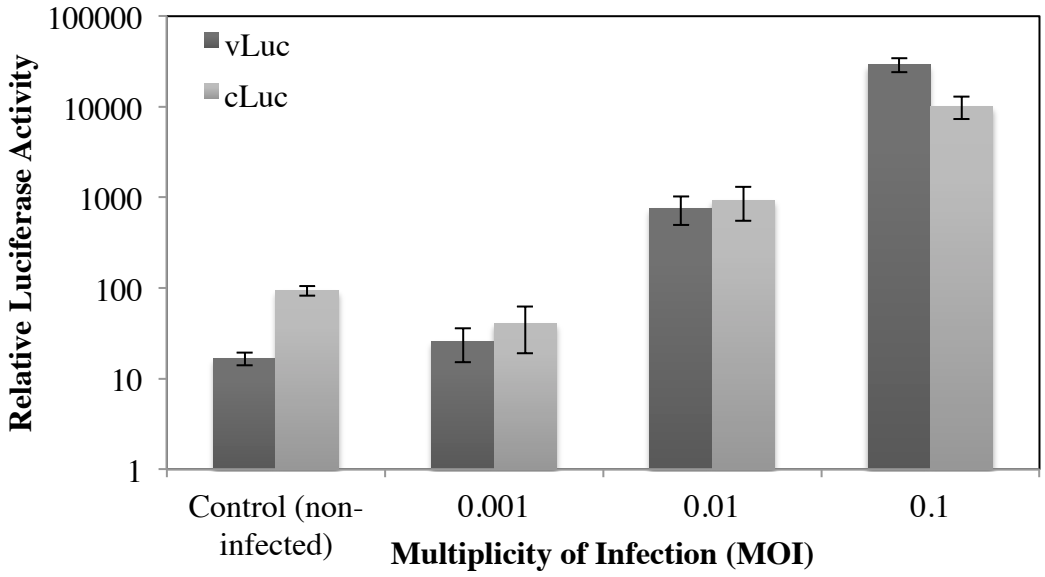
a.


10000
1000
100
10
1
vLuc
cLuc
Relative Luciferase Activity
Control (non-infected)
0.001
0.01
0.1
Multiplicity of Infection (MOI)

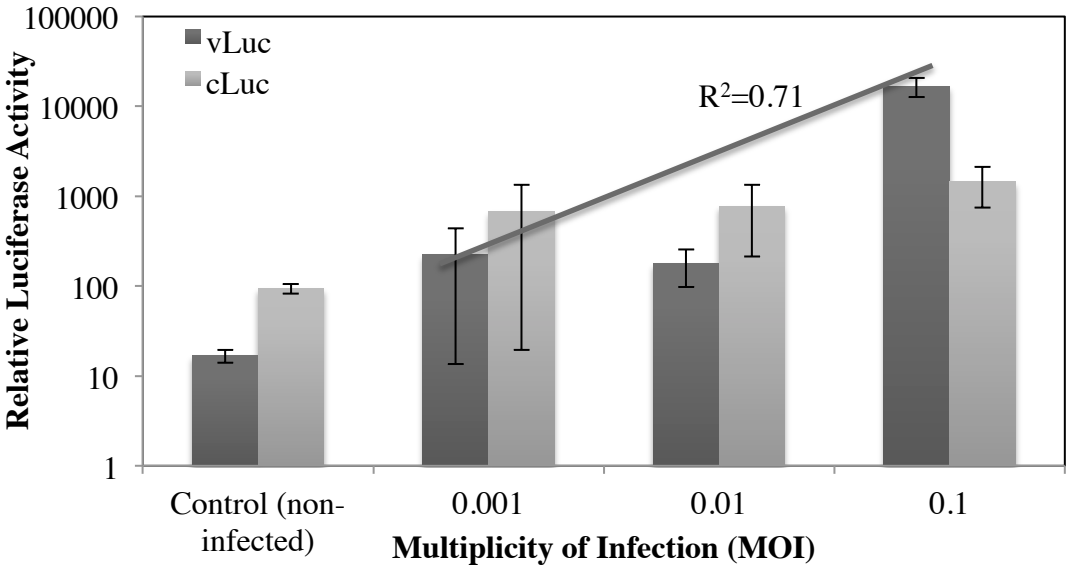
b.


1000000
100000
10000
1000
100
10
1
vLuc
cLuc
Relative Luciferase Activity
R2=0.91
R2=0.97
Control (non-infected)
0.001
0.01
0.1
1
Multiplicity of Infection (MOI)

c.


100000
10000
1000
100
10
1
vLuc
cLuc
Relative Luciferase Activity
Control (non-infected)
0.001
0.01
0.1
Multiplicity of Infection (MOI)

d.


100000
10000
1000
100
10
1
vLuc
cLuc
R²=0.71
Relative Luciferase Activity
Control (non-infected)
0.001
0.01
0.1
Multiplicity of Infection (MOI)

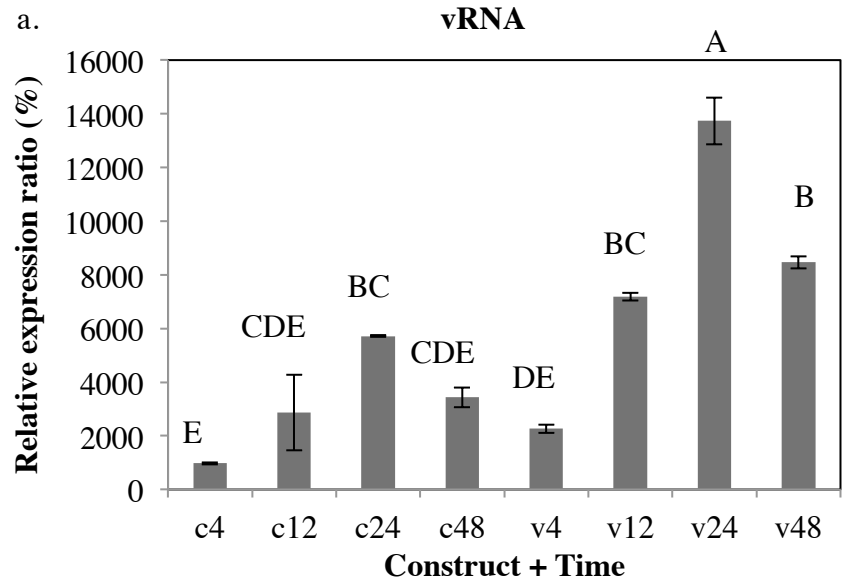
3.1.7 Time course analysis of level of vRNA, cRNA and mRNA for vLuc and cLuc constructs after infection using real time RT-PCR

To study the level of three RNA forms (vRNA, cRNA, mRNA) produced by two luciferase reporters Real Time RT-PCR was used to quantify RNA strands using strand specific RT Luc primers. Strand specific primer designs were modified from Ge *et. al* (163) to produce vLuc/cLuc strand-specific strands, and oligo-dT primers were used for mRNA reverse transcription (Appendix). For both the vLuc and cLuc reporter, high levels of vRNA were detected (Figure **3.7** a-c). The vLuc reporter had a 2.3 fold higher level of vRNA at 4 hpi and there was significant 6-fold increase in the level of vRNA at 24 hpi for both reporters ($p<0.05$). Overall distribution of vRNA level across time points was similar for both constructs.

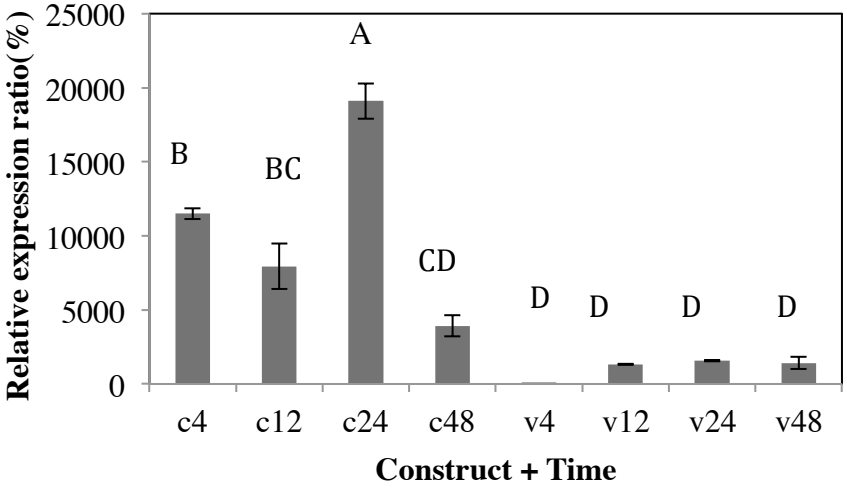
Overall distribution was very different for cRNA produced from vLuc and cLuc. Initial differences were much larger with the cLuc reporter, demonstrating 115-fold higher level of cRNA than vLuc reporter ($p<0.05$) (Figure **3.7** b). At 24 hpi cLuc reporter showed only 1.7 fold increase in the level of cRNA, while for vLuc construct there was 16 fold increase, resulting in overall 12 fold higher level of luciferase cRNA for cLuc reporter. Noticeably, cRNA expression from vLuc reporter plateaued after influenza infection. This might be explained by the previous proposed stabilization of replicative intermediate model, in which the cRNAs need newly synthesized influenza RNA polymerase and NP to be stabilized. While the constant production of vRNAs from vLuc construct by RNA polymerase I might compete for newly synthesized NP and RNA polymerase with cRNAs in order to be transcribed or packaged. So this might result in the delay and plateau of relative cRNA expression ratio. For cLuc reporter, the relative expression level of cRNAs stayed almost the same at early time

Figure 3.7 Time course analysis of level of vRNA, cRNA and mRNA for vLuc and cLuc constructs using Real Time RT-PCR. HEK293 cells were reverse transfected with constructs in 24 well plates and infected with A/Puerto Rico/8/1934 (MOI=0.1). At selected time points (4, 12 , 24, 48 hpi) the cells were collected, the total RNA was extracted and Real Time RT-PCR was used to quantify luciferase RNAs (vRNA, cRNA, mRNA) using strand specific RT primers. The RNA signal for cLuc cRNA 4 hpi was selected as 100%. All of the relative expression level ratios were normalized to that of vLuc cRNA 4hpi. The error bars represent standard error (N = 4 replicates). c4-c48, cLuc reporter at 4-48 hpi; v4-v48, vLuc reporter at 4-48 hpi. Tukey's test was used to compare level of RNA between different time points for both constructs. Different character (A, B, C, D, E) represents significant difference between corresponding RNA levels ($p<0.05$).

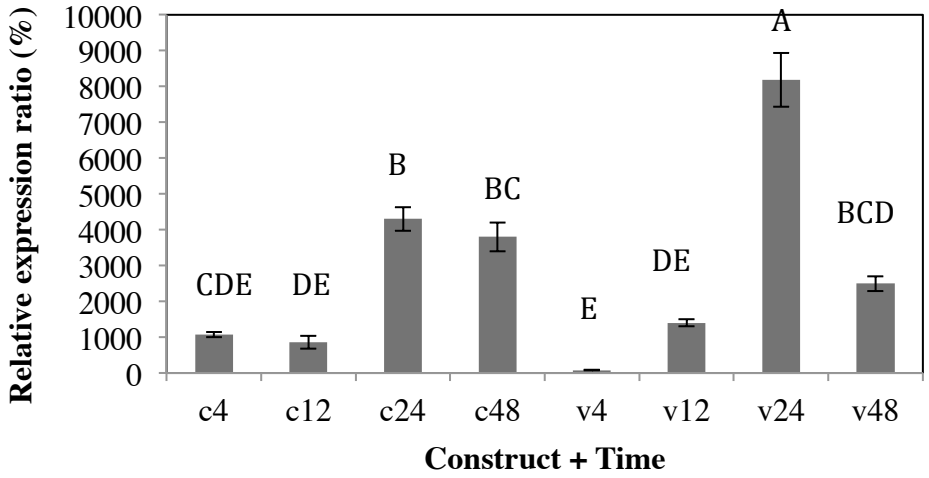
a.


vRNA
Relative expression ratio (%)
16000
14000
12000
10000
8000
6000
4000
2000
0
E
CDE
BC
CDE
DE
BC
A
B
c4
c12
c24
c48
v4
v12
v24
v48
Construct + Time

b.


cRNA
Relative expression ratio(%)
25000
20000
15000
10000
5000
0
B
BC
A
CD
D
D
D
D
c4
c12
c24
c48
v4
v12
v24
v48
Construct + Time

c. **mRNA**


Relative expression ratio (%)
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
CDE
DE
B
BC
E
DE
A
BCD
c4
c12
c24
c48
v4
v12
v24
v48
Construct + Time

post infection (4, 12hr) with a sharp increase at 24 h.p.i.. This might be the result of replication of vRNA->cRNA when progeny vRNA was stabilized 12 h.p.i. through the accumulation of newly synthesized NPs and RNA polymerase. At 48 hr post infection, the cRNA level dropped while mRNAs level stayed the same, which might indicate the degradation of cRNAs or a decreased replication level along with an increased transcription level. This corresponds to the primer extension assay result for cRNAs from cNA-Luc reporter in Regan *et al.* (2006) collected at 44h post infection, where mRNAs were most abundant and more cRNA strands could be detected than vRNA strands.

At 4 hpi the luciferase mRNA was 13 fold higher for cLuc reporter (Figure **3.7** c). There was 4-fold increase in the level of mRNA at 24 hpi for cLuc reporter and 100 fold for vLuc reporter ($p<0.05$). Overall 2 fold higher level of luciferase mRNA was found for vLuc reporter, which correlates well with detected differences in luciferase signal at 24 hpi. The vLuc reporter showed strong significant correlation between levels of vRNA, mRNA and luciferase (Table. **3.1**). The cLuc reporter showed lower but still significant correlation between levels of cRNA, mRNA and luciferase

Table 3.1 Pearson correlation analysis between levels of vRNA, cRNA, mRNA and luciferase protein expression

Variable	by Variable	Pearson correlation coefficient (r^2)	*p*-value
vLuc reporter			
cRNA	luciferase	0.29	0.39
cRNA	mRNA	0.41	0.14
mRNA	luciferase	0.97	<.0001*
vRNA	luciferase	0.88	0.0003*
vRNA	cRNA	0.49	0.09
vRNA	mRNA	0.94	<.0001*
cLuc reporter			
cRNA	luciferase	0.63	0.04*
cRNA	mRNA	0.37	0.19
mRNA	luciferase	0.71	0.01*
vRNA	cRNA	0.56	0.04*
vRNA	luciferase	0.54	0.09
vRNA	mRNA	0.45	0.11

*, indicates significant correlation.

3.1.8 Discussion

Previous studies have demonstrated applicability of vRNA-based reporters for sensitive detection of multiple influenza strains (69,233). In this study, for the first time we compared the detection of influenza infection (RdRp activity) using constructs with influenza vRNA or cRNA promoters controlling two popular reporter genes (EGFP and luciferase).

3.1.8.1 Comparison of vRNA and cRNA based reporters

The reporter constructs under the control of the ribosomal Pol I promoter and terminator produce uncapped non-polyadenylated transcripts with precise 5' and 3'ends containing influenza vRNA or cRNA promoters. We and others have demonstrated that such transcripts were recognized by viral RdRp and NP proteins and participated in viral transcription and replication (69,226,233). While both promoters span the same sequences at the end of vRNA and cRNA molecules, their function is different with distinct control mechanisms for positive and negative strand RNA synthesis. The vRNA promoter is responsible for both transcription of genomic vRNAs into mRNAs (vRNA > mRNAs) and for the first step of replication (vRNA > cRNA) via mechanism of terminal *de novo* initiation. The cRNA promoter is not able to generate functional mRNA transcript and is only responsible for the second step of replication leading to production of progeny vRNAs (cRNA>vRNA) via the internal initiation and realignment synthesis model(234). The progeny vRNAs are used as templates to produce more mRNAs in secondary transcription and are packaged into virions. In the course of influenza infection, the synthesis of cRNA occurs early and then plateau's at a relatively low level, while vRNA synthesis takes place later and increases exponentially leading to 10-100 fold higher level of vRNA compared to

cRNA (235). Biochemical and genetic evidence also suggests that distinct regions of the RdRp interact with vRNA and cRNA promoters, and that the vRNA and cRNA replication complexes are structurally and functional distinct, leading to the diverse kinetics of cRNA and vRNA synthesis during infection (236). Recent models suggest that mRNA synthesis occurs in *cis* using the vRNA-associated RdRp, while cRNA/vRNA synthesis occur in *trans* by distinct RdRp (63).

We hypothesized that there might be a higher level of expression of the reporter mRNA using the cRNA promoter. According to the recent replication model, it seems unlikely that Pol I expressed vRNAs can participate in primary transcription, as incoming NP and RdRp proteins were associated with parental influenza vRNAs. The vRNAs expressed by vLuc reporter or vRNAs produced by replication of cRNAs (cRNA>vRNA) expressed by cLuc reporter can only participate in secondary transcription, requiring newly synthesized NP and RdRp. Each molecule of cRNA is used to produce around 10-100 molecules of vRNAs, and it was expected that the level of progeny vRNA achieved with cRNA promoter might be higher leading to higher level of reporter mRNA (235). Also production of vRNA from strong ribosomal promoter may lead to the annealing of the *unencapsidated* (−) vRNA with the (+) mRNAs inducing the dsRNA mediated IFN response, apoptosis and cell death. The expression of (+) cRNAs using cRNA promoter might decrease such possibility.

Dose response and time course studies were conducted to compare the vRNA and cRNA based reporters. Results showed that vLuc reporter was more sensitive at early detection and at low titer of influenza A virus. The lower sensitivity of cLuc was due to its background expression; 2 fold lower expression of luciferase, and around 4 h delay in expression of luciferase. At the same time cLuc demonstrated more rapid

luciferase expression afterwards and at maximum there were no significant differences between both constructs.

The RNA produced by the Pol I ribosomal promoter is known to be uncapped and lack the polyA tail required for export into the cytoplasm and translation by ribosomes. But, contradictory to this, the background signal was detected with both cLuc and cEGFP reporters. This may be due to the cryptic Pol II promoter either overlapping or upstream of the ribosomal Pol I promoter(237). It has also been reported that a small percentage of Pol I ribosomal transcripts can be polyadenylated, transported out of the nucleus and translated at a low level (238–240). The background translation of cLuc can be decreased by the eliminating cryptic Pol II promoter, using more efficient terminator or using hepatitis delta virus ribozyme to cleave polyA tail and produce precise 3'end (241). Similar background transcription likely also happens in vLuc/vEGFP, but as the gene is in antisense orientation in this case, the functional protein cannot be produced in the absence of influenza RdRp. To further measure whether the strong background is produced by

The delayed production of luciferase from the cLuc promoter could be explained by the requirement for an additional replication step to produce progeny vRNA from cLuc reporter. Pol I promoter expressed vRNAs from vLuc reporter can directly participate in secondary transcription (vRNA>mRNA) after *encapsidation* in newly synthesized RdRp and NP proteins. Pol I promoter expressed cRNAs from pHH21-cLuc require an additional replication step (cRNA>vRNA) before secondary transcription. The influenza vRNA synthesis is known to occur late in the infection process, and the delay in luciferase expression for cLuc is equal to around one round of replication (4hr) (235). Our results seem to indicate a bottleneck in cRNA>vRNA

replication. Despite higher levels (3-115 fold) of cRNAs produced by cLuc reporters, and an expectation that 10-100 molecules of vRNA will be produced from each cRNA molecule, the levels of vRNA was consistently 2.4 fold higher for vLuc reporter. This indicates that for the cLuc reporter either not all expressed cRNAs were used for replication, or less vRNA molecules were produced from each cRNA. This might be caused by the deficiency in viral or host factors required for replication (242). Additionally high levels of expressed cRNAs might compete with newly replicated vRNAs for access to RdRp and NP proteins, decreasing stability of vRNAs and inhibiting secondary transcription. Thus it might offer an explanation for the higher production of vRNA and mRNA from vLuc reporter. Our observation of a lower expression of luciferase using the cRNA promoter might also explain the lower yield of infectious virus obtained using reverse genetics 'unidirectional' strategy producing positive sense cRNA (243)

During recent years, nucleic acid-based approaches, especially RNAi-based drugs became a popular tool to inhibit virus replication including influenza A virus (163,164,244,245). Despite the importance of the subject few tools are available to conduct high throughput screening for RNAi-based drugs for influenza. We have demonstrated the application of developing a vLuc reporter for the evaluation of kinetics of an siRNA-based influenza drug, and are planning to test some novel RNAi designs using developed reporters. Eventually high throughput systems with cell-based reporters can be developed for rapid screening and evaluation of novel RNAi based and traditional drugs (246). Currently only two families of antiviral drugs targeting either neuraminidase activity (oseltamivir, zanamivir) or viral ion channel protein M2 (amantadine, rimantadine) are used to treat human influenza infections.

Evolution of influenza strains resistant to both classes of drug has already occurred in nature and the development of novel therapeutic approaches is required. Developed reporters may be especially useful in screening the activity of novel drugs against specific stages of the viral cycle (vRNA>cRNA, cRNA>vRNA, vRNA>mRNA).

Two types of reporter proteins (EGFP, luciferase) were compared in the current study. Firefly luciferase (*luc2*) is easy to quantify and is more sensitive at earlier time points and at low titer, but conversely it require expensive reagents, dedicated equipment (luminometer) and does not allow real time monitoring of infection; as detection require cell lysis. The firefly luciferase also has a short half-life (~3 hours) and loses activity in the late phase of viral infection which might lead to incorrect estimation of viral titer. Application of other luciferases, such as secreted *Gaussia* luciferase, might be beneficial when real time detection is required (68). EGFP reporter can be easily detected under the microscope and allows repeated monitoring of signal from the same cells, but at the same time we observed extensive release of EGFP into cell culture which was not observed for luciferase. This seems to indicate that a high level of EGFP in cells leads to cell lysis. It has been demonstrated previously that high level of EGFP expression is cytotoxic to mammalian cells leading to cell apoptosis (247). As a result, EGFP might be unsuitable for some drug screenings that use cell death as a biomarker.

3.1.8.2 Secondary replication

A comparison of cRNA and vRNA reporters may also shed new light on the replication model of the influenza A virus. Contradictory to the traditional model, which suggest that only a single round of replication takes place and that the cRNA synthesis can use only the incoming parental vRNA not progeny vRNA as templates

(parental vRNA>cRNA>progeny vRNA) (63), we observed a significant 6 fold increase at 24 hpi in the level of vRNA produced by vLuc reporter. We hypothesized that in cLuc reporter transfected and infected cells, the subsequently produced vRNAs strands were all progeny vRNAs because they were produced from cRNAs rather than parental vRNAs , which should be covered by NPs and RdRp. This may have occurred through secondary replication, in which progeny vRNA participate in additional rounds of replication (vRNA>cRNA>vRNA>cRNA>vRNA). Even though such secondary replication has not been reported previously, it seems plausible. It is also well known that progeny vRNAs, after encapsidation by newly synthesized NP and RdRp proteins do participate in secondary transcription(248). Recently a study demonstrated that both mRNA and cRNA are produced at the same time from the same parental vRNA molecule (249), suggesting the possibility of simultaneous secondary transcription and replication from the same progeny vRNA. Our results might suggest the presence of luciferase cRNA produced in vLuc transfected cells arised through secondary replication using progeny vRNA. It is possible that in previous research which used a high MOI of 10, compared to the MOI of 0.1 in our study, that the virus concentration was too high to detect multiple rounds of replication. An alternative explanation is that Pol I expressed luciferase vRNAs might be unstable in the absence of RdRp and NP proteins, resulting in the low level of vRNA at the early phase of infection. Increased levels of newly synthesized RdRp and NP proteins in the late phase of infection might stabilize Pol I expressed vRNAs without a need for additional replication. Further studies would be needed to evaluate these hypotheses.

3.2 Developing SiRNAs Targeting Conserved Regions of Influenza A Viruses.

3.2.1 Introduction

RNA interference (RNAi) is a powerful technology that can knock down gene expression in a sequence-dependent fashion. Synthetic small interfering RNA (siRNA), a 19 bp long dsRNA with 3' overhang of two nucleotides, is one of the most efficient triggers to induce RNAi (108,250). It has been exploited as a therapeutic approach against human diseases such as cancer (251), neurodegenerative disorders (252) and shows promise against viral infections such as respiratory syncytial virus (RSV) (132), HIV(130), and hepatitis B virus (HBV) (131).

Mechanisms of RNA interference (RNAi) were recently explored for potential antiviral approaches to combat influenza virus in several studies. Both synthetic siRNAs (163,244) and vector-expressing shRNAs (253) and artificial microRNAs (146) have been developed against influenza virus replication. Major silencing targets were identified on the coding regions of segments 2, 3, 5 (163,165) and segment 7 of influenza A virus (254–256). These RNAi molecules and constructs effectively inhibit different strains including PR/8/34 (H1N1), WSN/33 (H1N1) and highly pathogenic avian influenza virus strain H5N1 with maximum ~ 40,000 fold reduction of infectious titer in the cell culture and ~10 fold reduction of fifty percent Egg Infectious Dose (EID_{50}) in the chicken embryos (163,257). These studies suggested that the RNAi molecules might directly target the viral mRNA resulting in the reduction of infectious viral titer (163).

One of the biggest concerns for the development of anti-influenza siRNAs is the possible emergence of resistant influenza strains. Although in Sui et al.'s study (166), they found that after 40 passages of the H5N1 strain in a shM2 transformed cell

line, no resistant mutations were developed, when it comes to a real life scenario of infection encompassing mutations and segment reassortment in a mixed influenza virus population, chances of viral escape from siRNA target through a single nucleotide polymorphism are still high. Possible solutions for viral escape include targeting conserved regions and developing siRNA cocktails against multiple conserved regions.

While highly conserved regions conferring important functionalities do exist on some segments of influenza virus genome, those regions are too short for targeting and the selections of those sequences for siRNAs design is highly restricted. Based on the criteria for rational siRNA design by Reynolds et al. (148), very few highly conserved regions could be used for targeting by conventional standard 19 bp siRNAs with 2 nt 3' overhang at each strand (19+2) (108,250) and especially for some of those that have an unfavorable thermodynamic asymmetry – a lower internal stability of 5'termini of the sense strand, compared to that of the antisense strand.

Recently, a variety of novel siRNAs designs having enhanced silencing potencies and reduced off-target effect have been reported. These novel siRNA designs include the introduction of sequence mismatches or bulge structures at the 3'-end of the sense strand or the 5'-end of the antisense strand (258,259), modulation of the 3'overhang sequence and structure (260–262), pre-cleavage of the sense strand at a specific site (263), or the introduction of dumbbell-shaped structure that can improve an siRNA's half life (264), shortened 16-nt duplex siRNA (265) and asymmetric RNA duplexes (266,267). Some of these designs also improved the targeting efficiency of siRNAs in a sequence-independent manner (258). All the structures mentioned above are demonstrated in Figure **3.8**. It was suggested that these designs enhance siRNA

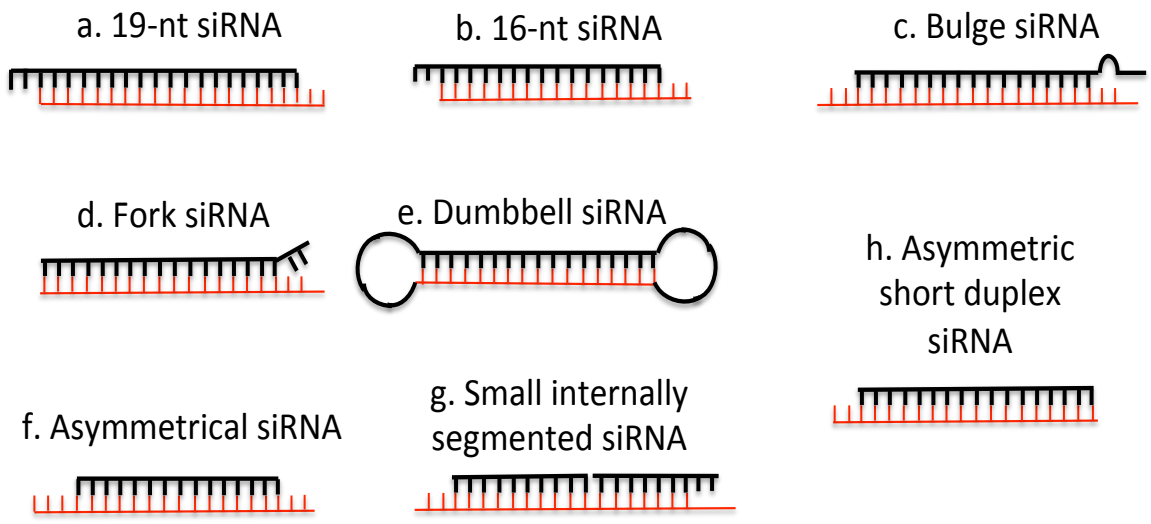


Figure 3.8 **Summerized novel structures of siRNAs.** The picture was adapted from Bramsen and Kjems, 2012 (268). The schematic diagram above described the recent developed novel designs for siRNA. (a) The canonical siRNA with 19-nt and 2-nt overhang (b) Short16-nt siRNA (c) Bulge siRNA (d) Fork siRNA (e) Dumbbell-shaped siRNA (f) Asymmetric short siRNA (g) Small internally segmented dsiRNA

potency by either increasing favorable thermodynamic asymmetries and RISC complex loading of the antisense strand or by giving a more stabilized structure.

In this section, we experimentally improved the siRNAs targeting efficiency of siRNA molecules with unfavorable thermodynamic stability profile. We tried novel modulations to the structure, size and sequence on siRNAs targeting several conserved regions on segments 3, 5 and 7. Those siRNAs were then thoroughly tested for efficacy, off-target effect and inhibition of influenza A virus replication. Antisense strand activities and off-target effects brought by sense strand was measured by reporter plasmids expressing fusion transcripts of luciferase. Finally, the actual inhibitory effect by the novel designs was tested on influenza A virus using the vLuc reporter developed in Chapter 3.1.

3.2.2 Identification of highly conserved regions on influenza A virus genomes

Full-length nucleotide sequences for all segments of influenza A virus strains from three different hosts – human, pig and bird, were retrieved from the NCBI Influenza Virus Database (269). The strains finally obtained were isolated from 1930 to 2009 (Figure **3.9**) and covered 11 hemagglutinin subtypes (H1-3, H5-9, H11, H13 and H16) and 9 neuraminidase subtypes (N1-N9) (Figure **3.10**). For each segment, around 1000 sequences were included for sequence analysis (Table **3.2**). The sequences for each segment were aligned through the multiple alignment program *MUSCLE* version 3.6 (270). Results of multiple sequence alignments were visualized through Jalview program (271) and gaps were removed. A conservation percentage for each nucleotide was calculated at each position based on multiple sequence alignment results. Highly conserved regions with >=10 consecutive nucleotide (including 10) with each position having >99% conservation were selected for siRNA designs.

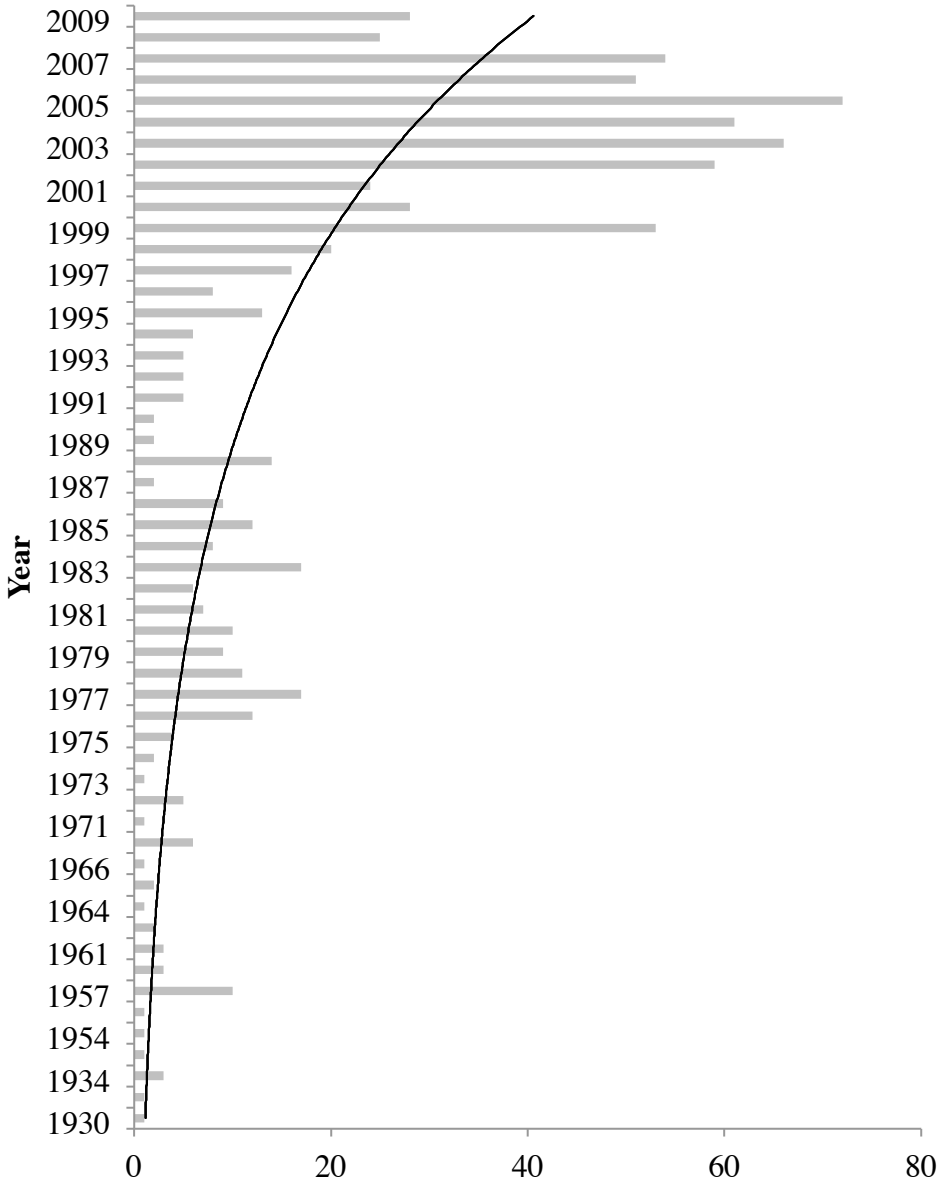


Figure 3.9 Distribution of year of isolation for all influenza A virus strains retrieved from NCBI Influenza Virus database.

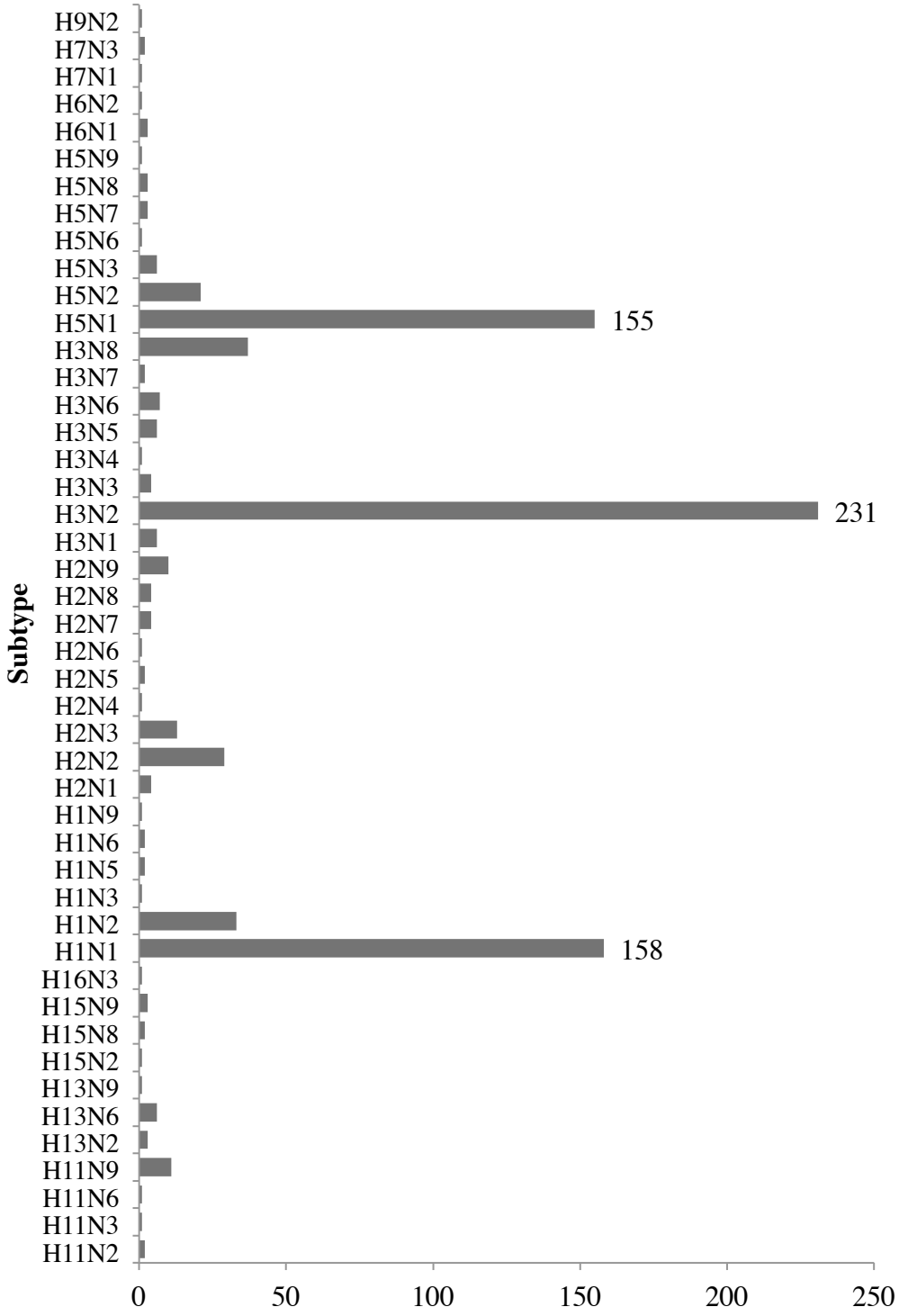


Figure 3.10 Distribution of each subtype of the influenza A virus strains retrieved from NCBI Influneza Virus database.

Table 3.2 Information of all influenza A virus strains retrieved from NCBI Flu database for the multiple sequence alignment analysis

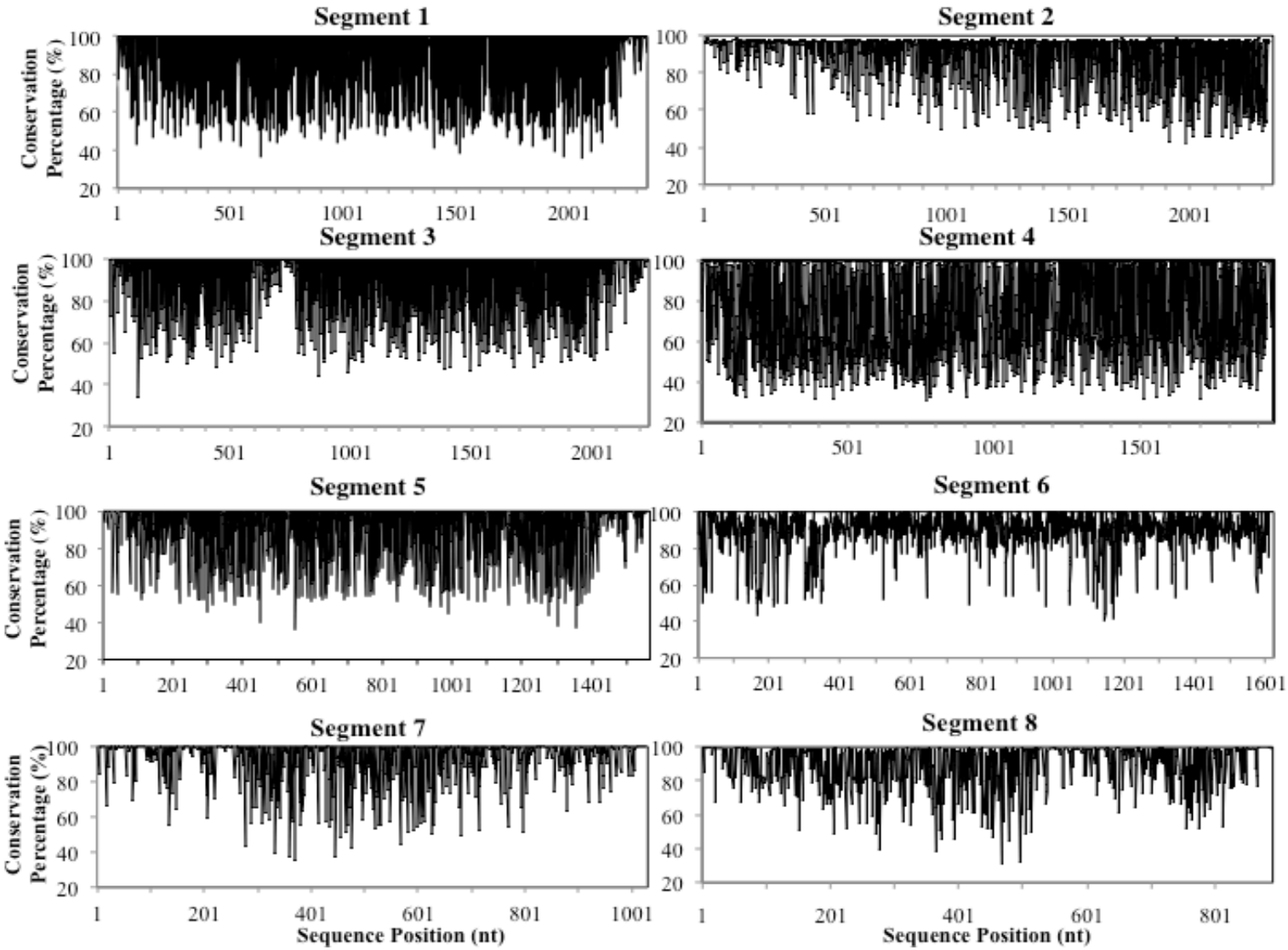
Segment of influenza	Sequences Retrieved	Year Covered	Subtypes Covered*	Human	Swine	Avian
PB2	820	54	81	115	61	644
PB1	752	35	40	123	71	558
PA	853	53	83	125	45	683
HA	794	53	48	297	118	379
M	923	58	84	185	76	662
NA	593	35	31	515	9	69
NS	975	35	89	228	79	668
NP	981	67	83	173	102	706

* Subtypes Covered include different combinations of hemagglutinin and neuraminidase subtypes.

3.2.3 Conservation profiles of inter- and intra- segments of influenza A virus.

Influenza A virus segment 7, encoding two spliced proteins NS1 and NS2, is the most conserved influenza A virus genome segment with a an average conservation of 94%, followed by PA, NP, PB1 and PB2 which are important components of the RdRp for influenza A virus. Segments 4 and 6, encoding the two surface antigens hemagglutinin and neuraminidase which classify influenza A viruses into 18 known HA subtypes and 11known NA subtypes, displayed the largest variation with segment 4 having the lowest average conservation of 73%. Although the average conservation varies across different segments, within each segment, they all had relatively conserved regions at the 5'termini and 3'termini of the genome segment, stretching from ten to fifty nucleotides. In addition, segment 3, encoding the PA protein, has a relatively conserved internal region around position 600 (Figure **3.11**) within the coding region. Highly conserved regions in these segments might be due to the presence of important motifs that could interact with the RNA-dependent RNA polymerase (RdRp) for the influenza virus to regulate replication (272), transcription(273–275) and segment-specific genome packaging (80). There was a large variation on the 4^{th} nucleotide (C4/U4) at the 5' termini of all segments except segment 2 and 6 (Figure **3.11**). This can be explained by its possible regulatory role in viral replication (276,277).

Figure 3.11 Conservation percentage charts for each influenza A virus segment based on results of the multiple sequence alignment. All eight segments were aligned for all strain sequences retrieved. Results were exported and charts were made using Microsoft Excel (2010) program. The X-axis stands for nucleotide position and the Y-axis represents the conservation percentage for each nucleotide.


Segment 1
Segment 2
Segment 3
Segment 4
Segment 5
Segment 6
Segment 7
Segment 8
Conservation Percentage (%)
Sequence Position (nt)

Highly conserved short regions were extracted by manually screening the consensus sequences from multiple sequence alignment results of each segment for regions equal to or longer than10 bp, with each nucleotide having a conservation percentage higher than 99%. Several regions were identified on segment 3, 5 and 7 as shown in Table **3.3**. Five conserved regions were identified on segment 5, six on segment 7 and five on segment 3. Some of the results obtained correspond to previous observations on codon conservation (278) and functional motif identification (279)

3.2.4 Design of siRNAs

To avoid viral escape, siRNAs were designed based on the short conserved regions identified on segments 3, 5 and 7. Five siRNAs (NP-1510, NP-1505, NP-452, M-35 and PA-700) targeting three segments (3, 5 and 7) were designed based on the conserved regions identified on those segments. All of the targeted sequences were blasted against chicken and human expressed sequence tag (EST) database (www.ncbi.nlm.nih.gov/BLAST/) in GenBank. No shared targets were identified in either human or chicken. The locations of all the targets are shown in Figure **3.12**. All of the targets fell within coding regions.

According to the nearest neighborhood methods (280), the thermodynamic properties for each designed siRNA were calculated by summing up the nearest-neighbor ΔG^{0}_{37} value of the first four nucleotides at each strand's 5' termini (Table **3.4**). All five conventional siRNAs were found to have thermodynamically unfavorable antisense strands (a more stable 5' termini on the antisense strand). Conventional 19 bp with 3' dTdT overhang designs along with their thermodynamic

Table 3.3 Regions of <99%(include 99%) conservation in <10bp continuous sequences in three highest conserved segments

Segment 5	Segment 7	Segment 3
5-14	32-54	5-14
17-27	74-93	707-732
55-71	162-174	2114-2130
1510-1523	180-192	2141-2157
1556-1566	224-258	2205-2226
	1007-1029	

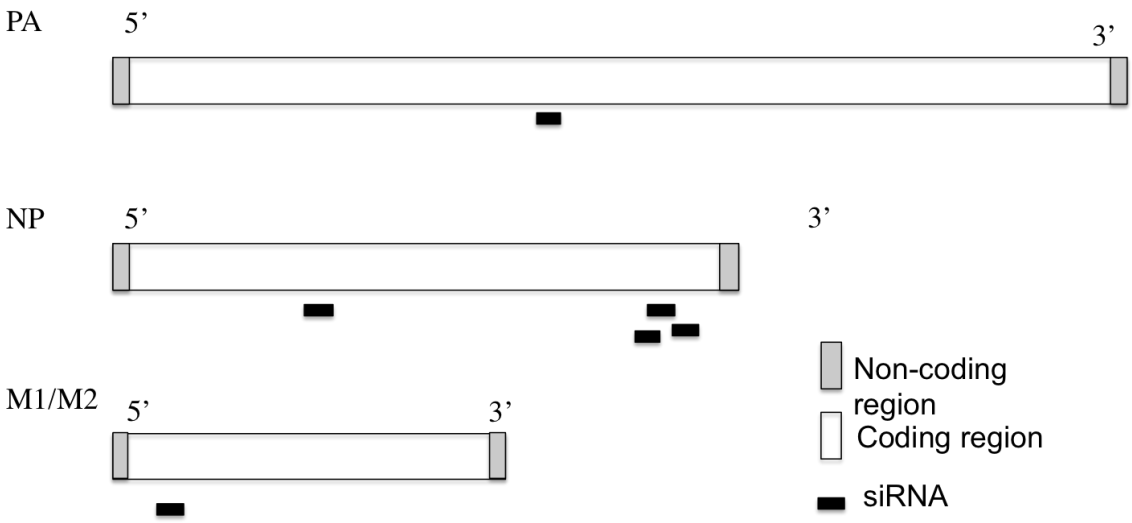


Figure 3.12 Schematic graph depicting the target locations of designed siRNAs on each segment of influenza

Table 3.4 Thermodynamic stability calculation for six conventionally designed siRNAs. The Gibbs free energy (ΔG) of 4nt at each end (5'end and 3'end) were calculated by using the nearest-neighbor method (280). The top strand is the sense strand and the bottom strand is the antisense strand. ΔΔG (A-S) stands for the differences between 5' and 3' ΔG of sense strand. The siRNAs with positive ΔΔG have a more stable 5' end of sense strand and the siRNAs with negative ΔΔG have a more stable 5' end of antisense strand.

			ΔΔG (A-S)	Tm
5'end	NP-1496	3'end		
	GGAUCUUAUUUCUUCGGAG[dTdT]			
ΔG=-10.4	[dTdT]CCUAGAAUAAAGAAGCCUC	ΔG=-10.1	0.3	56.67°C
	NP-1510			
	UUCGGAGACAAUGCAGAGG[dTdT]			
ΔG=-6.6	[dTdT]AAGCCUCUGUUACGUCUCC	ΔG=-8.8	-2.2	62.4 °C
	NP-1505			
	AUUUCUUCGGAGACAAUGC[dTdT]			
ΔG=-9.9	[dTdT]UAAAGAAGCCUCUGUUACG	ΔG=-10.4	-0.5	57.5 °C
	NP-452			
	UGAUGAUCUGGCAUUCCAA[dTdT]			
ΔG=-8.2	[dTdT]ACUACUAGACCGUAAGGUU	ΔG=-9.3	-1.1	60 °C
	M-35			
	CUAACCGAGGUCGAAACGU[dTdT]			
ΔG= - 6.8	[dTdT]GAUUGGCUCCAGCUUUGCA	ΔG= -8.1	-1.3	64 °C
	PA-700			
	UAGAGCCUAUGUGGAUGGA[dTdT]			
ΔG= -7.9	[dTdT]AUCUCGGAUACACCUACCU	ΔG= -9.3	-1.4	62 °C

properties are shown in Table **3.4**. The 5' termini thermodynamic stability of each strand was calculated. As expected, the positive control NP-1496, which was designed and demonstrated to have high effectiveness in inhibition of influenza virus by Ge et al. (163), has a relatively lower thermodynamic stability for the 5' antisense strand compared to the 5' sense strand, although the thermodynamic stability is quite close for the two strands ($\Delta\Delta G$=0.3). However, for the five designs we made targeting other conserved regions on segment 5 (NP-1505, NP-1510 and NP-452), segment 3 (PA-700) and segment 7 (M-35), none of them showed such a preferred thermodynamic stability for the antisense strand (5'ΔG < 3' ΔG).

Based on a conventional 19-bp symmetric siRNA structure, two novel designs, one with the dTdT overhang removed from the 3' terminus of the sense strand and the other with two nucleotides removed from the 5'terminus of the sense strand were applied to NP-1505, NP-1510 as well as a previously demonstrated highly potent siRNA, NP-1496. More novel designs, including the introduction of mismatches (AC mismatch, AG mismatch and UG mismatch), a bulge into the 3' terminus of sense strand, a nucleotide deletion from the 5' terminus of the sense strand and a dumbbell design, were applied to NP-1505. Also, a UG mismatch and AC mismatch were appied to three siRNA NP-452, PA-700 and M-35 (Table **3.5**).

Table 3.5 siRNAs synthesized in this project

Name	Sequences
anti-GFP	5'-GCAGCACGACUUCUUCAAG[dTdT]-3' 3'-[dTdT]CGTCGTGCTGAAGAAGTTC-5'
NP-1496*	5'-GGAUCUUAUUUCUUCGGAG[dTdT]-3' 3'-[dTdT]CCUAGAAUAAAGAAGCCUC-5'
NP-1505	5’-AUUUCUUCGGAGACAAUGC[dTdT]-3’ 3’-[dTdT]UAAAGAAGCCUCUGUUACG-5’
NP-1510	5’-UUCGGAGACAAUGCAGAGG[dTdT]-3’ 3’-[dTdT]AAGCCUCUGUUACGUCUCC-5’
NP-1496 AS	5’-AUCUUAUUUCUUCGGAG[dTdT]-3’ 3’-[dTdT]CCUAGAAUAAAGAAGCCUC-5’
NP-1496 NH	5’- GGAUCUUAUUUCUUCGGAG-3’ 3’-[dTdT]CCUAGAAUAAAGAAGCCUC-5’
NP-1505 AS	5’-UUCGGAGACAAUGCAGAGG[dTdT]-3’ 3’-[dTdT]UAAAGAAGCCUCUGUUACG-5’
NP-1505 NH	5’-AUUUCUUCGGAGACAAUGC[dTdT]-3’ 3’-[dTdT]UAAAGAAGCCUCUGUUACG-5
NP-1510 AS	5’-CGGAGACAAUGCAGAGG[dTdT]-3’ 3’-[dTdT]AAGCCUCUGUUACGUCUCC-5’
NP-1510 NH	5’-UUCGGAGACAAUGCAGAGG-3’ 3’-[dTdT]AAGCCUCUGUUACGUCUCC-5’
NP-1505 sisi	5’-AUUUCUUCGG-AGACAAUGC[dTdT]-3’** 3’-[dTdT]UAAAGAAGCC UCUGUUACG-3’
NP-1505 AG mis	5’-AUUUCUUCGGAGACAAUGA[dTdT]-3’ 3’-[dTdT]UAAAGAAGCCUCUGUUACG-5’
NP-1505 UG mis	5’-AUUUCUUCGGAGACAAUGU[dTdT]-3’ 3’-[dTdT]UAAAGAAGCCUCUGUUACG-5’
NP-1505 AC mis	5’-AUUUCUUCGGAGACAAUAC[dTdT]-3’ 3’-[dTdT]UAAAGAAGCCUCUGUUACG-5’
NP-1505 bulge	5’-AUUUCUUCGGAGACAAUGAC[dTdT]-3’ 3’-[dTdT]UAAAGAAGCCUCUGUUACG-5’
NP-1505 deletion	5’-AUUUCUUCGGAGACAAU-3’ 3’-[dTdT]UAAAGAAGCCUCUGUUACG-5’
NP-1505 dumbbell	A G A G AUAUUUCUUCG-3’ 5’-GAGACAAUGCAGAU U C A A A C U UAUAAAGAAGC CUCUGUUACGUCUA G A G
NP-452	5’- UGAUGAUCUGGCAUUCCAA[dTdT]

	[dTdT]ACUACUAGACCGUAAGGUU
	UAGAGCCUAUGUGGAUGGA[dTdT]
PA-700	[dTdT]AUCUCGGAUACACCUACCU
	CUAACCGAGGUCGAAACGU[dTdT]
M-35	[dTdT]GAUUGGCUCCAGCUUUGCA
	UGAUGAUCUGGCAUUCUAA[dTdT]
NP-452 UG mis	[dTdT]ACUACUAGACCGUAAGGUU
	UGAUGAUCUGGCAUUCC
NP-452 deletion	[dTdT]ACUACUAGACCGUAAGGUU
	UAGAGCCUAUGUGGAUGAA[dTdT]
PA-700 AC mis	[dTdT]AUCUCGGAUACACCUACCU
	UAGAGCCUAUGUGGAUG
PA-700 deletion	[dTdT]AUCUCGGAUACACCUACCU
	CUAACCGAGGUCGAAACAU[dTdT]
M-35 AC mis	[dTdT]GAUUGGCUCCAGCUUUGCA
	CUAACCGAGGUCGAAAC
M-35 deletion	[dTdT]GAUUGGCUCCAGCUUUGCA

* NP-1496 sequence was obtained from Ge et al. (2003) (163)

**sisiRNA has a cleavage at the sense strand. Slash means where the cleavage site is.

3.2.5 Construction of reporter plasmids

For ease in testing siRNA efficiency, two types of reporter plasmids were constructed: Plasmids expressing luciferase-nucleoprotein fusion transcript and vLuc reporter plasmid developed in Chapter 3.1 used as indicator for influenza replication activity. Fusion transcript expressing recombinant proteins were constructed based on the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Inc.). The plasmid map of pmirGLO is shown in Figure **3.13**. The full-length nucleoprotein (NP) gene from influenza A/PR/8/34 (H1N1) was cloned into the *Xho*I site on pmirGLO plasmid at the 3'-untranslated region (UTR) of the luciferase gene. First strand cDNAs of all viral mRNAs were obtained by reverse transcription of extracted viral RNA using primer RT-PR8, a non-specific oligo-dT primer tagged with *Xho*I restriction site (Appendix). Second strand was then PCR amplified using an NP-specific forward cloning primer Luc-NP/PN-F with overlapping sequence at 5'end of NP gene and a reverse cloning primer Luc-NP/PN-R similar to RT-PR8, both of which were tagged with *Xho*I restriction sites (Appendix).

Double stranded full-length NP cDNA was digested by *Xho*I following manufacturer's protocols and ligated to *Xho*I digested pmirGLO plasmid by T4 ligase as described in Chapter 2.2. The presence and orientation of the NP gene insertion were checked by colony PCR using Luc-test-F1 and Luc-test-R1 for the sense orientation and Luc-test-F2 and Luc-test-R2 for the antisense orientation. The completed plasmids, Luc-NP (sense insertion of NP) and Luc-PN (antisense insertion of NP), were confirmed by sequencing (Figure **3.13**). The expression level of firefly luciferase is the indicator of miRNA/siRNA targeting efficiency.

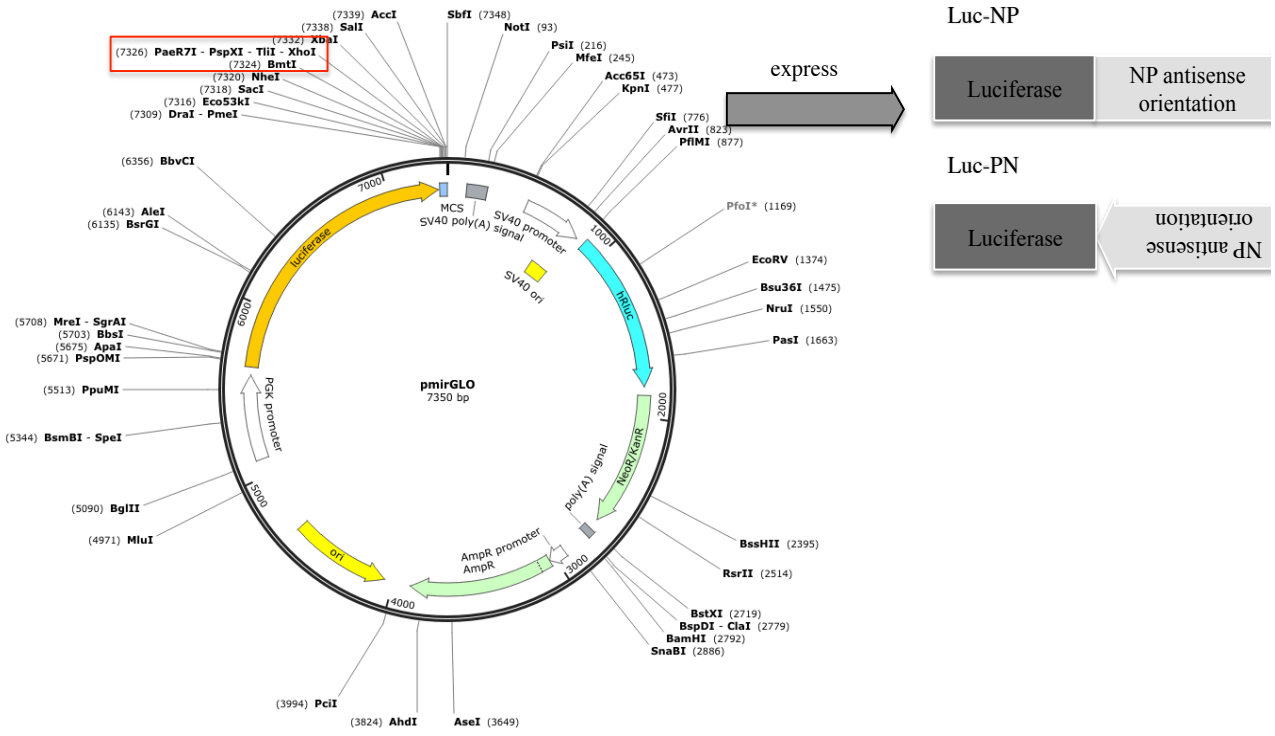
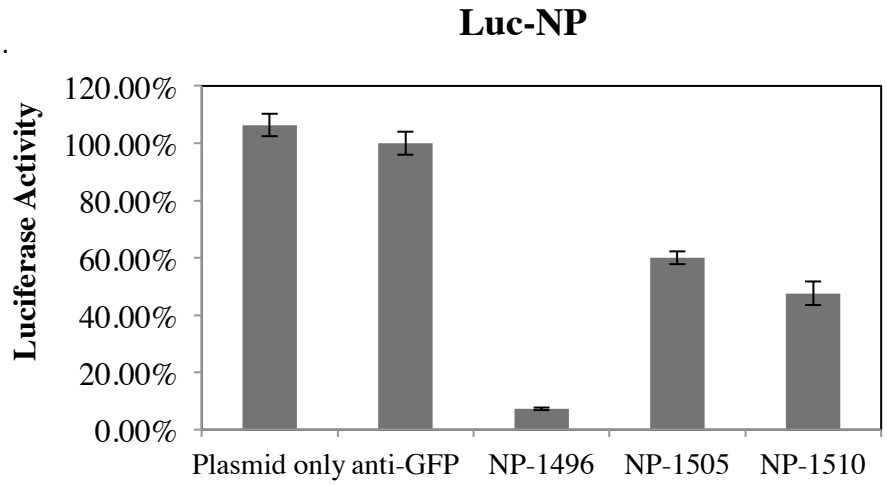


Figure 3.13 Schematic diagram showing pmirGLO plasmid map and its expression of luciferase-nucleoprotein fusion transcripts. The plasmid map was drawn using SnapGene® software plasmid map software (from GSL Biotech; available at snapgene.com). The pmirGLO contains two luciferase gene, one primary reporter gene, firefly luciferase gene (*luc2*) and one control reporter gene, *Renilla* luciferase gene (*hRluc*-neo) for normalization and selection. The nucleoprotein (NP) gene (in sense or antisense orientation) at the *Xho*I site (marked with red box in the graph) was cloned from influenza A/PR/8/34(H1N1) strain. The recombinant plasmid can produce a luciferase-sense NP or luciferase-antisense NP fusion transcript.

3.2.6 Optimization of siRNA designs and an examination of the targeting efficiency of reporter plasmids Luc-NP and Luc-PN

Three conventionally-designed siRNAs, NP-1496, NP-1505 and NP-1510 targeting three overlapped regions at the 3' end of the nucleoprotein coding region, were examined on plasmids Luc-NP and Luc-PN, which produced fusion transcripts with the full-length nucleoprotein (NP) cDNA cloned at the 3' untranslated region (UTR) of a luciferase gene in either sense (Luc-NP) or antisense orientation (Luc-PN). These two reporters allowed us to monitor both 'desired' silencing (by the antisense strand of the siRNA) and 'off-target' silencing of fusion transcript (by the passenger strand) by measuring luciferase gene expression levels. A anti-GFP siRNA served as a negative control. It does not have any predicted target on either nucleoprotein or luciferase gene. Twenty pmol of anti-GFP, NP-1496, NP-1505 or NP-1510 were co-transfected individually with 100 ng Luc-NP/Luc-PN into HEK293 cells into a 96-well plate. Cells were also mock transfected with Lipofectamine^{TM} 2000 as a cell toxicity control. Luc-NP/Luc-PN were also transfected alone into HEK293 cells as a control for non-specific silencing by siRNAs. After 48-hr incubation, cells were lysed for the luciferase assay. Results are shown in Figure **3.14**. As a negative control, cells co-transfected with the negative control anti-GFP siRNA and with Luc-NP/Luc-PN exhibited no significant reduction of luciferase level compared to cells transfected with Luc-NP/Luc-PN only. This indicated that there were no non-specific siRNA effects on luciferase activity. For the three conventional-designed siRNAs, although they share 30%-70% sequence similarity, due to their different thermodynamic stability profiles, each exhibited large differential inhibition effect on luciferase production by plasmid Luc-NP.

a.



b.

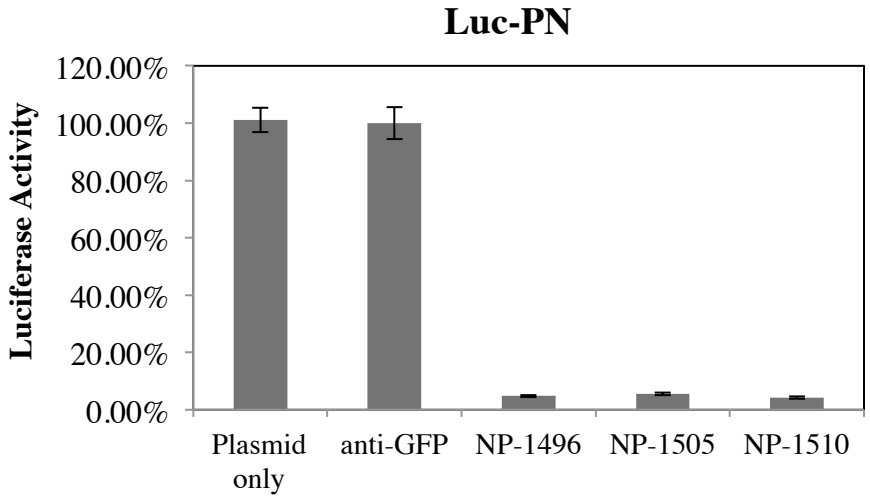


Figure 3.14 Luciferase assay for conventional siRNA designs on plasmid Luc-NP for antisense/guide strand activity) and Luc-PN for sense/passenger strand activity. HEK293 cells were cotransfected with Luc-NP (a) or Luc-PN (b) and conventional-designed siRNAs. Luciferase activity was measured after 48 hr post transfection. The error bars represent standard error (N=4 replicates). The value for anti-GFP was considered 100%. Plasmid only, plasmid Luc-NP/Luc-PN was transfected alone into cells; anti-GFP, reporter plasmid was co-transfected with anti-GFP siRNA. 20 pmol/well siRNAs and 100 ng reporter plasmids Luc-NP/Luc -PN were used in all experiments.

The positive control NP-1496 showed high efficacy with a >90% reduction of the luciferase signal compared to only ~40% and ~50% reduction by siRNAs NP-1505 and NP-1510 on Luc-NP. On the other hand, for the sense strand targeting efficiency examined by Luc-PN, all of the three designs exhibited similarly high potency with >90% luciferase reduction. This might be explained by the close thermodynamic stability of NP-1496 and the reversed thermodynamic asymmetry by NP-1505 and NP-1510. Two termini with close thermodynamic stability could possibly make each strand equivalently potent in silencing while the reversed thermodynamic asymmetry of siRNAs might render the sense strand more potent in silencing. Based on previous researches on novel designs of siRNAs, preliminary design optimizations were applied to NP-1510 and NP-1505. Asymmetric siRNA designs with sense strand having two nucleotides trimmed from 5' end (AS) or with sense strand having 3' overhang removal (NH) were made for targeting sites NP-1505, NP-1510 as well as NP-1496 for the control purpose. Six novel siRNA designs are shown in Table **3.5** and were tested similarly on Luc-NP/Luc-PN plasmids. Results were shown in Figure **3.15**. For highly active NP-1496, both asymmetric designs (AS and NH) led to 2-3 fold decreased activity of the antisense strand as well as for the passenger strand (2-fold for NH design) (Figure **3.15** a). For poorly active NP-1505 both asymmetric designs led to an increased activity of antisense strand (1.5 fold for AS, and 2.2 fold for NH design; $p<0.05$). Little changes were observed for sense strand (seen in Figure **3.15** b). For poorly active NP-1510 both asymmetric designs led to increased activity of the antisense strand (~2-fold, $p<0.05$), while 3-fold decreased activity of sense strand with the AS design ($p<0.05$) (seen in Figure **3.15** c).

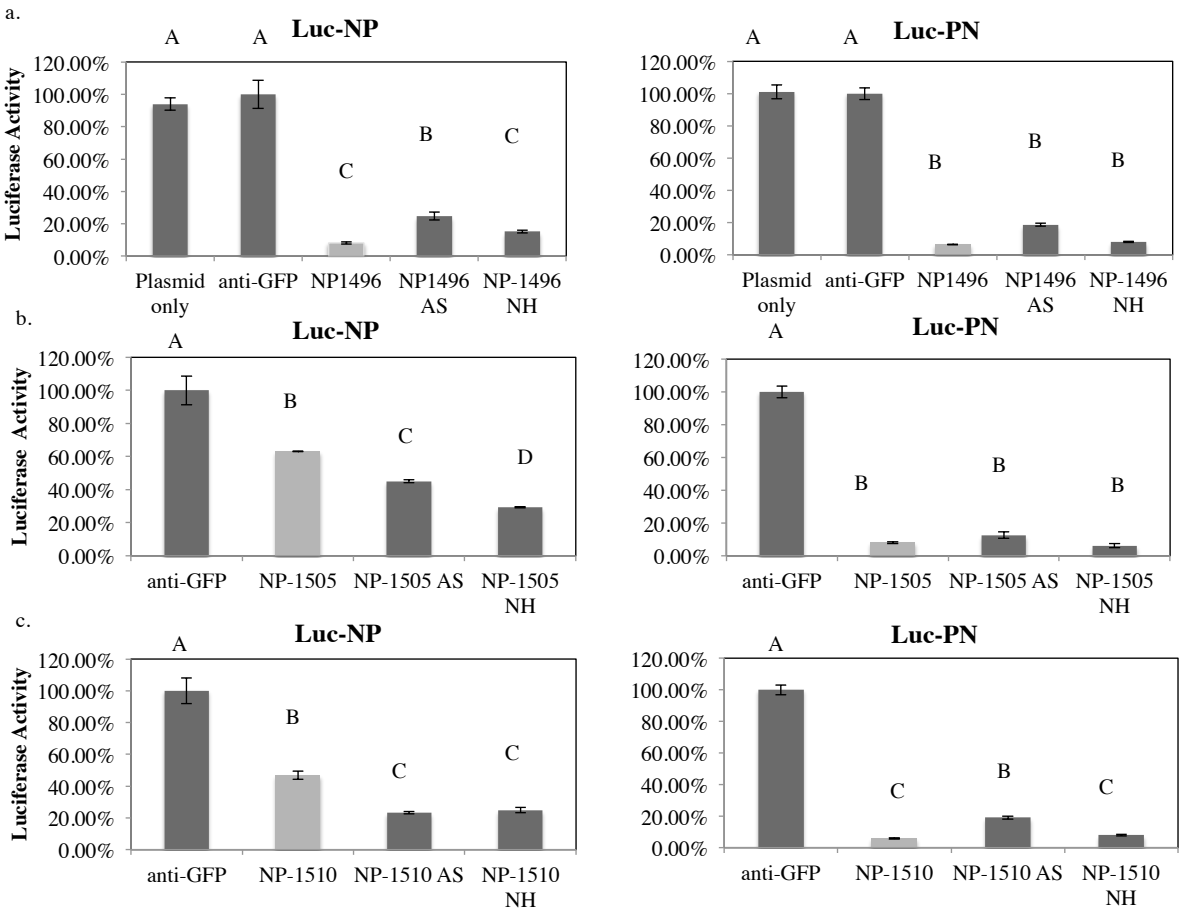


Figure 3.15 Luciferase assay test for silencing activity of conventional and novel designs of siRNA NP-1496, NP-1505 and NP-1510. HEK293 cells were co-transfected with Luc-NP/Luc-PN and siRNAs targeting three overlapping sites with different novel designs, NP-1496 (a), NP-1505 (b) and NP-1510 (c). Luciferase activity was measured after 48 hr post transfection. The error bars represent standard error (N=4 replicates). The luciferase activity from anti-GFP treated cells was considered 100%. Plasmid only, plasmid Luc-NP/Luc-PN was transfected alone; anti-GFP, plasmids was co-transfected with anti-GFP siRNA; Grey bars, plasmids were cotransfected with conventional designs; NP-1496/1505/1510 AS, plasmids was co-transfected with siRNA designed with two nucleotides trimmed from 5'end of sense strand based on the conventional design; NP-1496/1505/1510 NH, siRNA design with 3' overhang removed from sense strand based on the conventional design. Samples with different letters (A, B, C) were statistically different using Tukey's test (p<0.05).

This might mean that for highly active siRNAs (NP-1496) any modifications might be deleterious, while for siRNAs with reversed thermodynamic stability profiles (stable 5'end and unstable 3'end of sense strand) and poor activity of antisense strand, it is possible to increase the activity of the desired antisense strand at least 2 fold and to decrease the 'off-target' effects of the passenger strand by using asymmetric designs. Similar results were reported by Hirohiko et al.(258), where it was found that the introduction of mismatches at the 3'end of siRNA duplexes enhanced siRNA silencing with moderate RNAi activity but not siRNA with strong RNAi activity. Still even after optimization NP-1505 and NP-1510 siRNAs did not reach the activity of the highly effective NP-1496.

3.2.7 Further optimization of siRNA NP-1505 by novel designs

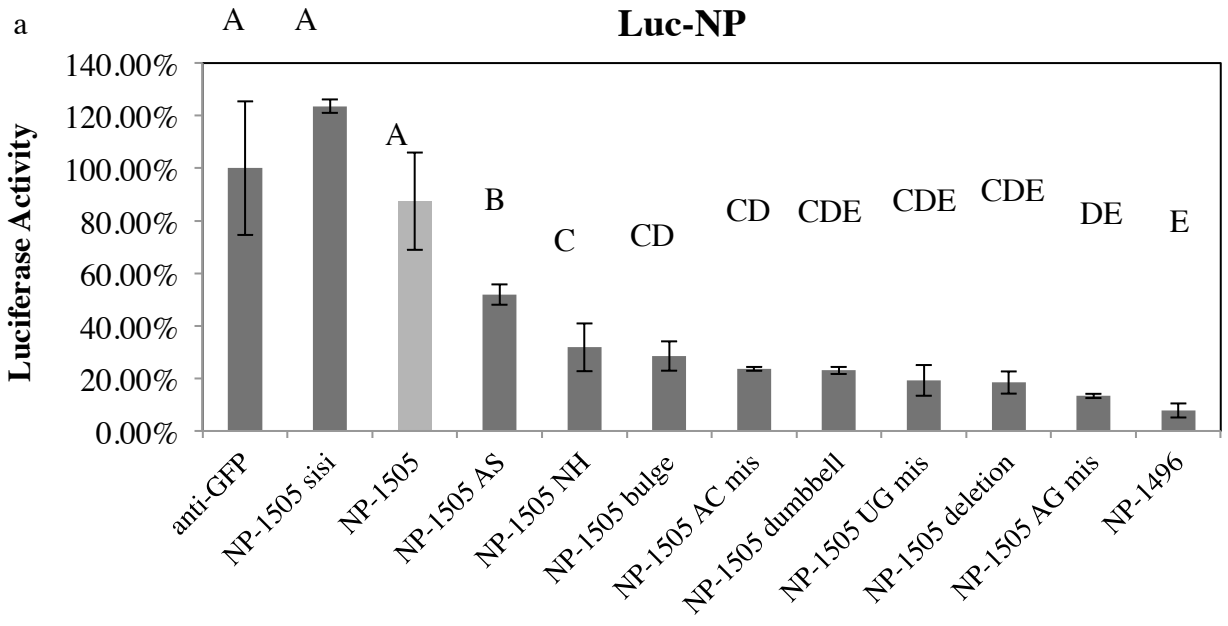
The most poorly active antisense strand, NP-1505 was chosen for further optimization: AG, UG and AC mismatches, a bulge or a 5' end deletion (2 nt and overhang) were introduced into 3' sense strand in order to destabilize the 5' antisense strand. Small internal segmented siRNAs (sisi) NP-1505 was designed carrying a cleavage at the sense strand between the 10-11bp in order to reduce the off-target effect from the passenger strand. The novel NP-1505 dumbbell-shaped siRNAs was designed similarly to NP-1505 sisi pre-cleavage at 9-10bp but it also possess an elongated stem and two 9-nt loops at each end to stabilize the siRNA cleaved structure (Table **3.5**). It was designed based upon Abe et al.'s study (264) that showed such a shaped siRNA could be efficiently processed by Dicer with prolonged RNAi activity. Then we tested all the NP-1505 novel designs along with the asymmetric designs and conventional NP-1505 designs on Luc-NP and Luc-PN constructs using anti-GFP as a negative control and NP-1496 as positive control. Experiments were done as described

above. The results (Figure **3.16**) showed that, not surprisingly, NP-1505 with the sisiRNA design was ineffective. It might be the annealing difficulties for small segmented RNAs or their instability in cell culture, which corresponds to the previous experiments that showed sisiRNA molecules, without any modifications, could be completely degraded when incubated with 10% FCS (263).

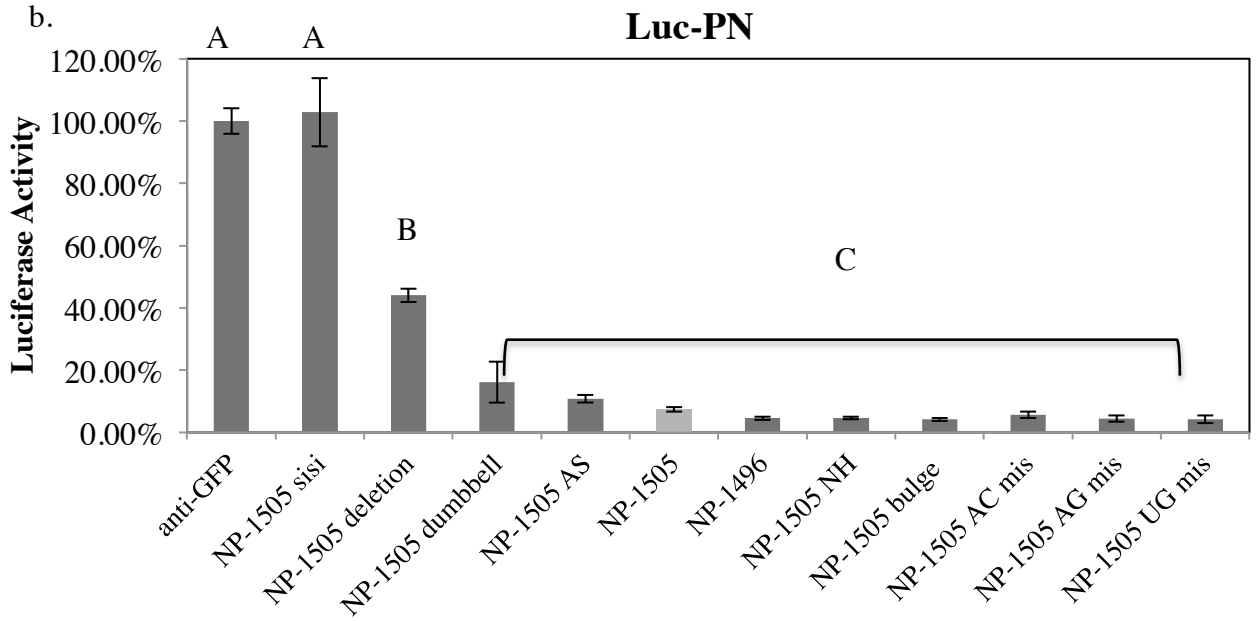
Except for the sisiRNAs, all of the novel designs showed significant improvement in antisense strand activity ($p<0.05$), about 3-6.7 fold when compared to conventional NP-1505 design and about 1.5-3.7 fold significant increase ($p<0.05$) when compared to the asymmetric NP-1505. Moreover, some of the designs, NP-1505 dumbbell, UG mismatch, AG mismatch and deletion, exhibited comparable effectiveness to the positive control NP-1496 according to the Tukey's statistical test (Figure **3.16**). However, for the sense strand targeting test, when compared to the conventional NP-1505, only the NP-1505 deletion design significantly reduced activity of the sense strand (6-fold, $p<0.05$) and the NP-1505 dumbbell design seems to be reduced by a 2-fold ($p>0.05$). This probably suggests that the only NP-1505 deletion design successfully reversed the effect of the thermodynamic asymmetry at each end with a highly active antisense strand and a less active sense strand. However, the other novel designs improved antisense strand activity without compromising sense strand activity. Interestingly, the dumbbell designs with pre-cleavage did not eliminate the sense strand's 'off-target' as effect predicted in Bramsen et al.'s work (263). This might be due to the elongated stem (23 bp) that made each of the segmented sense strand active again.

Figure 3.16 Luciferase assay test for silencing activity of all NP-1505 siRNA novel designs on plasmid Luc-NP and Luc-PN. HEK293 cells were cotransfected with the NP-1505 novel designs and Luc-NP(a) or Luc-PN(b) and cells were lysed at 48 hpi for luciferase assay. The error bars represent standard error (N=4 replicates and 3 individual tests). The luciferase activity from anti-GFP treated cells was considered 100%. Anti-GFP, reporter plasmid was co-transfected with anti-GFP siRNA; sisi-1505, NP-1505 siRNA design with a cleavage at 10-11nt at the sense strand; Deletion, with 2nt deletion at the 3'end of the sense strand and no overhang; Dumbbell, novel NP-1505 design annealed from a single RNA strand; Bugle, design with an extra A nucleotide between 1st and 2nd nucleotide of sense strand; AC, design with AC mismatch at the 3' sense strand; AG, design with AG mismatch at the 3' sense strand; UG, design with a UG mismatch at the 3'sense strand. Grey bars, NP-1505 conventional design. Statistical analysis was done by Tukey's test. Different characters represent significant difference between different siRNA treatments ($p<0.05$).

a


Luc-NP
Luciferase Activity
140.00%
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
A A A B C CD CD CDE CDE CDE DE E
anti-GFP
NP-1505 sisi
NP-1505
NP-1505 AS
NP-1505 NH
NP-1505 bulge
NP-1505 AC mis
NP-1505 dumbbell
NP-1505 UG mis
NP-1505 deletion
NP-1505 AG mis
NP-1496

b.


Luc-PN
Luciferase Activity
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
A A B C
anti-GFP
NP-1505 sisi
NP-1505 deletion
NP-1505 dumbbell
NP-1505 AS
NP-1505
NP-1496
NP-1505 NH
NP-1505 bulge
NP-1505 AC mis
NP-1505 AG mis
NP-1505 UG mis

3.2.8 Dose response study for NP-1505 novel designs

As shown above, different novel designs have improved the NP-1505 antisense strand targeting efficiency. All novel designs, except NP-1505 sisi, were chosen for a dose response study. To determine the most effective dose of each novel-designed siRNAs conferring maximum antisense strand activity without over-saturation of RISC complex and toxicity to cells, a dose response study for novel designs was conducted using Luc-NP as an indicator. Experiments were conducted as described above. Two to ten pmol, 2 pmol or 20 pmol of anti-GFP, novel NP-1505 or positive control NP-1496 siRNAs were cotransfected with plasmid Luc-NP individually into a 96-well plate containing 150 μL medium with final concentrations as 0.001 μM, 0.01 μM and 0.1 μM of siRNAs. Similar to the above experimental procedures, cells were lysed after a 48-hr incubation and luciferase activity was determined. Transfected cells were observed before lysis and no significant cell toxicity effect was observed compared to the mock-transfected cells. Results showed that (Figure **3.17**).
The 0.1 μM siRNA appear to be the most effective dose for all of the novel designs. All novel designs achieved maximum inhibition of luciferase production and no obvious toxicity for cells were observed for HEK293 cells under microscope after 48-hr incubation at this dosage.

Besides, it is clear to see that different designs perform differently under dosage changes. Conventional NP-1505, the novel NP-1505 AS, NP-1505 NH, NP-1505 Bulge and the NP-1505 UG mis designs completely lost their silencing activity for antisense strand when the dosage was dropped 10-fold to 0.01 μM, while NP-1505 AC, NP-1505 Dumbbell, NP-1505 Deletion, NP-1505 AG and the positive control NP-1496 still effectively inhibited luciferase expression on Luc-NP by ~10% -50%.

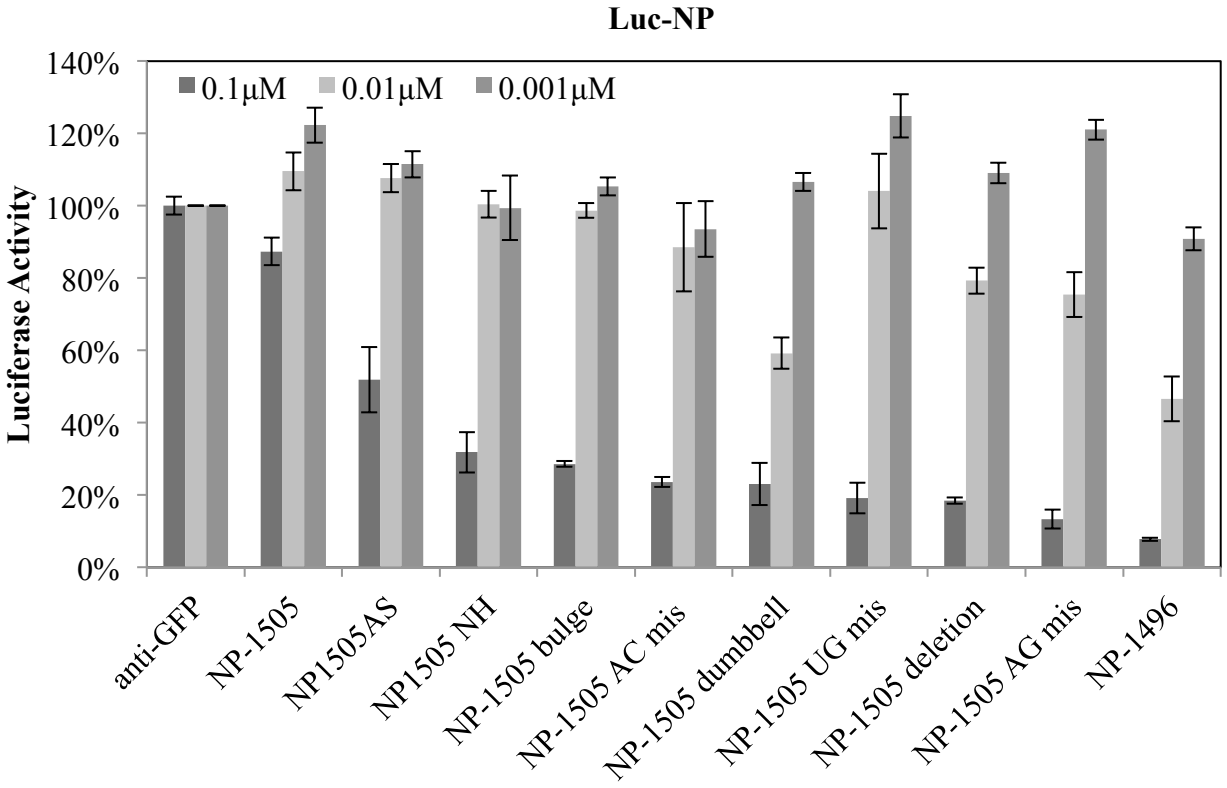


Figure 3.17 Dose response study based on luciferase test on Luc-NP for different novel designs of NP-1505. Luc-NP was co-transfected with chosen NP-1505 novel designs at different concentrations (0.1μM, 0.01μM and 0.001μM). Luciferase assay was conducted 48 hr post transfection. Error bars represent standard error (N=4 replicates).

When the dosage decreased further to 0.001 μM, all of the designs lost their silencing activities against Luc-NP. This indicates that the antisense strand is not effective at low dosages.

3.2.9 Kinetic study of anti-influenza NP-1496 siRNA using vLuc reporter construct

The vLuc reporter constructs were tested as a tool to evaluate *in vitro* silencing kinetics of siRNAs. The NP-1496 siRNA targeting the NP segment (Ge et al., 2003) was chosen for test. The vLuc reporter demonstrated that NP-1496 siRNA significantly decreased the influenza RdRp activity by around 335 fold for 5 pmoles ($p<0.05$) and 862 fold for 10 pmoles ($p<0.05$) of siRNA by 24 hpi compared to anti-GFP treatment (Figure **3.18**). There were no significant differences between 5 pmoles and 10 pmoles siRNA treatments ($p>0.05$).

3.2.10 Inhibition of actual influenza virus replication by novel siRNAs

To study the actual inhibition of siRNAs on influenza virus,
The reporter vLuc developed was utilized as an indicator for influenza virus replication. The experiments were conducted by cotransfecting vLuc along with different novel designs into HEK293 cells in a 96-well plate. Cells were then infected with A/PR/8/34 (H1N1) strain at a MOI of 0.1 18h post transfection. At different time post infection, cells were lysed and assayed for luciferase activity.

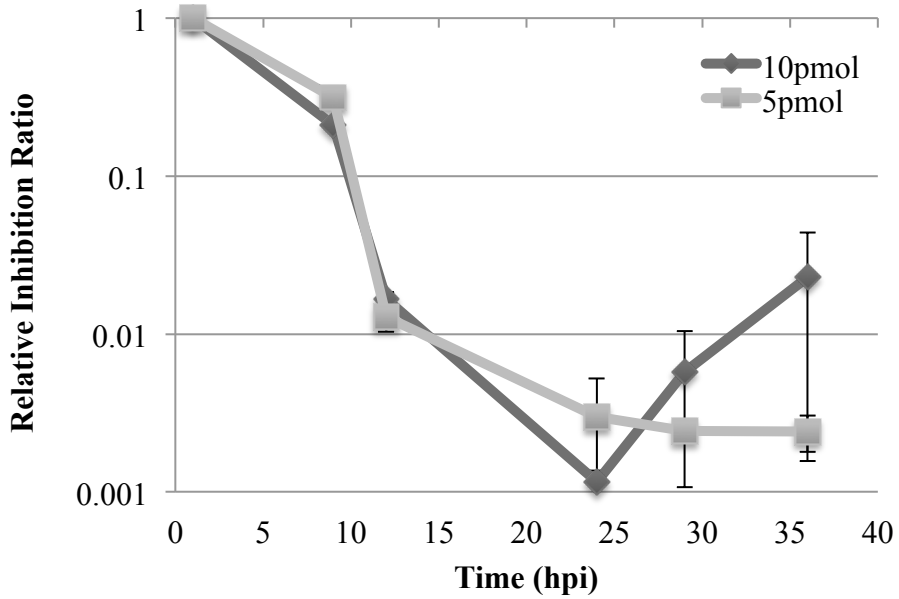


Figure 3.18 Kinetic study of anti-influenza NP-1496 siRNA using vLuc reporter construct. HEK293 cells were reverse co-transfected with vLuc constructs and NP-1496 or anti-GFP siRNA (5 or 10 pmoles per well) in 96 well plate and infected with A/Puerto Rico/8/1934 at M.O.I = 0.01 at 18 hr post infection. Cells were lysed at different time post infection and luciferase activity was determined. Relative inhibition of influenza RNA polymerase was determined by dividing activity of NP-1496 by activity of anti-GFP siRNA treated cells. Each treatment was done in triplicates and statistical analysis was done by Student t-test. Indicate significant differences ($p<0.05$) between NP-1496 and anti-GFP treated cells: *, 5 pmoles per well; #, 10 pmoles per well.

Results were shown as Figure **3.19**. Anti-GFP did not reduced luciferase activity, indicating that there were non-specific inhibition effects on influenza virus replication. Clearly, all designs showed different trends in inhibiting influenza though they demonstrated strong inhibition of luciferase activity of the Luc-NP plasmid. In general, most of the novel designs did not show high silencing activity. NP-1505 NH and bulge, which conferred high activity on Luc-NP silencing, had no significant inhibition effect on influenza virus replication. NP-1505 AC, UG mismatch and deletion had reduced inhibition potency of influenza replication compared to that on Luc-NP. On the other hand, the dumbbell shape designed NP-1505 even showed higher potency than it had on Luc-NP in the prior test. Notably, NP-1505 Dumbbell has a much more stable structure than the other novel designs. This suggests that during influenza replication, siRNAs with high potency might lose efficacy. Although all of the designs target the same region on the same segment and showed similar potency in silencing, other factors might affect their inhibitory effect on influenza replication

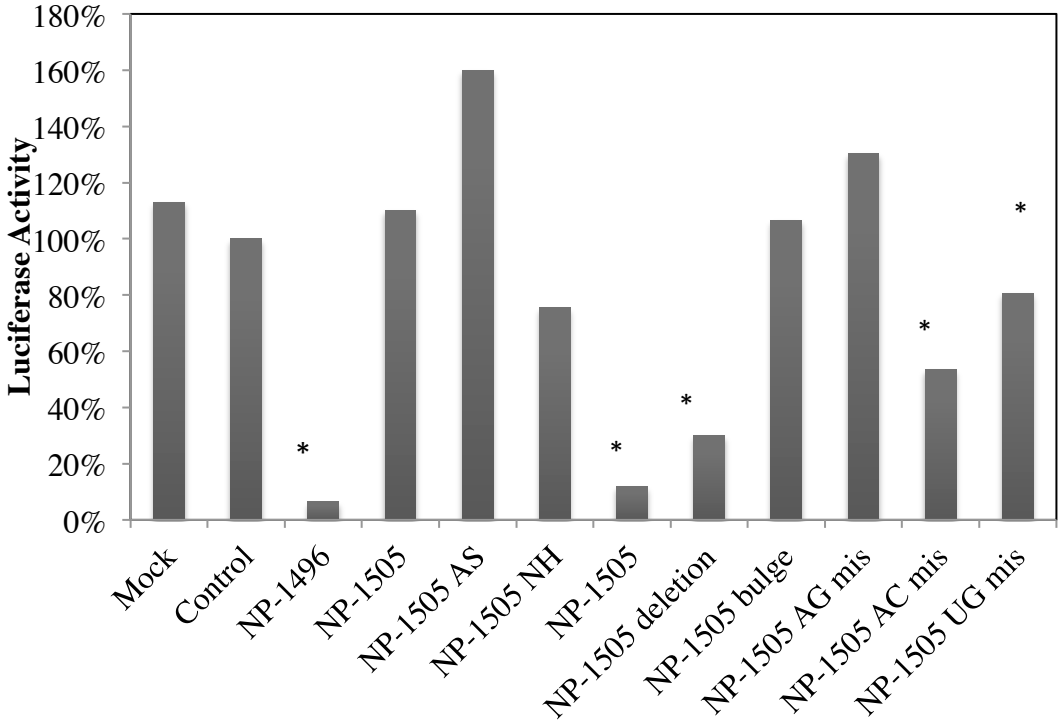


Figure 3.19 Luciferase assay test of conventional/novel designed siRNA NP-1505 inhibitory effect on actual influenza virus using reporter plasmid vLuc as an indicator for activity of viral replication. HEK293 cells were cotransfected with novel designs of NP-1505 and vLuc reporter plasmid. 18 hr after transfection, cells were infected with influenza PR/8/34 (H1N1) at MOI = 0.1. 24 hr. Error bars represent standard error (N=4 replicates). Statistical analysis was done by Tukey's test. Different characters represent significant difference between different siRNA treatments (p<0.05).

3.2.11 Selection and design optimization of potent siRNAs targeting other conserved regions

Based on the performance in the antisense strand activity and inhibition of actual influenza virus replication of novel designs for siRNA NP-1505 and the ease of application, the three best designs, AC, UG mismatch and deletion were applied to three other conserved sites: NP-452, PA-700 and M-35, all of which had the unfavorable thermodynamic asymmetries as described in Table **3.4**. Based on the sequence differences, different designs were applied. An AC mismatch was applied to the second nucleotide of the PA-700 5' sense strand and the second nucleotide of M-35 5' sense strand, while a UG mismatch was applied to the third nucleotide of the NP-452 5' sense strand. Deletion designs were made universally for all three sites by removing two nucleotides along with the dTdT overhangs from the 5' sense strand. In total six novel designs along with their original conventional designs were tested for their inhibitory effect on influenza replication. NP-452 and its derivatives were also tested with the Luc-NP plasmid (data not shown).

To test their efficiency, vLuc was co-transfected individually with the newly designs siRNAs mentioned above as well as with the negative control anti-GFP into HEK293 cells and then cells were infected with the A/PR/8/34 (H1N1) strain at an MOI of 0.1 18-hr post transfection. Finally, 24-hr post infection, cells were lysed and evaluated for luciferase assay. The luciferase expression level indicated the influenza replication activity. As shown in Figure **3.20**, different effects were observed for the three individual targets. For NP-452, it is noteworthy that with a relatively unfavorable thermodynamic asymmetry (Table **3.4**), the conventional siRNA NP-452 showed relatively high silencing activity with ~80% inhibition on Luc-NP (Figure **3.20**) and ~70% inhibition on influenza virus replication in terms of luciferase expression from

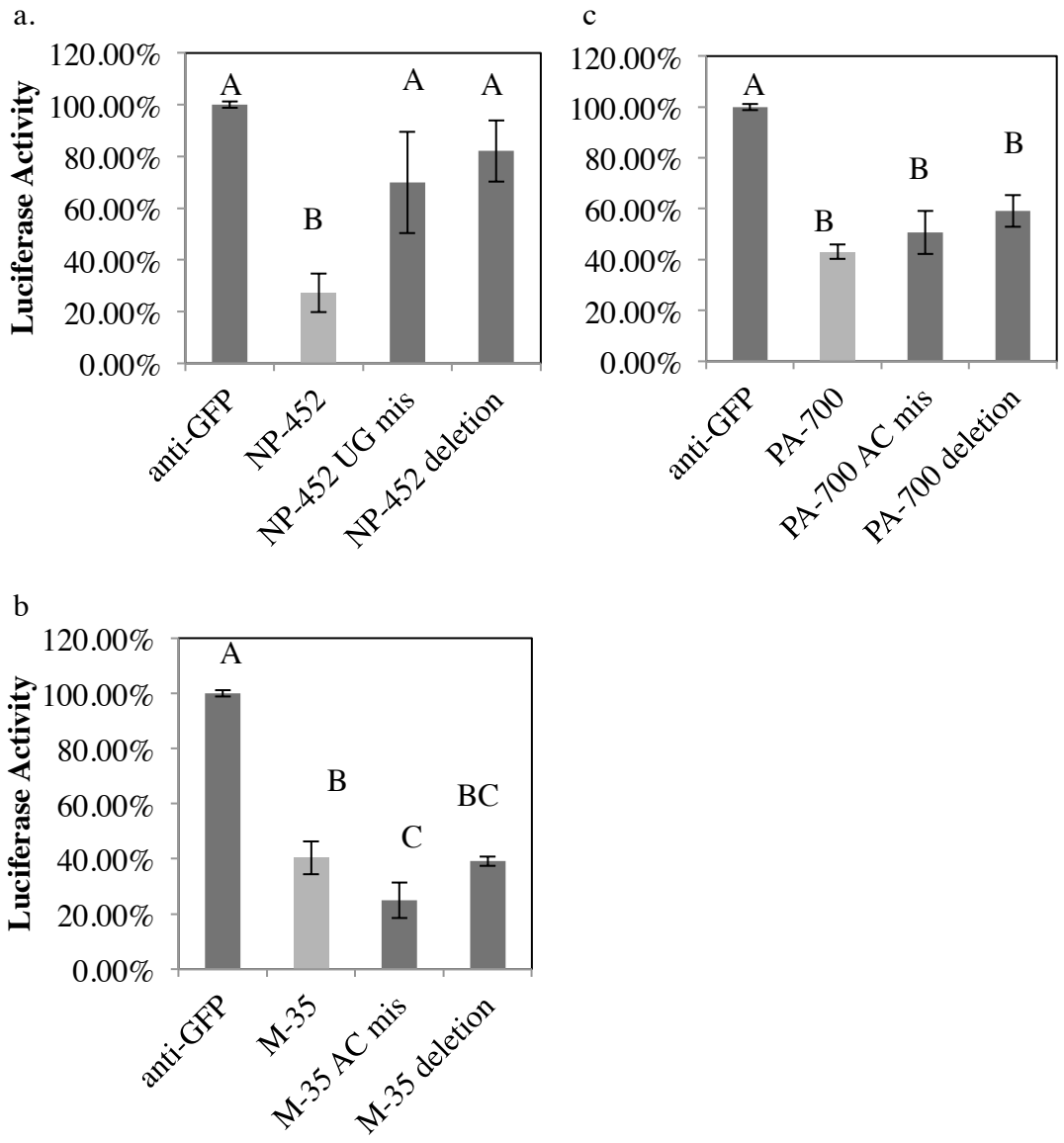


Figure 3.20 Luciferase assay test of conventional/novel designed siRNA NP-452, PA-700 and M-35's inhibitory effect on actual influenza virus using reporter plasmid vLuc as an indicator for activity of viral replication. HEK293 cells were cotransfected with novel designs of NP-452/PA-700/M-35 and vLuc reporter plasmid. 18 hr after transfection, cells were infected with influenza PR/8/34 (H1N1) at MOI = 0.1. 24 hr 1error bars represent standard error (N=4 replicates). Statistical analysis was done by Tukey's test. Different characters represent significant difference between different siRNA treatments (p<0.05).

vLuc plasmid. Neither of the novel designs managed to improve its silencing activities but rather be deleterious. For PA-700, similarly, both novel designs failed to improve its silencing activities to a significant level. Finally for M-23, compared to the conventional M-35, the AC mismatch design showed significant silencing by increasing inhibition of influenza virus in terms of luciferase expression to 15%, while the deletion design did not show any significant change in activity. In summary, the six best designs with different modifications that both had good silencing efficacy on Luc-NP and inhibition of influenza replication were selected (Table **3.6**).

3.2.12 Discussion

We have designed several siRNAs based on conserved regions on the influenza virus genome and improved their silencing efficacy through various novel designs. Applications of almost all these novel designs to the target sites NP-1505 showed an improved knockdown activity for siRNA antisense strand and enhanced inhibitory effect on influenza virus replications compared to the conventional designs. The most potent designs also showed comparable efficiency to the positive control siRNA NP-1496. Although the followed applications of selected novel designs on NP-452, PA-700 and M-35 showed differential effects on inhibition of influenza virus, compared to conventional designs, we still managed to select relatively potent siRNA NP-452 and siRNA M-35 AC mis.

Table 3.6 Summary of best designs and their inhibitory effect on both Luc-NP and actual influenza virus

Sites	Modifications	Luc-NP	Inhibition of Influenza virus infection based on vLuc
NP-1496	None	7%	8%
NP-1505	AC mismatch	23.60%	62%
NP-1505	UG	18%	86%
NP-1505	Dumbbell	23%	14%
NP-452	None	24%	27%
M-23	AC mismatch		25%

Several conventional siRNAs or expressed shRNAs were developed previously targeting nucleoprotein segment (163,165,257,281), components of the virus RNA polymerase (163,257) or matrix protein (166,282). These all showed effective inhibition on influenza virus replication both *in vitro* or *in vivo*. Among these experiments, only in Ge et al.'s experiment were siRNAs designed against conserved regions across multiple strains. All the other rationally designed siRNAs were based on sequence from only one or two specific strains. In our experiment, we explored the most updated influenza virus sequence database, including recently isolated strains. In our studies we showed that regardless of the rational design rules for siRNAs, the potency of siRNAs with sequence restricted within the conserved regions of influenza virus could be improved by novel sequence or structural designs. Highly conserved regions were located at the coding regions on three segments (3, 5 and 7), which were also selected for siRNAs development in previous studies. Ge et al. (163) suggested the nucleoprotein or the component of RNA polymerase as potent inhibitors of influenza virus due to their essential roles in influenza virus replication and their broad inhibition effect on all viral RNA production from other segments, unlike siRNAs that targeted segment 7, which can only cause M-specific mRNA degradation. However, we and others (166,282) showed that siRNAs targeting segment 7 exibited potential therapeutic application for influenza virus. Hui et al. showed that lentivirus-delivered shRNA M-331, targeting the matrix gene, could be a robust inhibitor for influenza virus and Sui et al.'s also found that shRNA targeting the M2 ion channel could cause broad inhibition on viral RNA production. If efficient delivery methods was the possible contributing factors above, in our study, we found that the matrix protein-targeted siRNA M-35 with novel 5' AC mismatches designs conferred effective

inhibition on influenza virus with transient transfection. Moreover, the efficiency of siRNA M-35 AC mis was assessed by luciferase expression level from vLuc reporter plasmids, which served as an indicator for influenza replication activity. This means that inhibiting matrix protein production could interfere with influenza replication activity. Previous studies showed that as a structural protein, M1 also plays important roles in multiple influenza virus steps. It participates in blocking viral mRNA transcription (283) nuclear export of vRNPs (284), viral assembly and budding (285,286). So the reduced matrix protein production could possibly affect the packaging and release of new vRNPs and resulted in reduction of the new vRNPs replication activity.

Previous studies showed siRNAs with novel designs, such as a shortened sense strand, acquired efficacious antisense strand silencing correlated with reduced off-target effect of the sense strand (266,267,287,288). However, we did not observe similar effects for novel designs NP-1505 NH, bulge and three 5' mismatch designs. One of the mechanisms proposed for the increased antisense strand targeting activity by structural asymmetric designs was the increased RISC loading efficiency for the antisense strand by eliminating loading of the sense strand (287). It seems to be not the case for these novel designs. Moreover, the sense strand-mediated 'off-target' effect brought by the sense strand might be manipulated as a positive factor for anti-influenza studies. Among the three strands produced during influenza virus replication, mRNA has been suggested as the direct target for siRNAs in Ge et al's study (163). vRNA, on the other hand, usually exists in the from of vRNPs in the cytoplasm and is wrapped by the nucleoproteins. This made vRNA hard to get access to for targeting. Ge et al. (214) showed that phosphorodiamidate morpholino

oligomers (PMO) targeting the terminal regions of NP vRNA or cRNA could also inhibit influenza virus efficiently, which suggests that the nucleic acid analogs could access to the vRNA possibly by entering the nucleus. Similarly, for siRNAs, if one strand had been transported into nucleus, it would have the same effect on influenza virus. So this 'off-target' effect brought by sense strand might not necessarily be a detrimental effect. siRNAs with both strands highly active might be more efficient in inhibiting influenza virus replication.

Notably, some of the novel designs improved siRNA silencing also in a sequence-dependent manner. Although two modulations (mismatch and sequence deletion) both managed to enhance the RNAi activity of the NP-1505 siRNA duplex, they seem to affect the silencing activity of siRNAs at three target sites, NP-452, PA-700 and M-35 differentially. First, NP-452, although conferring thermodynamic unfavorable stability (Table **3.4**) still exhibited high activity with ~80% inhibition on influenza virus replication (Figure **3.20**). Looking at the five siRNA duplexes with unfavorable thermodynamic stabilities, only NP-452 had two consecutive AU base pairs on the 5' antisense strand and this might further enhance its antisense strand's potency according to previously identified rules in distribution of 5' end A/U and G/C content with two strands (289). Besides, as described in the rational design rules (148), terminal stability is not the only determinant for RNAi activity of siRNA duplexes. So overall it is possible that the two novel NP-452 siRNAs did not enhance its RNAi activity as expected, which was also observed for NP-1496. It is hard to explain how the same mismatches (UG/AC) and deletions that significantly improved siRNA NP-1505 silencing activity did not show similar effects on target sites PA-700 and M-35. Since the effect on thermodynamic stability by RNA single mismatch is sequence and

position (290), it is possible that the AC mismatch on siRNA PA-700 did not destabilize the 5' antisense strand enough to be compared to the 5' sense strand. For the deletion design, neither M-35 deletion nor PA-700 deletion showed enhanced inhibition effects as observed for NP-1505. Previous studies reported that siRNA with a trimmed sense strand had a reduced off-target effect (266,287) and improved potency (287) by producing a structural asymmetry for siRNAs. However this observation also seems to be sequence dependent. An examination of the 3' sense strand of the deletion design in NP-1505, PA-700 and M-35 revealed that there was a AU pair in NP-1505 but a GC pair in PA-700 and M-35 and this novel design failed to reverse the unfavorable thermodynamic profile just through trimming of 5' sense strand. Two potent siRNAs, siRNA NP-452 and siRNA M-35 mis were constructed and evaluated in this study,

3.3 Developing Amplifiable MicroRNAs During Influenza A Virus Replication

3.3.1 Introduction

RNAi triggers include chemically synthesized siRNAs and vector-expressed short-hairpin RNAs and microRNAs. shRNAs were originally designed based on the assumption that endogenous miRNAs form a stem-loop structure and are processed alone by Dicer alone in the cytoplasm (116,291). As the biogenesis process of endogenous microRNAs in animal cells was better studied, it was found that endogenous miRNAs were usually transcribed by RNA Polymerase II into a longer transcript

In light of the natural processing of microRNAs, artificial microRNAs were developed in the form of a naturally occurred primary miRNA structure (142). Silva et

al. have also shown that artificial miRNAs are much more efficient in producing mature miRNAs by Dicer cleavage than shRNAs (142). The expression of artificial microRNAs can be either driven by RNA polymerase III (pol III) or polymerase II (pol II) on DNA-based plasmids or viral vectors. These are commonly based on adeno-associated viruses and lentiviruses (292). H1 and U6 (small nuclear RNA promoters), are the two most common pol III promoters used to express miRNAs as well as shRNAs (136,142). The H1 or U6-driven expression of artificial microRNAs usually produces a single transcript with a defined 5' start site and a 3' terminal end with a stretch of 4-5 uridines (293,294). On the other hand, pol II-driven expression could produce a 5' capped and 3' polyadenylated transcript, which has a structure closely resembling the naturally occurred miRNAs. Furthermore, the pol II promoter can also be utilized to express polycistronic miRNAs targeting different sites for combinatorial RNAi treatment.

To achieve sustainable inhibition of influenza virus growth in cell lines and animal models, several studies utilized vector-expressing shRNAs (166,282,295) or miRNAs (146) delivered via plasmid DNA or viral vectors. These constructs conferred with comparable inhibitory effects on influenza virus replication with that of chemically synthesized siRNAs (163). In one recent study, Chen et al. (2011) successfully developed a lentiviral vector expressing three tandem miRNAs targeting PB1, PA and NP under the control of a pol II promoter, which showed significantly higher efficiency in inhibiting influenza virus growth compared to the vector expressing anti-NP miRNA alone. However, pol II and pol III can only drive constitutive expression of miRNAs. Long-term constitutive expression of RNAi molecules might post risks to specific cellular functions and animal health. These

include off-target effect on host genes due to partial sequence complementarity (202,203), inhibition of endogenous miRNA production (205) due to RNAi system and induction of interferon and inflammatory response (201). To reduce these risk factors, one of corresponding strategies is to develop inducible promoters that could limit the RNAi molecule expression only in presence of virus infections. Several recent studies against some other viruses have made progress in developing inducible RNAi. Strayers et al. tried to construct inducible siRNA expression system against HCV by cloning the HIV-1 LTR upstream of anti-HCV RNAi. During infection, the HCV infection induced NF-κB expression could in turn stimulate LTR and trigger the expression of anti-HCV RNAi (206). Another example is a HIV-1 TAT inducible anti-rev RNAi expression system under the control of the HIV-1 LTR-like promoter. The expression of TAT could induce the corresponding production of anti-rev RNAi in a negative feedback mechanism.

Previously, we and others (68,69,296) have developed reporter constructs expressing influenza virus mini-genome segment with viral protein coding regions replaced by reporter proteins. It was demonstrated that the constructs could have inducible expression of reporter proteins upon influenza virus infection via recognition by influenza virus polymerase. Besides, several recent studies have shown that recombinant influenza A viruses carrying microRNA expression cassette are capable of expressing functional microRNAs targeting different host genes (297–299). This provides possibilities for development of inducible anti-influenza virus RNAi.

3.3.2 Design and construct of anti-influenza microRNA based on siRNA NP-1496

To construct virus-inducible artificial microRNA expression construct, we first tried to design efficient artificial microRNA targeting influenza virus. We employed the sequence of NP-1496, the highly efficient anti-influenza virus siRNA as a positive control to develop the anti-influenza virus artificial microRNA. The design was made according to the protocol given by Hannon's lab (300) and shown in Figure **3.21**. The corresponding DNA oligo miR-30-NP-1496 was synthesized by Integrated DNA Technologies Inc. Sequence is shown in Appendix.

The artificial microRNA was first cloned into the pCAG-mir30 plasmid, which contains the miR-30 5' and 3' flanking sequences at multiple cloning site and tested for efficacy on inhibition of influenza virus replication under the control of the CAG promoter, a constitutive RNA polymerase II promoter. In order to construct human miR-30 based artificial microRNA NP-1496 target, the artificial microRNA cassette was PCR amplified from the DNA oligo miR-30-NP-1496 as shown in Figure **3.21**, using two primers tagged with X*ho*I and *Eco*RI multiple cloning site. After restriction enzyme digestion, the PCR amplicon was ligated into pCAG-mir30 plasmid in the multiple cloning sites. The finished construct pCAG-mir30-NP-1496 was confirmed by sequencing.

To test the silencing efficiency of the artificial miR-30-NP-1496 against NP segment, the pCAG-mir30-NP-1496 was cotransfected with Luc-NP developed in Chapter 3.2 in HEK293 cells. After 48hr post transfection, cells were lysed and subjected to luciferase assay. As shown in Figure **3.22**, compared to cells transfected with Luc-NP alone, cells cotransfected with pCAG-mir30-NP-1496 expressing artificial microRNA and Luc-NP indeed showed ~30% reduction in luciferase activity.

a.

```
Initial ΔG = -45.70
          10          20          30
        A       C                                   ----------|        A
AGUG   GCG   AGGAUCUUAUUUCUUCGGAGACA                        GUG     A
UCAU   CGU   UCCUAGAAUAAAGAAGCCUCUGU                        CAC     G
        C       U                                   GUAGA^        C
          70          60          50          40
```

b.

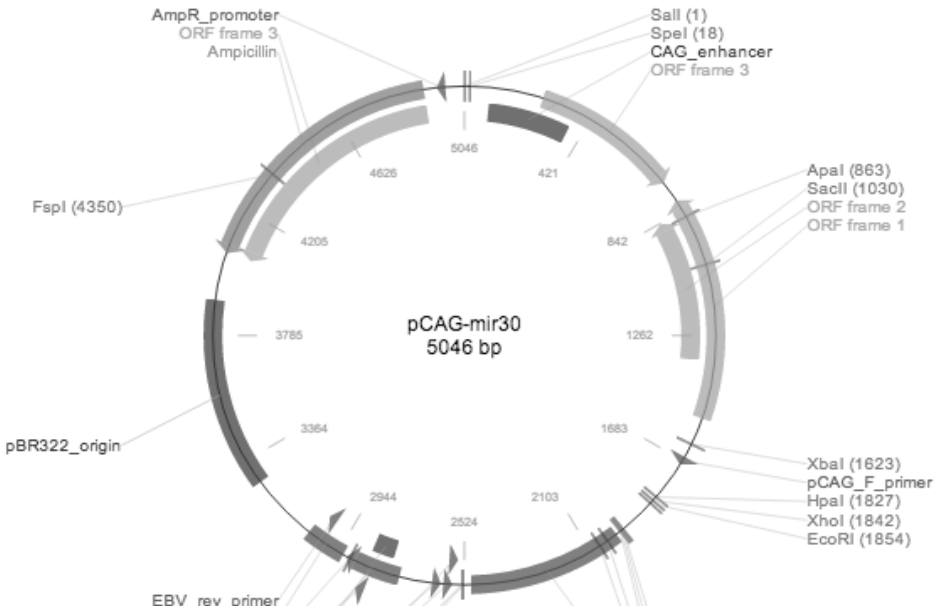


Figure 3.21 Schematic graph design of artificial microRNA based on siRNA NP-1496 targeting influenza. This plasmid map was originally constructed by Matsuda and Cepko (301) The sequence of artificial microRNA design (a) was based on siRNA NP-1496 and human miR-30 backbone. The top strand shown in blue is NP-1496 sense strand, the bottom strand is the antisense strand. The plasmid map for pCAG-mir30 is shown in (b). pCAG-mir30 carries a human mir-30 backbone. Artificial microRNA could be cloned between the X*hoI* and E*coRI* site as described by Paddison et al.(300).

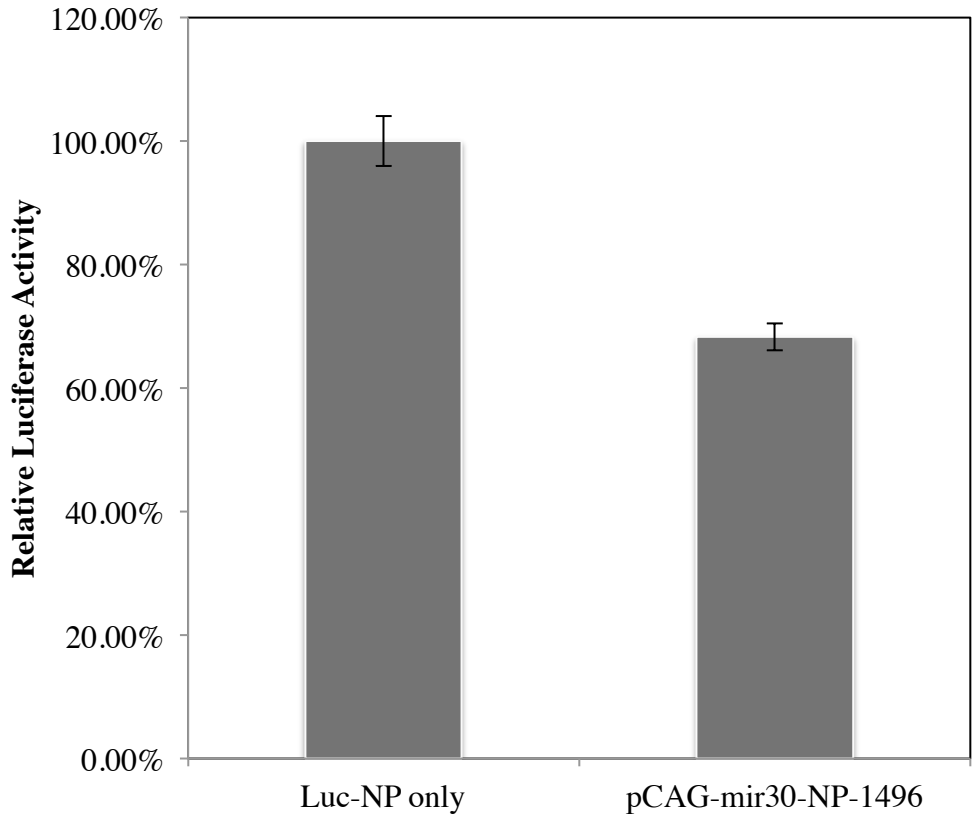


Figure 3.22 Inhibition of Luc-NP luciferase activity by artificial microRNA targeting influenza NP segment based on human mir-30 backbone. HEK293 cells were cotransfected with pCAG-mir30-NP01496 and Luc-NP or Luc-NP alone. Cells were lysed and subjected for luciferase assay after 24 hr post infection. Luciferase activity was normalized to cells transfected with Luc-NP alone.

However, this reduction is not potent as the previous developed NP-1496 sequence-based shRNAs and siRNAs.

3.3.3 Construct of virus-inducible artificial microRNA (vMicroRNA)

Chen et al. developed several artificial microRNAs based on chicken gga-mir-126 as backbone. Among these, two artificial microRNAs with single or multiple miRNAs exhibited potent inhibitory effect on luciferase construct and also influenza virus growth. As shown in Figure **3.23**, pLB2-NP expresses a single miRNA targeting NP protein and pLB2-PB1-NP-PA expresses three tandem miRNAs that target PB1, NP and PA protein. We decided to select these two artificial microRNAs as backbone for vMicroRNAs. The vMicroRNA would be cloned with 5' and 3' flanked influenza virus vRNA promoter sequence into the pHH21 vector. In pHH21, the expression is under control of the RNA polymerase I promoter, which produces transcripts with defined 5' and 3' termini.

The proposed model for the infection-induced artificial microRNA expression is as followed. Once the construct was transfected inside of cells, upon influenza infection, with the assistance of nucleoprotein (NP), the viral polymerase protein (PA, PB1 and PA) could recognize the vRNA promoter located on the 5' and 3' flank of the artificial microRNA and amplify or transcribe the entire modified artificial microRNA. The amplified artificial microRNA could be recognized by Drosha inside nucleus and be processed into pre-miRNA followed by transportation into cytoplasm and Dicer cleavage (Figure **3.24**). The processed miRNA duplex would then participate in the subsequent RNAi pathway. On the other hand, there might be three RNA species (vRNA, cRNA and mRNA) produced from the inducible artificial microRNA construct due to the replication and transcription activity of the viral

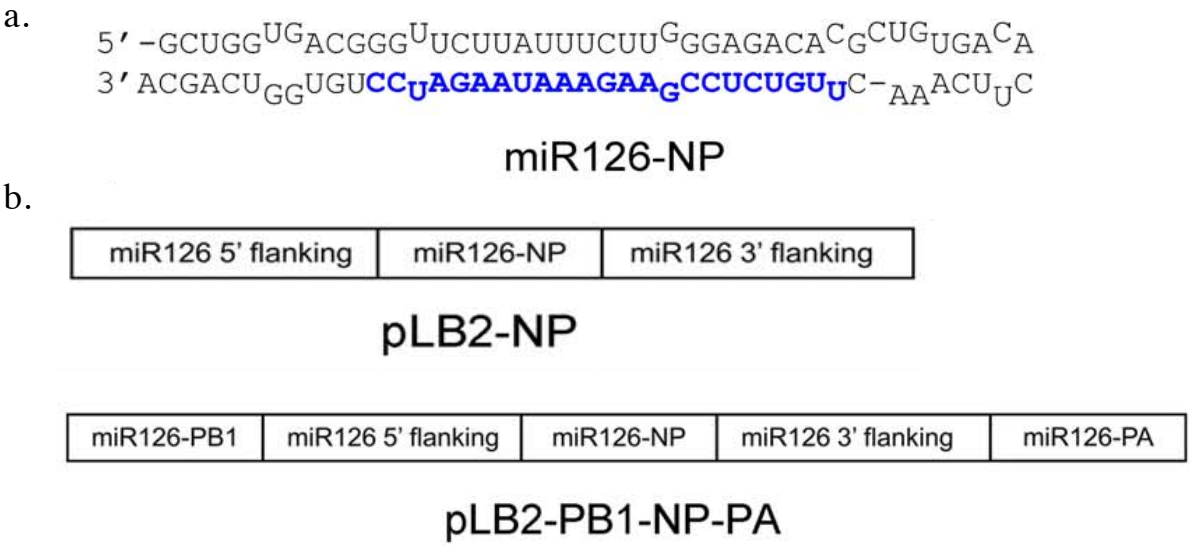


Figure 3.23 Schematic diagram depicting artificial microRNA with single miRNA or multiple miRNA The diagram was adapted from Chen et al.2011 (146)) The sequence of artificial gga-miR-126 expressing anti-influenza NP is shown in (a). The bottom strand marked with blue color stands for the antisense strand targeting NP segment. Schematic diagram of two artificial microRNA expressed by pLB2-NP and pLB-PB1-NP-PA is shown in (b). The pLB2-NP expresses single miRNA (NP) and the pLB2-PB1-NP-PA expresses three tandem miRNAs (PB1-NP-PA). They were PCR amplified for construct of viral inducible artificial microRNAs.

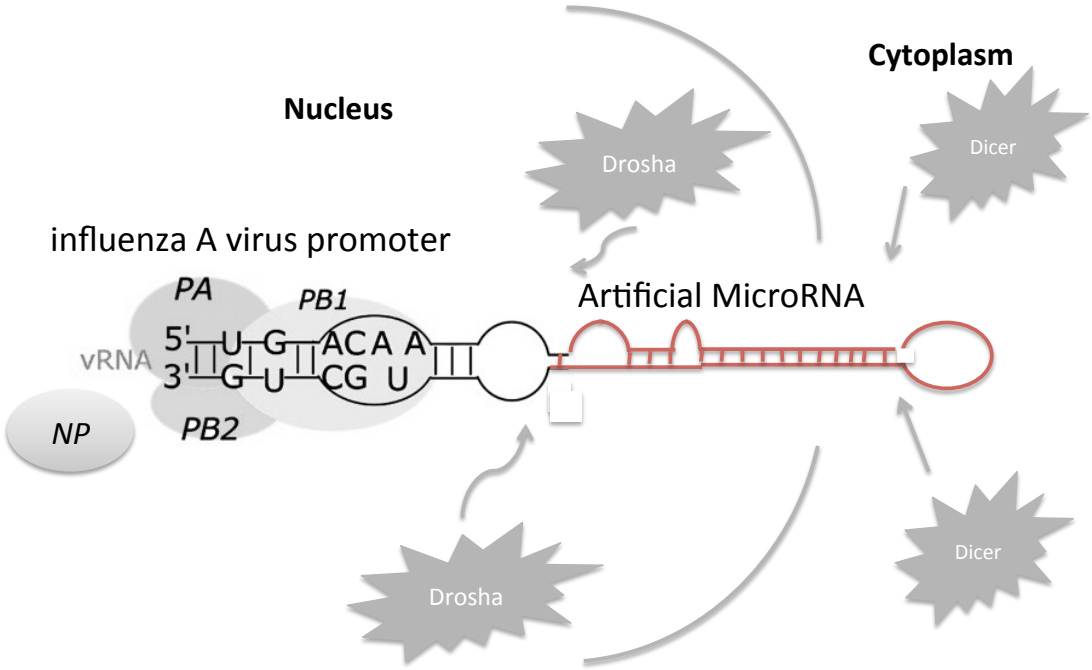


Figure 3.24 Schematic graph depicting the proposed model for virus-inducible expression of artificial microRNA. The artificial microRNA targeting influenza virus (red) is flanked by the influenza vRNA promoter. In the proposed model, during infection, the influenza promoter could be recognized by RNA polymerase and NP, amplified and processed by Drosha inside of the nucleus. The pre-miRNAs will be transported into cytoplasm and processed by Dicer for subsequent RNAi activity.

RNA polymerase (Figure **3.25**). So the artificial microRNA might be processed by Drosha into pre-miRNAs in any of the three different species in plus or negative sense. In order to develop the most active artificial microRNA among the three RNA species, we decided to construct the artificial microRNAs as well as its reverse complementary counterpart flanked by the vRNA promoters in the pHH21 vector. The artificial microRNAs without flanking vRNA promoters were also constructed in the pHH21 for control purposes. In that case, the artificial microRNA expression is driven by RNA polymerase I for constitutive expression.

In total six vMicroRNA expression plasmids were constructed. The design was based on miR-126-NP sequence expressed by pLB2-NP plasmid and multiple microRNAs targeting NP, PB1 and PA, expressed by pLB2-PB1-NP-PA. Both plasmids were developed by Chen et al. (146) and kindly provided by Dr. Jianzhu Chen (Massachusetts Institute of Technology, MA). The schematic diagrams of those two plasmids were shown in Figure **3.23**. Four vMicroRNA expression vector (pHH21-NPi(+)/NPi(-), pHH21-3i(+)/3i(-)) along with two controls without promoter sequences (pHH21-NPc and pHH21-3c) were constructed based on the two artificial microRNAs. Following similar procedures described in Chapter 3.2.5, to construct pHH21-NPc/NPi(+)/NPi(-) expressing artificial microRNA targeting NP, the gga-miR-126-NP was PCR amplified from pLB2-NP using cloning primers NPc-F/R, NPi(+)-F/R and NPi(-)-F/R accordingly, digested with *Bsm*BI and ligated with pHH21 plasmid pre-digested with *Bsm*BI restriction enzyme. The pHH21-NPc was constructed expressing gga-miR-NP alone for control purposes. The pHH21-NPi(+) and pHH2-NPi(-) were constructed to express gga-miR-NP flanked by 5' and 3'

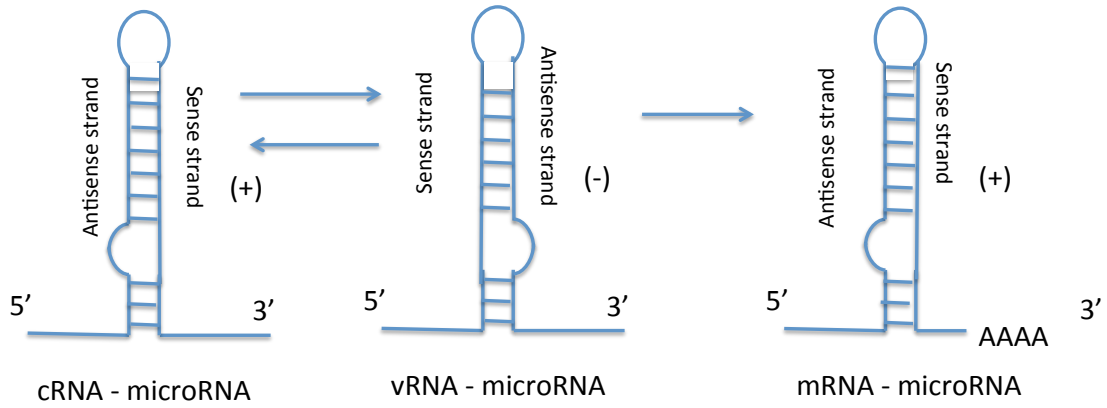


Figure 3.25 Three possible RNA species produced from the inducible artificial microRNA construct. Once the vRNA promoter that flank the artificial microRNA is recognized by the viral RNA polymerase, it might be able to replicated into cRNA-microRNA transcript which is reverse complementary to the original transcript or transcribed into mRNA with 3'- polyadenylation. Any of the three RNA species could possibly be processed by Drosha.

promoter of influenza A/WSN/33(H1N1) strain (same to that of vGFP and vLuc constructs in Chapter 3.2.5) with either directions. On the other hand, to construct pHH21-3c/3i(+)/3i(-) expressing three tandem microRNAs, the artificial microRNA was amplified from pLB2-NP-PB1-PA plasmid using cloning primers 3c-F/R, 3i(+)-F/R and 3i(-)-F/R similarly as described above. pHH21-3c was constructed for control purposes for pHH21-3i(+) and pHH21-3i(-). The finished constructs were confirmed by sequencing.

3.3.4 Time course and dose response study on vMicroRNA expression's inhibition of influenza virus replication using vLuc as an indicator.

To test the inhibition efficiency of vMicroRNAs against influenza viruses in response to their infection, time course and dose response study was conducted against influenza virus. The reporter plasmid vLuc developed in Chapter 3.1 was employed as an indicator for the influenza virus replication activity. Basically, the vMicroRNA constructs (pHH21-NPi(+)/NPi(-) and pHH21-3i(+)/pHH21-3i(-)) or the control constructs were cotransfected with vLuc into HEK293 cells and cells were then infected with influenza A PR/8/34 H1N1 strains at different MOIs (0.02, 0.1, 0.5). After different time (16, 24, 48 hr) post infections, cells were lysed and subjected to luciferase assay. The firefly luciferase activities were normalized to cells transfected with vLuc alone in each group of experiment. However, as described in Chapter 3.1, the vLuc plasmid also contains the influenza virus vRNA promoter and this might cause competitive inhibition of RNA polymerase in transcribing vMicroRNAs when there are not enough production of the polymerase. To test this possibility, we cotransfected the vGFP, another reporter plasmid that contains vRNA promoter

sequence constructed in Chapter 3.1, and vLuc into HEK293 cells and compared with cells transfected with vLuc alone.

As shown in Figure **3.26**, there was no significant difference in the luciferase activity between cells transfected with vLuc alone and cells cotransfected with the vLuc and vGFP which might have competitive use of RNA polymerase. This indicated that no competitive inhibition of RNA polymerase activity on one or another when cotransfected with two plasmids that both contain the influenza virus vRNA promoter when MOI was as low as 0.02. Besides, we also tested whether there was any off-target effect by artificial microRNAs directly on the vLuc luciferase transcript, we cotransfected the artificial microRNA expression construct made above individually with pmirGLO plasmid DNA, which express the luciferase protein and no non-specific inhibition on luciferase expression was identified (data not shown).

As suggested in Chapter 3.1, the firefly luciferase produced from vLuc plasmid transcripts could reflect the activity of influenza replication. So the inhibitory effect on influenza virus replication by the constitutive miRNA or vMicroRNA expression construct could be compared from the level of luciferase activity. HEK293 cells were cotransfected with vLuc and six microRNA expressing plasmid constructed above, infected at three different MOIs (0.02, 0.1 and 0.5) and lysed at different time post infection. All final luciferase activity was normalized to the 16 hpi vLuc only control.

As shown in Figure **3.27**, according to the level of luciferase activity, cells transfected with constitutive multiple miRNA-expressing constructs (pHH21-3c) showed significant inhibitory effect on influenza virus reproduction at all MOIs, while cells transfected with constitutive single anti-NP miRNA-expressing construct did not have any inhibitory effect except at MOI = 0.5. To compare the inhibition effect on

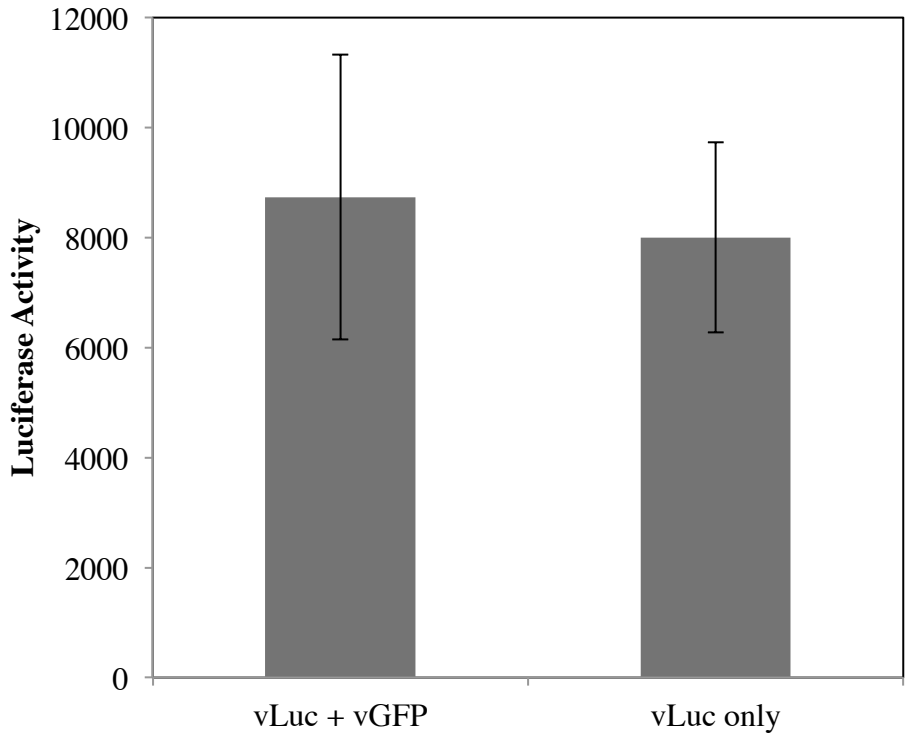
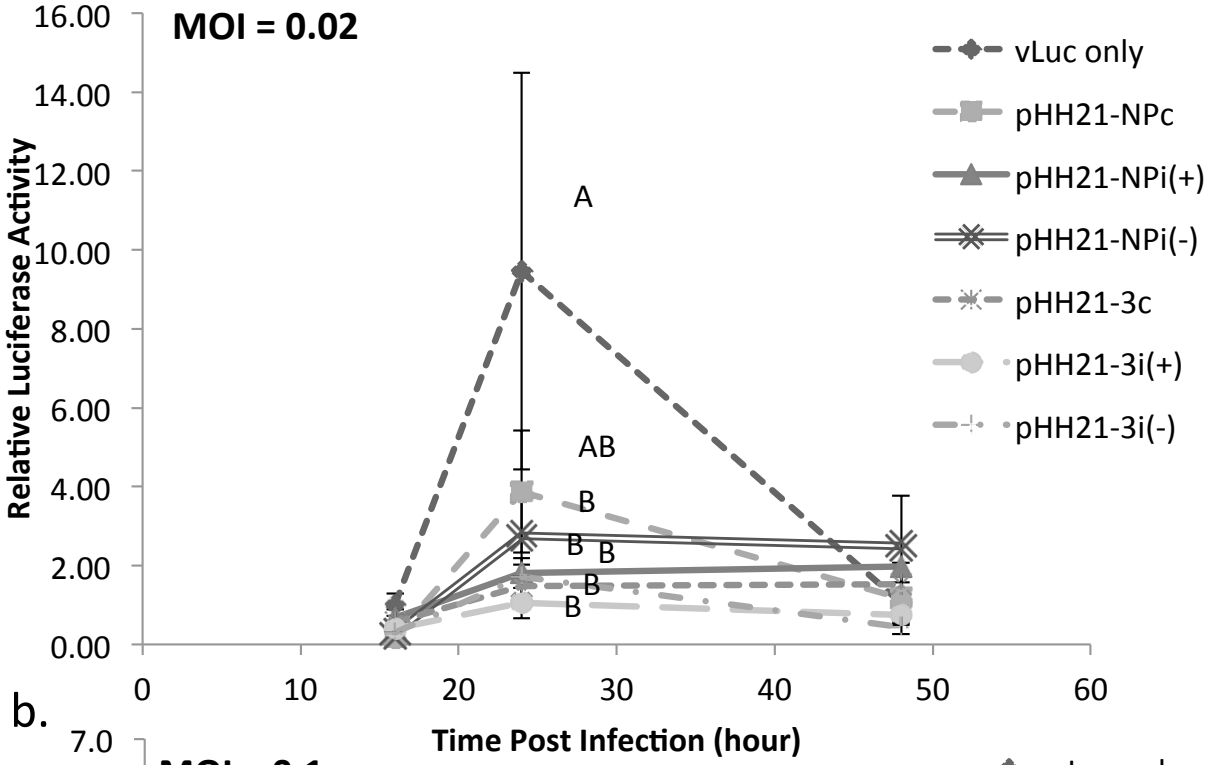


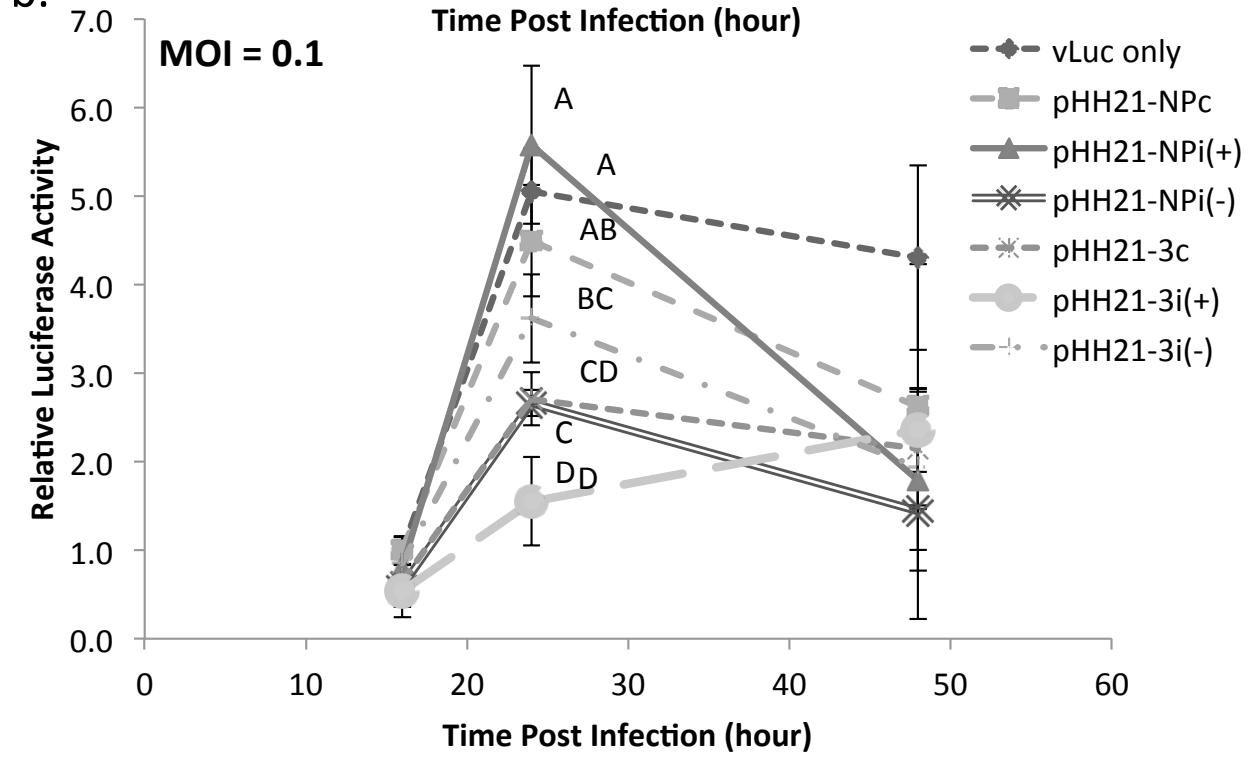
Figure 3.26 Test of competitive inhibitory effect on RNA polymerase when cells were co-transfected with constructs that both contained influenza vRNA promoter. HEK293 cells were cotransfected with vLuc and vGFP (vLuc+vGFP) or vLuc alone (vLuc only) and infected with influenza A PR/8/34 H1N1 virus at MOI = 0.02. Cells were lysed for luciferase assay after 24 hr post infection (hpi).

Figure 3.27 Indirect inhibition of vLuc luciferase activity by vMicroRNAs construct. HEK-293 cells were cotransfected with 100 ng vLuc plasmid and 100 ng constitutive microRNA (pHH21-NPc/3c) or vMicroRNA (pHH21-NPi(+)/(-) and pHH21-3i(+)/(-)) expression constructs. After 18hpi post transfection, cells were infected with influenza A PR/8/34 H1N1 virus at MOI = 0.02 (a), MOI = 0.1 (b) and MOI = 0.5 (c). At 16 hr, 24 hr and 48 hpi, cells were lysed for luciferase assay. The luciferase activities were all normalized to the activity of cells transfected with vLuc plasmid DNA alone (vLuc only) at 16 hpi (set as 1). Pairwise student *t*-test was made for statistical analysis of relative luciferase activity at 24 hpi. Luciferase level marked with no shared character stands for significant differences between each other *($p < 0.05$)*

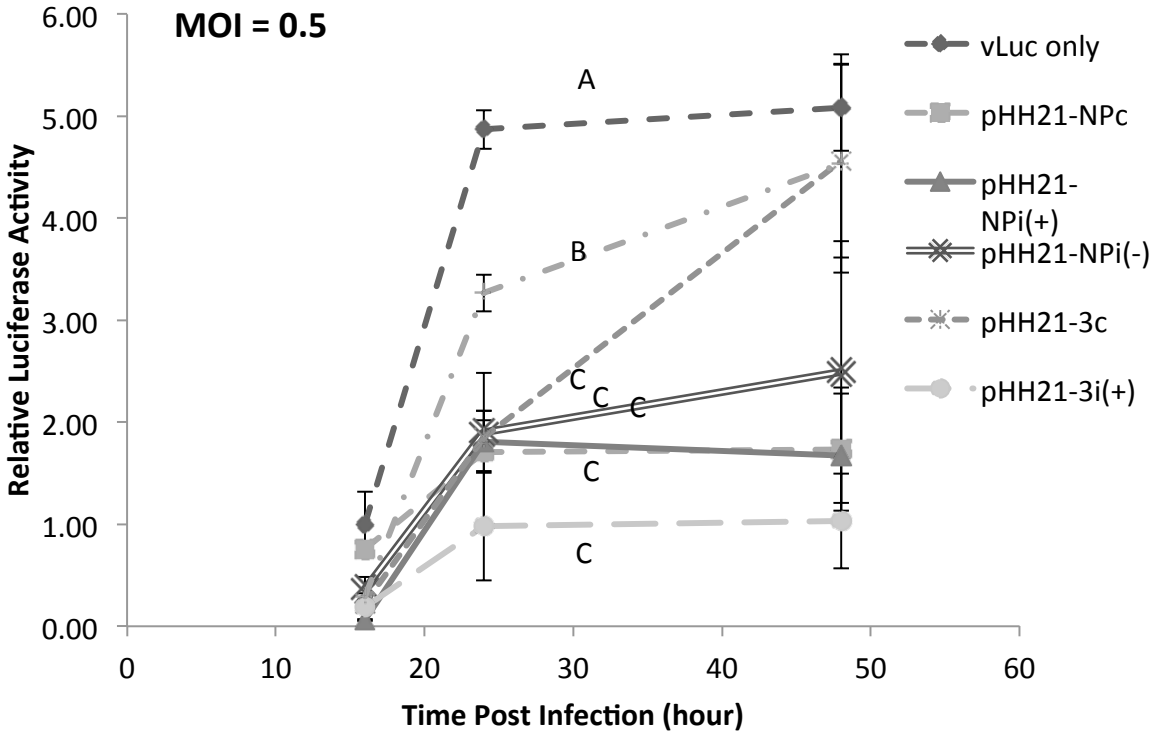
a.


MOI = 0.02
Relative Luciferase Activity
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
0
10
20
30
40
50
60
Time Post Infection (hour)
A
AB
B
B
B
B
B
vLuc only
pHH21-NPc
pHH21-NPi(+)
pHH21-NPi(-)
pHH21-3c
pHH21-3i(+)
pHH21-3i(-)

b.


MOI = 0.1
Relative Luciferase Activity
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
10
20
30
40
50
60
Time Post Infection (hour)
A
A
AB
BC
CD
C
D
D
vLuc only
pHH21-NPc
pHH21-NPi(+)
pHH21-NPi(-)
pHH21-3c
pHH21-3i(+)
pHH21-3i(-)

C.


MOI = 0.5
Relative Luciferase Activity
Time Post Infection (hour)
6.00
5.00
4.00
3.00
2.00
1.00
0.00
0
10
20
30
40
50
60
A
B
C
C
C
C
C
vLuc only
pHH21-NPc
pHH21-NPi(+)
pHH21-NPi(-)
pHH21-3c
pHH21-3i(+)

influenza virus replication by the constitutive expressing-miRNA and inducible vMicroRNA at 24 hpi, the pattern also varied between the construct with single anti-NP miRNA and the one with multiple tandem miRNA. Cells transfected with constructs expressing inducible anti-NP exhibited equally potent inhibitory effect on influenza at high MOI (0.5) in both directions (+/-) when compared to cells transfected with pHH21-NPc. However, pHH21-NPi(-) transfected cells indeed showed more potent reduction (~1.7-fold, $p<0.05$) in virus reproduction compared to pHH21-NPc transfected cells when MOI dropped to 0.1 while pHH-21-NPi(+) transfected cells did not have any inhibition effect on virus reproduction compared to the vLuc only control. When MOI dropped further to 0.02 the inducible vMicroRNAs seem have more potent effect (~1.4-2 fold) but it is not significant. This suggested that at different MOIs, viruses manipulate the construct expression differently. Also, The two vMicroRNA constructs also exhibited differential inhibitory effect, with pHH21-NPi(-) construct constantly having relative potent inhibition effect and possibly being amplified .at MOI = 0.1. On the other hand, for the construct expressing multiple miRNA in one cassette, the inducible vMicroRNA exhibited a different trend than. Cells transfected with pHH21-3i(+) constantly had a more potent inhibition effect (~1.4-2-fold) than the ones transfected with pHH21-3c on influenza at all MOIs although not significantly different. However, cells transfected with pHH21-3i(-) had less reduction on viral reproduction at MOI = 0.5 ($p<0.5$) and MOI = 0.1 compared to cells transfected with pHH21-3c, which trend is the opposite to the vMicroRNA with single anti-NP miRNA expressed.

3.3.5 Discussion

Previously, we and others (146,166,282,295) have developed constitutively expressed shRNAs/miRNAs as well as transduced cells lines that showed sustainable potent inhibition of influenza virus replication. Here in this project, based on the previously developed chicken miR126-based artificial single miRNA or multiple miRNAs cassette against influenza virus, we tried to establish an artificial virus-inducible microRNA (vMicroRNA) system by expressing them with flanked influenza virus promoter at each terminus. Based on the time course and dose response study for the six miRNA-expressing plasmids, it is suggested that the artificial vMicroRNA might be virally amplified and processed into functional miRNA that target influenza virus but in a dose-dependent fashion. As shown, reflected from the level of luciferase activity, cells transfected with constitutive multiple miRNA-expressing constructs (pHH21-3c) showed significant inhibitory effect on influenza virus reproduction at all MOIs, while cells transfected with constitutive single anti-NP miRNA-expressing construct did not have any inhibitory effect except at MOI = 0.5. This is similar to the results obtained by Chen et al. (146), where the multiple miRNA expression cassette exhibited a more potent (~2-fold) inhibition than the single miRNA expression cassette on influenza replication when expressed in cells, although in their study, the anti-NP miRNA expression is driven by a RNA polymerase II (146).

To look at the overall trend and compare the virus inducible vMicroRNA and the constitutive microRNA expression on the inhibition of influenza virus reproduction at all three MOIs, it seems that in the plasmid expressing single microRNA anti-NP, the vMicroRNA with minus direction (pHH21-NPi(-)) exhibited constant potent inhibition while among the plasmid expressing multiple microRNAs, the vMicroRNA with plus direction (pHH21-3i(+)) and the constitutive pHH21-3c had

equal potency on influenza inhibition. As we discussed in Chapter 0, cloning vMicroRNA in two different directions (+/-) is due to the possible recognition by Drosha at different RNA species produced by the pHH21 transcript (Figure **3.25**). For the vMicroRNA (+), the active microRNA should be present in vRNA species, which is the direct transcript from pHH21, while for vMicroRNA (-), the active microRNA would be present in cRNA species and mRNA species, which needed a second step for replication/transcript. Due to that the cRNA species amplified by the influenza polymerase is very little compared to the other two RNA species (as described in Chapter 3.1.7), mRNA should be the major RNA species carrying the active anti-influenza artificial miRNA. Moreover, indicated by previous studies of Varble et al. (302), the vRNA might not be a favored substrate by Drosha because it is usually encapsidated with nucleoproteins. So here, we hypothesized that the direct transcript from vMicroRNA (+), once it is recognized by influenza RNA polymerase, the further amplified vRNA species cannot be further recognized by Drosha. So hypothetically, their silencing efficacy should be equivalent to the corresponding constitutive expression plasmid pHH21-NPc or pHH21-3c. This explained the equal potency between those vMicroRNA(+)s and the constitutive miRNA expression plasmid. However, for vMicroRNA (-), which would have mRNA species carrying active miRNAs, only pHH21-NPi(-) exhibited increased potency at MOI=0.2 while pHH21-3i(-) seemed to have an decreased potency at all MOIs. This might be explained by their secondary structures. As we could tell, the vMicroRNA expressing multiple artificial microRNAs had a more complex secondary structure compared to the vMicroRNA expressing single anti-NP miRNA, which might make the recognition by RNA polymerase for the vMicroRNAs much harder and the amplification might be

hindered. Overall, the opposite result suggested that the pHH21-NPi(-) might be amplified and its mRNA form were processed into miRNAs while neither of the vMicroRNA expressing multiple miRNAs were amplified.

Besides, as we could tell from the result, the possibly amplified pHH21-NPi(-) did not exhibit a large increase in its inhibition effect (~2-fold). This might be due to a negative feedback by targeting NP. NP is an important protein involved in replication (303) and it is also required for the amplification of vMicroRNAs. To target NP might also destruct the vMicroRNA production by influenza RNA polymerase. To construct effective vMicroRNA, avoiding NP, PB1, PB2 and NP for targets might be necessary.

Chapter 4

CONCLUSIONS AND FUTURE WORK

Overall, we have successfully developed and evaluated vRNA and cRNA-based reporter systems expressing GFP or luciferase for efficient anti-influenza RNAi screening and utilized the vLuc reporter throughout the entire project for the rapid detection of influenza virus replication. Second, we have designed and developed potent RNAi molecules targeting conserved regions in the NP, M and PA segment of the influenza A virus genome. Moreover, by employing a variety of novel siRNA designs, we have improved the efficiency of an siRNA targeting NP segment, NP-1505, by 7-fold in antisense strand targeting and by 8-fold in inhibiting on influenza virus reproduction. We also applied those novel designs to siRNAs targeting different sites and finally obtained three highly potent siRNAs against conserved regions on different segments (NP, PA and M), which could be used for further siRNA cocktail studies.

Finally, to sustainably inhibit on influenza virus in cell lines as well as animal models in the future, we tried to develop a virus-inducible artificial microRNA expression system by expressing a microRNA from a viral promoter. Time course and dose response studies suggested that the virus-inducible anti-NP artificial microRNA cloned in the negative sense might have been recognized by the viral RNA polymerase and been amplified by the RNA polymerase. The anti-NP artificial miRNA transcribed from pHH21-NPi(-) showed ~ a 2-fold increase in its inhibitory effect on influenza virus replication at a MOI of 0.1. Nevertheless, cells transfected with constructs

carrying multiple miRNAs did not demonstrate significantly improved inhibition of replication of influenza virus compared with constructs constitutively-expressing multiple miRNAs, This might be due to the secondary structure hindrances that prevented influenza RNA polymerase from recognizing the flanking promoter sequence.

The future directions to utilize those efficient reporter plasmids could be development of transduced cell lines with vGFP/cGFP or vLuc/cLuc and utilize these cell lines for screening of antiviral agents without being affected by different transfection efficiency. We have shown the siRNA designs inhibition efficacy on influenza virus in cell culture using vLuc as indicator. Further examination of them for their *in vivo* inhibition effect of influenza virus by using the traditional influenza infectious assay or the RT-PCR quantitation of viral mRNA production might be applied. SiRNA cocktails could also be developed for the prophylaxis and treatment of influenza virus infection in human or poultry. Furthermore, more work might need to be done on development of vMicroRNA constructs. The amplification and correct processing of miRNAs would need to be confirmed by molecular biology tools such as Real-Time PCR. The targets of the designed miRNAs also might need to be manipulated to avoid the negative feedback mechanism.

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Appendix

OLIGOS USED IN THIS PROJECT

Name	Sequence	Descriptions
RT-PR8	CCGCTCGAGTTTTTTTTTTTTTTTTT	Reverse transcription primer for PR/8/34 strain NP gene
Luc-NP/PN-F	CCGCTCGAGAGCAAAAGCAGGGTAGATAATCACT	Forward cloning primer for Luc-NP/Luc-PN
Luc-NP/PN-R	CCGCTCGAGTTTTTTTTTTTT	Reverse cloning primer for Luc-NP/Luc-PN
Luc-test-F1	GTGGTGTTGTGTTCGTGGAC	Forward primer for colony PCR testing sense orientation of insertion
Luc-test-R1	CGCCATGATTTTGATGTCAC	Reverse primer for colony PCR testing sense orientation of insertion
Luc-test-F2	GTGGTGTTGTGTTCGTGGAC	Forward primer for colony PCR testing antisense orientation of insertion
Luc-test-R2	GAGCTCTCGGACGAAAAGG	Reverse primer for colony PCR testing antisense orientation of insertion
vGFP-F	ATA*CGTCTCATATT*AGTAGAAACAAGGGTATTTTTCTTTACTTG	Forward cloning primer for vGFP
vGFP-R	ATA*CGTCTCGGGG*AGTAGAAACAGGGTAGATAATCACTCACTGAGTGACATCGGTGCCACCATGGTGA*	Reverse cloning primers for vGFP
cGFP-F	ATA*CGTCTCATATT*AGTAGAAACAGGGTAGATAATCACTCACTGAGTGACATCGGTGCCACCATGGTGA*	Forward cloning primer for cGFP
cGFP-R	ATA*CGTCTCGGGG*AGTAGAAACAAGGGTATTTTTCTTTACTTG*	Reverse cloning primers for vGFP
vLuc-F	ATA*CGTCTCATATT*AGTAGAAACAAGGGTATTTTTCTTTACACGGCGATCTTGC*	Forward cloning primers for vLuc
vLuc-R	ATA*CGTCTCGGGG*AGTAGAAACAGGGTAGATAATCACTCACTGAGTGACATCGGTGCCACCATGGAAGATGCCAAA	Reverse cloning primers for vLuc

cLuc-F	ATA*CGTCTCATATT*AGTAGAAACAGGG TAGATAATCACTCACTGAGTGACATCG GTGCCACCATGGAAGATGCCAAA*	Forward cloning primers for cLuc
cLuc-R	ATA*CGTCTCGGGG*AGTAGAAACAAGG GTATTTTTCTTTACACGGCGATCTTGC*	Reverse cloning primers for cLuc
vLuc RT	GGTGCCACCATGGAAGAT	Reverse transcription primer for vRNA quantification from vLuc/cLuc
cLuc RT	ATATCGTCTCGTATTAGTAGAAACAAG GGTATTTTTCTTT	Reverse transcription primer for cRNA quantification from vLuc
mLuc RT	Oligo $(dT)_{15}$	Reverse transcription primer for mRNA quantification from vLuc/cLuc
Luc F PCR	GTACACCTTCGTGACTTCCCATTT	Real-time PCR forward primer for quantitation of luciferase transcripts.
Luc R PCR	TTTTGTCCCGGTCGAAGCT	Real-time PCR reverse primers for quantitation of luciferase transcripts.
GAPDH F PCR	ACCCCTTCATTGACCTCAAC	Real-time PCR forward primers for GAPDH gene
GAPDH R PCR	CACTCCTGGAAGATGGTGATG	Real-time PCR reverse primers for GAPDH gene
miR-30 F	CAGAAGGCTCGAGAAGGTATATTGCT GTTGACAGTGAGCG	Cloning forward primer for construct of artificial NP-targeting microRNA with miR-30 backbone
miR-30 R	CTAAAGTAGCCCCTTGAATTCCGAGGC AGTAGGCA	Cloning reverse primer for construct of NP-targeting artificial with miR-30 backbone
miR-30-NP-1496	AGTGAGCGCAGGATCTTATTTCTTCGG AGACAGTGAAGCCACAGATGTGTCTCC GAAGAAATAAGATCCTTTGCCTACT	DNA oligo for construct of pCAG-mir30-NP-1496

NPc-F	ATACGTCTCATATTCAGGGTGGCTAGAGAAGGAC	pHH21-NPc forward cloning primer
NPc-R	ATACGTCTCGGGGAGAATAAAGTGAGGGAGTTTCTTAGG	pHH21-NPc reverse cloning primer
NPi(+)-F	ATACGTCTCATATTAGTAGAAACAAGGGTATTTTTCTCAGGGTGGCTAGAGAAGGAC	pHH21-NPi(+) forward cloning primer
NPi(+)-R	ATACGTCTCGGGGAGTAGAAACAGGGTAGATAATCACTCACTGAGTGACATCGGTGAATAAAGTGAGGGAGTTTCTTAGG	pHH21-NPi(+) reverse cloning primer
NPi(-)-F	ATACGTCTCATATTAGTAGAAACAAGGGTATTTTTCTGAATAAAGTGAGGGAGTTTCTTAGG	pHH21-NPi(-) forward cloning primer
NPi(-)-R	ATACGTCTCGGGGAGTAGAAACAGGGTAGATAATCACTCACTGAGTGACATCGGTCAGGGTGGCTAGAGAAGGAC	pHH21-NPi(-) reverse cloning primer
3c-F	ATACGTCTCATATTGCTGGTGACGTGTAGATCTGTT	pHH21-3c forward cloning primer
3c-R	ATACGTCTCGGGGATGCTGACCACAGCAATTGAG	pHH21-3c reverse cloning primer
3i(+)-F	ATACGTCTCATATTAGTAGAAACAAGGGTATTTTTCTGCTGGTGACGTGTAGATCTGTT	pHH21-3i(+) forward cloning primer
3i(+)-R	ATACGTCTCGGGGAGTAGAAACAGGGTAGATAATCACTCACTGAGTGACATCGGTTGCTGACCACAGCAATTGAG	pHH21-3i(+) reverse cloning primer
3i(-)-F	ATACGTCTCATATTAGTAGAAACAAGGGTATTTTTCTTGCTGACCACAGCAATTGAG	pHH21-3i(-) forward cloning primer
3i(-)-R	ATACGTCTCGGGGAGTAGAAACAGGGTAGATAATCACTCACTGAGTGACATCGGTGCTGGTGACGTGTAGATCTGTT	pHH21-3i(-) reverse cloning primer

* The Italic sequences are multiple cloning site and the underlined sequences are the promoter sequence from NP segment of PR/8/34 strain.