Title
Engineering Influenza A/WSN/33 for In vivo Bioluminescent Imaging

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Publication Date
2014

Peer reviewed|Thesis/dissertation

University of California

Los Angeles

Engineering Influenza A/WSN/33 for In vivo Bioluminescent Imaging

A thesis submitted in partial satisfaction

of the requirements for the degree

Master of Science in Biomedical Engineering

by

Chetan Raj Lingaraju

2014

Abstract of the Thesis

Engineering Influenza A/WSN/33 for In vivo Bioluminescent Imaging

by

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Master of Science in Biomedical Engineering

University of California, Los Angeles, 2014

Professor Yong Chen, Co-chair

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Influenza A virus is a major causative agent of respiratory diseases in humans. It causes significant morbidity, mortality and economic losses each year worldwide with about 3-5 million clinical infections per annum. Its ability to mutate rapidly leads to seasonal epidemics and with its high frequency of genetic reassortment it causes pandemics. Conventional methods of studying viral pathogenesis do not allow for monitoring viral spread in real time during an infection. Whole body bioluminescent imaging of infected animals will overcome many of the problems associated with the current methods. In this work the wild type Influenza A/WSN /33 was engineered to carry a luciferase reporter gene in segment 1, based on a well established reverse genetics system for Influenza A viruses. The novel reporter WSN virus will enable less expensive, non-invasive in vivo imaging of viral replication and better evaluation of novel therapeutics.

The thesis of Chetan Raj Lingaraju is approved.

Genhong Cheng

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University of California, Los Angeles

2014

To my family

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Acknowledgments

Firstly, I am grateful to Prof. Cheng for allowing me to explore different topics of study and for providing a flexible environment for my research. I would like to thank Prof. Chen, Prof. Cheng, Prof. Chow and Prof. Deming for serving as the committee members for my MS thesis. Their mentorship has made me a better a student of science.

I am deeply thankful to Dr. Yao Wang and my lab manager, Dr. Saba Aliyari whose guidance was invaluable for this work. I am also thankful to all the other members of the lab for providing me with a great environment to learn.

Special thanks to my roommates Hitesh Mittal, Pranay Doshi, Sameed Hameed and Shailesh Chaudhari for being incredibly supportive during tough times and for making me feel at home in a foreign land.

CHAPTER 1

Introduction

Influenza A virus is a major causative agent of respiratory diseases in humans which can be fatal to young children, seniors and immunocompromised individuals. It causes a significantly high morbidity and mortality each year worldwide with about 3-5 million clinical infections and 250,000 - 500,000 fatal cases per annum [1]. With its rapid mutability and a high frequency of genetic reassortment it causes seasonal epidemics and pandemic outbreaks respectively [2]. The worst known influenza pandemic was the one recorded in 1918 caused by a particular strain of H1N1 Influenza A virus and it killed over 50 million people [3]. The recent flu pandemic was caused by a novel strain of H1N1 virus called the 2009 Influenza A (H1N1) [4]. In order to design better control methods for this disease, it is important to understand the pathogenesis of the virus.

Treating influenza virus infection is difficult due to an incomplete understanding of the pathogenesis of influenza viruses. The main factor affecting the spread of influenza A virus within an animal is its tissue tropism [1, 5]. Influenza viruses bind to sialic acids via their hemagglutinin antigen. Two major types of linkages are found between sialic acid and the galactose sugar present in the glycoprotein α (2,6) and α (2,3). Human IAV preferentially binds to sialic acid attached to the penultimate galactose sugar by an α (2,6) linkage as most epithelial cells in the human respiratory tract have this type of linkage [6]. The natural reservoirs of influenza A viruses are wild aquatic birds; and the gut epithelium of wild aquatic birds contain mostly α (2,3) linkage. However, due to high rates of mutation of

the hemagglutinin surface protein, the virus can adapt itself to receptors on cells from different species as well as from different parts of the body expressing different sialic acid-galactose linkages [7]. This allows some highly virulent strains of the virus to cross from one species of host animal to another and to even go beyond the respiratory tract and infect the central nervous system which is far more lethal to the infected animal [8].

Until recently, there was no method to monitor the virus infection in real time [9]. Conventional studies in mice require the monitoring of the virus in numerous animals, euthanizing them at specific time points and slicing various tissues to test for viral presence and the efficacy of a novel drug candidate. This results in less data per animal in each experiment, increased costs and reduced repeatability because of the inherent animal-to-animal variation. A technique for whole-body imaging in living animals can circumvent many of the problems associated with conventional methods to study viral pathogenesis [10]. Optical imaging (via fluorescence or bioluminescence) has been proven to be a good approach in monitoring different diseases [10, 11].

Previous efforts have shown that integrating the gene encoding GFP (green fluorescent protein) into one of the eight segments of the IAV genome is not a very efficient method for creating a reporter influenza virus due to high background from the surrounding tissue [12]. A few research groups tried to integrate the gene encoding luciferase or GFP in different segments of IAV, but that resulted in attenuated viruses. Other attempts to include an extra segment encoding the luciferase gene resulted in the production of an unstable reporter virus. This is due to the stringent packaging requirements of influenza during assembly. The work presented in this thesis is mainly based on the work by Heaton et al reported recently which shows that IAV can be engineered to contain a luciferase reporter gene which enables its use for in vivo tracking of the virus [9]. However, the strain used in that study was Influenza A/PR8 (H1N1) and in this study another

dangerous lab strain called Influenza A/WSN/33 (H1N1), an important derivative of the 1933 H1N1 isolate, was engineered to contain a Gaussia luciferase gene.

CHAPTER 2

Background

Viruses that belong to the family *Orthomyxoviridae* are enveloped, single-stranded, negative-sense RNA viruses. Negative sense RNA viruses have their genome that is complementary to the mRNA, which is defined as positive sense [13]. Due to this the naked genome of a negative sense RNA virus is non-infectious. There are five genera under *Orthomyxoviridae*, namely - Influenza A, Influenza B & Influenza C viruses; Thogotovirus and Isavirus.

Influenza is an acute infectious disease caused by a member of the Orthomyxovirus family influenza virus A, B or, to a much lesser extent, influenza virus C [15]. Each of these three genera has one species by the same name as the respective genus. Influenza type A exhibits the highest mutability and hence, it can adapt to hosts from different species most frequently. Influenza type B also shows some antigenic changes and sometimes causes epidemics. Influenza type C is the most stable and causes only mild illness in immunocompetent individuals. Thus, Influenza A virus is of prime importance in terms of human health.

2.1 Influenza A virus (IAV)

Wild aquatic birds are the natural hosts for influenza A [14]. They are subdivided into different serotypes based on their response to antibodies specific to H or N antigens. There are eighteen different Hemagglutinin (H1 to H18) and ten different Neuraminidase (N1 to N10) antigens known today. Genes coding for H

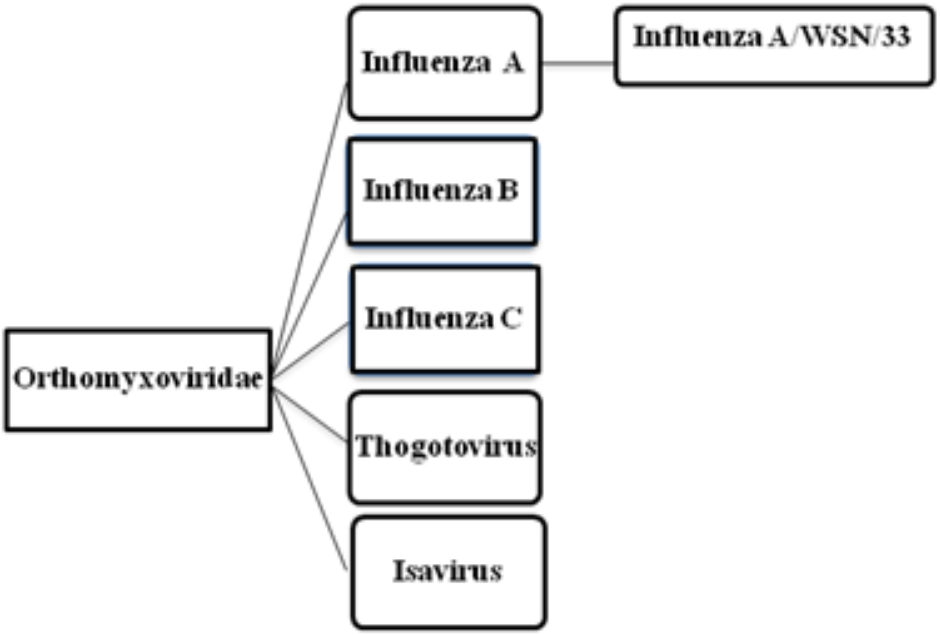

Figure 2.1: Taxonomy of Orthomyxoviruses

and N antigens undergo rapid point mutations avoiding recognition from host immunity. This phenomenon termed antigenic drift causes seasonal epidemics. The gene segments encoding the H antigen (segment 4) and the N antigen (segment 6) from different serotypes can reassort, resulting in novel recombinant viruses. This phenomenon termed antigenic shift is known to cause deadly pandemics and thus, is more dangerous than the point mutations in these two viral antigens.

2.1.1 Nomenclature of Influenza A viruses

Every genus of influenza viruses has one species each. Different influenza viruses are named according to their species (type), the species from which the virus was isolated, location of isolate or the research group who identified it, the year of isolation and in the case of Influenza A viruses, the hemagglutinin (H) and neuraminidase (N) subtypes (serotypes) [16]. For example: Influenza A/WSN/33 (H1N1) belongs to the species Influenza A, isolated by Wilson-Smith in the year of 1933 and expresses H1 and N1 antigens on its surface. It is often called WSN/33

or WSN for simplicity.

2.2 Structure overview

The virus is pleomorphic - it is usually spherical in shape with a diameter of about 100nm [17]. When it is elongated the filamentous particle measures at around 300nm. The genome of the influenza A virus has 8 (-) ssRNA segments.

Each RNA molecule is folded into a rod-shaped, partial double-helical ribonucleoprotein (vRNP) complex. Each of the eight vRNP complexes has a viral RNA segment wound around multiple nucleoprotein (NP) molecules in a stoichiometric manner and this is in turn associated with one copy of the heterotrimeric polymerase complex [17]. Each polymerase complex consists of a polymerase acidic protein (PA), polymerase basic 1 protein (PB1) and a polymerase basic 2 protein (PB2).

The vRNP complex is the core of the virus particle. This is held intact by a protein shell. The protein shell is made of matrix protein (M1), this is surrounded by a host-derived lipid membrane. The lipid membrane has HA, NA and M2 surface proteins. Each of the eight vRNP complexes acts as a separate transcriptional & replicative unit.

2.3 Proteins encoded by the viral genes

Each of the eight segments of Influenza A virus codes for at least one protein. When more than one protein is encoded by a single RNA segment, the virus hijacks the host-cell splicing machinery. The thirteen known viral proteins are summarized in table 2.1.

Segment	Protein	Primary function
1	Polymerase basic protein 2 (PB2)	Viral RNA transcription, host cell mRNA cap recognition
2	Polymerase basic protein 1 (PB1); Polymerase basic 1 –F2 (PB1-F2); Truncated PB1 protein N-40	v RNA transcription & replication, endonuclease activity; Pro-apoptic activity; enhances pathogenicity; Implicated in modulation of pathogenesis of the virus
3	Polymerase acidic protein (PA); Polymerase acidic protein X (PA-X)	vRNA replication, protease activity; Virus-induced host shut-off, enhances virulence
4	Hemagglutinin (HA OR H)	Viral attachment to host cell via receptor binding, enables vRNP release from endosome via fusion peptide
5	Nucleocapsid protein (NP)	vRNA binding, vRNA synthesis, vRNA nuclear import
6	Neuraminidase (NA OR N)	Viral budding
7	Matrix protein 1 (M1); Membrane protein 2 (M2)	Constructs the matrix, vRNP nuclear export; H+ ion channel
8	Non-structural protein 1 (NS1) ; Non-structural protein 2/ nuclear export protein (NS2/NEP)	Viral IFN (interferon) antagonist; vRNP nuclear export, regulation of transcription & replication

Table 2.1: The thirteen IAV proteins and their functions.

2.4 An overview of replication cycle of IAV

The replication cycle of IAV can be roughly divided into 5 stages [18]; adsorption; entry; fusion & uncoating; replication, transcription & translation and assembly & budding as represented in Fig. 2.2.

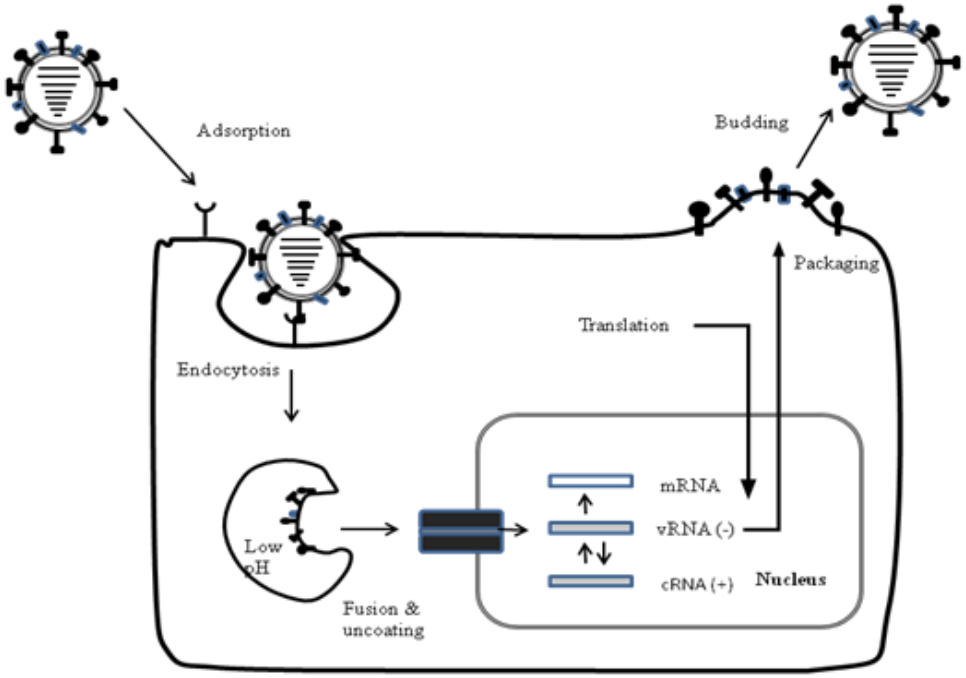

Figure 2.2: The life cycle of Influenza A virus

- Adsorption or attachment of the virus onto sialic acid containing receptors of host cells via HA protein on virus membrane.
- The whole virus enters the host cell mostly by clathrin-coated pit mediated Endocytosis.
- Fusion of viral lipid membrane to host cell membrane and uncoating of vRNP to release it into the cytoplasm.
- vRNP is imported to host cell nucleus mostly via CRM-1 mediated pathway. vRNA replication & transcription then occurs in the host nucleus. Viral

mRNA is then translated in the cytoplasm of the host cell. The newly made polymerase complex and NP is translocated into the nucleus which is followed by their association with the vRNA replicates resulting in new vRNPs.

- Newly synthesized vRNP is exported to cytoplasm where it is packaged into new viral particles with the M1 coat protein.
- Assembly of new viral particles is completed at the cell membrane along with the surface proteins H, N and M2 in a stoichiometric manner followed by budding. New viral particles are then ready to infect neighboring cells.

2.5 Pathogenesis of IAV

The virus spreads from one person to another by airborne droplets with a diameter of 10uM. The virus particles in the droplet once inhaled by a healthy person can initiate infection if they escape the sneeze-cough response and neutralization by preexisting IgA antibodies. It attaches to the respiratory epithelium through its HA surface protein and quickly starts making progeny virions that spread to neighboring cells. The virus has a short incubation period of 18-72 hours. Usually, only the epithelial cells in the upper respiratory tract are infected and destroyed in healthy and immunocompetent individuals. The main factor affecting its pathogenesis is tissue tropism of the virus. Eighteen different HA antigens and 10 different NA antigens, which are responsible for host-cell attachment and budding respectively, implies different IAV serotypes show different tissue tropism. Another important factor affecting the viral spread is the host immune response (both cell-mediated and humoral immunity are activated during IAV infection) [1]. The effect of this factor is clearly seen in infection of young children and the elderly, and in all previously immunocompromised individuals; the virus spreads to the lower respiratory tract and can be lethal. H1N1 virus, unlike most other

IAV strains can spread to the central nervous system of the infected which further worsens the patient condition. Thus it is of prime importance to study viral spread within an infected animal in real time.

2.6 Studying the pathogenesis of IAV

Current methods to study pathogenesis require euthanasia of infected animals and then performing plaque assays in homogenized tissue samples to calculate viral titres. This is a time-consuming method which gives no data in real time.

To study the pathogenesis of IAV in its entirety has remained a challenge. Whole-body imaging in living animals provides many advantages over conventional methods that necessitate euthanizing of animals. Optical imaging has been employed in other disease models such as cancer and Herpes virus infections [19].

Optical imaging has two main approaches - fluorescence and bioluminescence.

The advantages of having an integrated reporter gene are:

- Rapid quantification of virus.
- Whole-body, non-invasive imaging of living animals to monitor virus growth and spread in real time.
- Easy to assess treatment efficacy.
- Reduced cost.
- Better controls – each animal can serve as its own control. Less variability in results as it enables longitudinal studies.

2.7 Bioluminescent imaging (BLI)

BLI exploits the light-emitting properties of luciferase enzymes for monitoring cells and biomolecular processes in living subjects . The basic principle of BLI is as follows:

- The luciferase enzyme integrated into the host cell/animal genome acts on a substrate to produce light. For example, Gaussia luciferase catalyses the oxidation of coelenterazine to produce coelenteramide, CO_2 and light with an emission maxima at around 480 nm [20].
- The light emitted is proportional to enzyme activity.
- As enzyme production is controlled by a viral promoter, enzyme activity in a particular region reflects the concentration of infectious virus particles in that region.

CHAPTER 3

Designing the Virus

Classical forward genetics refers to the group of techniques where random mutations are induced in the genome of an organism and the mutant with a favorable phenotype is selected. The genome of this mutant is then analyzed using standard molecular techniques to identify the change in the genetic code that gave rise to the desired phenotype, thus enabling the identification of the gene(s) responsible for that phenotype. With the improvements in gene (and whole genome) sequencing methods, geneticists and molecular biologists began to approach their study of genes in an opposite manner i.e., by inducing specific alterations to the sequence of a known gene and testing for the new phenotype and hence identify the function of novel gene(s). This approach, termed reverse genetics', is a crucial complement to forward genetics in the study of genes.

Reverse genetics is particularly useful in virology as the small genome of a virus is relatively easier to manipulate compared to the genome of say, a worm or a mouse. Moreover, viruses are easier to work with owing to their short replication cycles. As a result, reverse genetics has been widely employed to study viral genes and also to create recombinant viruses. Different techniques in reverse genetics have been developed for different purposes since its inception.

In order to study RNA viruses using reverse genetics, the genome of an RNA virus is first reverse transcribed into a cDNA (complementary DNA) clone. This cDNA is inserted into suitable expression vectors that can be easily modified. Creating (or rescuing) a recombinant virus that has a positive sense RNA genome

is more straight-forward compared to a virus with negative sense RNA as its genome. This is because, unlike positive sense RNA, negative sense RNA of a virus is not recognized by the host cell's transcription or translation machineries. No viral mRNA, genomic RNA or viral protein will be produced by transfecting a eukaryotic cell with the genome of a negative sense RNA virus. Thus, viral enzymes that do the transcription and translation of RNA viral genomes must be added to the eukaryotic cell during transfection. This can be done by using a helper virus which infects the eukaryotic cells and provides the viral proteins necessary for production of new viral RNA and proteins. This method is not 100% safe as the helper virus, albeit attenuated, is still infectious. New developments led to the use of cDNA alone in bidirectional plasmids as described Neumann et al in a 2000 PNAS paper. This increases the transfection efficiency and safety of the whole procedure. Several other attempts of reducing the total number of plasmids used for transfection have been made which have resulted in new approaches to engineering viral vectors [23].

In this work a popular lab strain of Influenza A virus was engineered to contain a luciferase reporter gene based on the technique of the eight plasmid transfection system as reported by Neumann et al, 2000 [22].

3.1 The eight-plasmid pol Ipol II transfection system.

In this system one viral cDNA template can be read in two opposite directions by specific eukaryotic RNA polymerases to produce viral genomic RNA and viral mRNA. Each of the eight viral RNA segments is reverse transcribed as a cDNA clone into an expression vector (plasmid pHW). The sequence for RNA *pol* I promoter is placed immediately downstream of each cDNA template and the immediate upstream region has the sequence for RNA pol I terminator. The host RNA polymerase I enzyme recognizes the pol I promoter and transcribes the viral

cDNA of a particular segment into the negative sense vRNA (viral genomic RNA) of that segment.

This unit is flanked by a CMV promoter on the 5' end of cDNA and a BGH polyadenylation signal on its 3' end. The host RNA polymerase II enzyme recognizes the CMV promoter and transcribes the cDNA into viral mRNA (viral messenger RNA). Unlike pol I, this enzyme will add a 5' cap and a 3' polyadenylation signal to the transcript. This mRNA transcript will be translated into viral protein by ribosomes in the cytoplasm of the host cell. Fig. 3.2 shows the basic idea of transfection of a eukaryotic cell with the plasmids containing the cDNA of all eight vRNA segments of IAV. While most viral proteins remain in the cytoplasm, the proteins NP, PA, PB1 and PB2 translocate back to the nucleus in order to make new vRNP molecules which are then exported to the cytoplasm and assembled with the remaining proteins. This way the virus is replicated using the transfection of eukaryotic cells using only cDNA for the eight viral RNA segments. The new virions then bud off to infect neighboring cells and hence get amplified.

3.2 Difficulty in making the reporter virus

Due to the small size and segmented nature of the genomes of influenza A viruses it is a great challenge to integrate a reporter gene into any of the eight segments [23].

3.2.1 Structure of an RNA segment of IAV

There is a common basic structure to all the 8 RNA segments of IAV [24]. Each vRNA segment has a long central coding region, flanked by short untranslated regions (UTRs) specific to each vRNA segment. This, in turn, is flanked by a very short, highly conserved stretch of nucletiodes (12 on 3' end and 13 bases on 5' end) that are partially self-complementary and are common to all the eight

segments. The total length of UTRs in all Influenza A viral segments vary from 19 nt on the 3' end to 58 nt on the 5' end [25].

Each vRNA segment is associated with the four viral proteins (NP, PA, PB1 and PB2) to form eight types of vRNPs which are translocated to the cytoplasm and packaged into a progeny virion before it buds from the apical plasma membrane of the host cell. At least one copy of all the eight vRNPs have to be assembled for a virion to be infectious. Thus, the segmented nature of IAV poses a significant problem to engineer the virus.

Two models - random incorporation model and specific incorporation model - have been proposed to explain the assembly of vRNPs into progeny virions and there is increasing evidence to support the model for specific incorporation of the viral genome [24]. Several studies have shown that the ends of each segment have a unique cis-acting packaging signal. These packaging signals include the highly conserved termini for each segment (the 12 and 13 bases at 3' and 5' end of vRNA respectively), a variable length of the segment-specific conserved region and variable numbers of nucleotides in the adjacent coding region. If the integrity of the packaging signals is lost while engineering the virus, no infectious viral particles will be obtained [26].

Moreover, even point mutations in specific areas of the UTRs or the coding region have been shown to attenuate viral activity greatly [24]. Thus, it is essential to identify the safest region on the vRNA for insertion of a transgene.

3.2.2 Why segment 1?

Extensive studies have been done to map the packaging signals on segment 1. The following highly conserved nucleotides of segment 1 have been shown to be crucial for proper packaging of WSN PB2 vRNA - nucleotides 2218 to 2220, nucleotides 2221 to 2224 and nucleotides 2296 to 2298 [27]. Fig. 3.1 illustrates the noncoding

regions and the position of the 5' packaging signal on PB2 vRNA.

The packaging signals on Segment 1 encoding PB2 seem to mostly reside at the 5' end of the vRNA [27]. It is about 120nt from the 5' end which includes the 34nt UTR. Based on previous studies segment 1 of IAV has been shown to tolerate small insertions at the 5' end of the viral gene encoding PB2 protein [9]. Since segment 1 (like segment 2) is one of the longest of all 8 segments (2341 bases long), it is more likely to tolerate insertions of whole genes and still produce a replication-competent, stable influenza virus. Hence this segment was chosen for insertion of the gene encoding GLuc enzyme such that 120 nt from 5' end of PB2 vRNA was duplicated at the 3' end of GLuc gene.

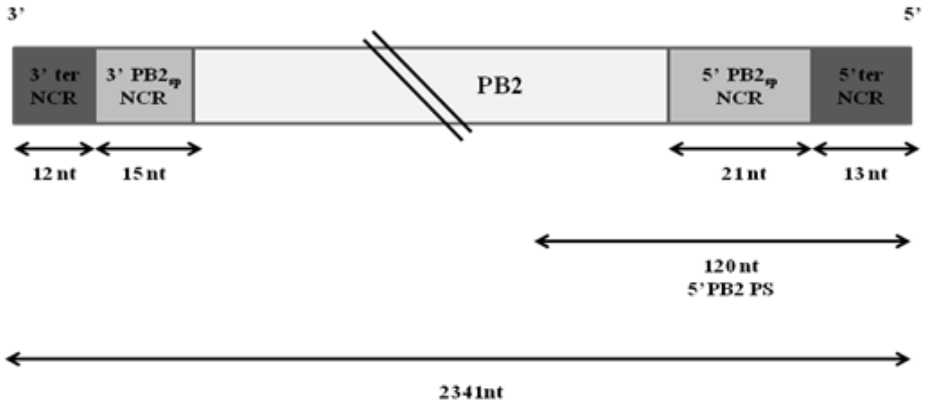

Figure 3.1: Schematic representation of wt PB2 vRNA of WSN virus. The total length of WSN segment 1 is 2341nt. The 3' and 5' terminal, highly conserved part of non-coding region is 12nt long (3' ter NCR) and 13nt long resp. (5' ter NCR). The 3' and 5' segment specific non-coding regions are 15nt (5' PBsp NCR) and 21nt long resp. (5' PBsp NCR). The crucial 5' packaging signal of PB2 vRNA is about 120nt long (5' PB2 PS). The coding region is denoted as PB2 with a crossed bar to indicate the segment is not drawn to scale.

3.2.3 Why Gaussia luciferase (GLuc)?

It is difficult to create a recombinant influenza virus which carries a full transgene due to its small and segmented nature of the genome. Each of the eight segments is not more than a couple kilobases long and hence it is difficult to make stable insertions in any given segment. Since it is segmented, it is difficult to ensure the new segments are being picked up during viral assembly. Gaussia luciferase - the smallest known luciferase protein with a molecular weight of 19.9 k Da and a short coding sequence of 555 bp, seemed to be an attractive candidate for this work [20]. GLuc has a broad emission spectrum with an emission maximum at 480 nm. Moreover, being a naturally secreted protein, GLuc has become an increasingly popular reporter enzyme for *in vitro* experiments. *In vivo* studies have also demonstrated that this luciferase has more sensitivity and brightness than Renilla luciferase or Firefly luciferase. Hence, GLuc was chosen as the reporter enzyme for this work.

3.3 The recombinant IAV for in vivo bioluminescent imaging was engineered in two steps:

Only one of the eight RNA segments of IAV WSN was to be integrated with the gene for a reporter enzyme. As segment 1 (PB2) was chosen, two similar recombinant plasmids were made from the wt plasmid pHW-PB2 with the aim of using one plasmid (which can make extracellular Gaussia luciferase) to rescue a recombinant WSN virus for *in vitro* tracking of the virus; and the second plasmid (which can make intracellular Gaussia luciferase) to create a recombinant WSN virus specifically for use in *in vivo* imaging.

First, the recombinant PB2 plasmid which produces extracellular GLuc (**pHW-PB2-GLuc**) was made and then the recombinant PB2 plasmid which produces

intracellular GLuc (**pHW-PB2-GLuc-KDEL**) was made. The two recombinant PB2 plasmids were then transfected into 293T cells in different plates to check for their respective GLuc activity. Then each of the two recombinant PB2 plasmids were co-transfected in different plates with the wild type plasmids for the seven other WSN RNA segments in the WSN eight-plasmid pol I-pol II system to make the recombinant IAV with extracellular and intracellular GLuc separately.

3.3.1 General design of the plasmid encoding WSN-PB2 gene.

The wild type plasmid pHW-PB2 has the insert for encoding wild type WSN-PB2 gene (segment 1 of WSN/33). This is represented by the lowest bar - insert (i) in Fig. 3.2. The size of insert (i) i.e. segment 1 is 2.3 kbp and the overall size of the wild type plasmid pHW-PB2 is 6.5 kbp.

Insert (i) encoding PB2 gene is flanked by polymerase I promoter and CMV promoter on opposite sides, such that the negative sense vRNA for PB2 is synthesised by RNA polymerase I and mRNA for PB2 is transcribed by RNA polymerase II of the host cell.

The insert added to the backbone of wild type plasmid pHW-PB2 to make the recombinant plasmid pHW-PB2-GLuc is represented by insert (ii) in Fig. 3.4. This insert consists of the PB2 gene fused with Gaussia luciferase gene with a sequence for P2A self-cleaving peptide. This ensures that the two polypeptides PB2 and GLuc will be separated immediately after translation. The original 5' packaging signal of segment 1 is silently mutated and denoted as PS* in Fig. 3.4. This packaging signal (PS) is duplicated at the 3' end of GLuc gene. Duplication of the 5' packaging signal at the end of the new insert (ii) is required for the packaging of the vRNP for mutant segment 1 synthesized from this recombinant plasmid into progeny virions. As GLuc is a naturally secreted protein; the recombinant IAV with this plasmid can be used for in vitro tracking of viral replication simply by

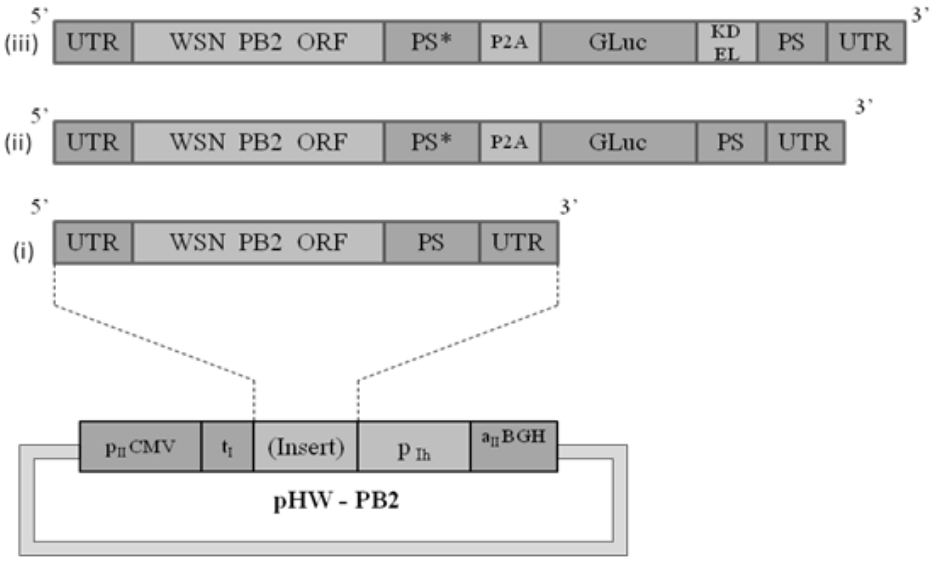

Figure 3.2: Schematic representation of the bi-directional plasmid pHW-PB2. (i) Insert for the wild type WSN-PB2 gene; (ii) Insert for the recombinant plasmid encoding PB2-Gluc fusion gene with a P2A self-cleaving peptide 5' to GLuc; (iii) Insert for the recombinant plasmid encoding PB2-Gluc fusion gene with a P2A self-cleaving peptide 5' to GLuc plus the sequence for KDEL ER retention sequence 3' to GLuc; UTR= untranslated region of segment 1, PS= 5' packaging signal of segment 1, PS*=silent mutation of 5' packaging signal of segment 1.

collecting the supernatant of the media in which the infected cells are cultured.

The insert added to the backbone of wild type plasmid pHW-PB2 to make the recombinant plasmid pHW-PB2-GLuc-KDEL is represented by insert (ii) in Fig. 3.4. Insert (iii) is very similar in construction to insert (ii). It has an additional stretch of nucleotides encoding the endoplasmic reticulum (ER) retention sequence - the KDEL peptide - fused to the 3' end of GLuc gene. This peptide binds to a specific receptor in the ER of the host cell and hence allows the protein which has this peptide to be retained within the cell. With this construct, the Gaussia luciferase enzyme becomes an intracellular protein and thus can be used for in vivo imaging of the recombinant IAV.

3.3.2 Designing the plasmid pHW-PB2-GLuc

Five different PCR reactions in two separate steps were done to produce the linear insert (ii) as shown in Fig. 3.3. Each primer was designed in such a way that half of the sequence (20 bases) was complementary to the template it was amplifying, and the overhanging half (15-25 bases) was complementary to the neighboring segment. This is essential for the overlapping PCR to work. Section 4.3 of chapter 4 - "Materials and methods" provides the sequence for each primer.

Step 1:

- PCR #1: the insert encoding wt WSN PB2 was amplified from wt plasmid pHW-PB2 using the primers (a) and (b). Result: amplicons encoding wt PB2 with a 3' end that overlaps with the 5' end of GLuc from PCR#2, and a 5' end that overlaps with the 3' end of linearized empty vector pHW to be made in the second PCR reaction of step 2.

- PCR #2: the insert encoding GLuc was amplified from the plasmid pGLuc using the primers (c) and (d). Result: amplicons encoding GLuc with a 3' end that overlaps with the 5' end of PB2 PS and a 5' end that overlaps with

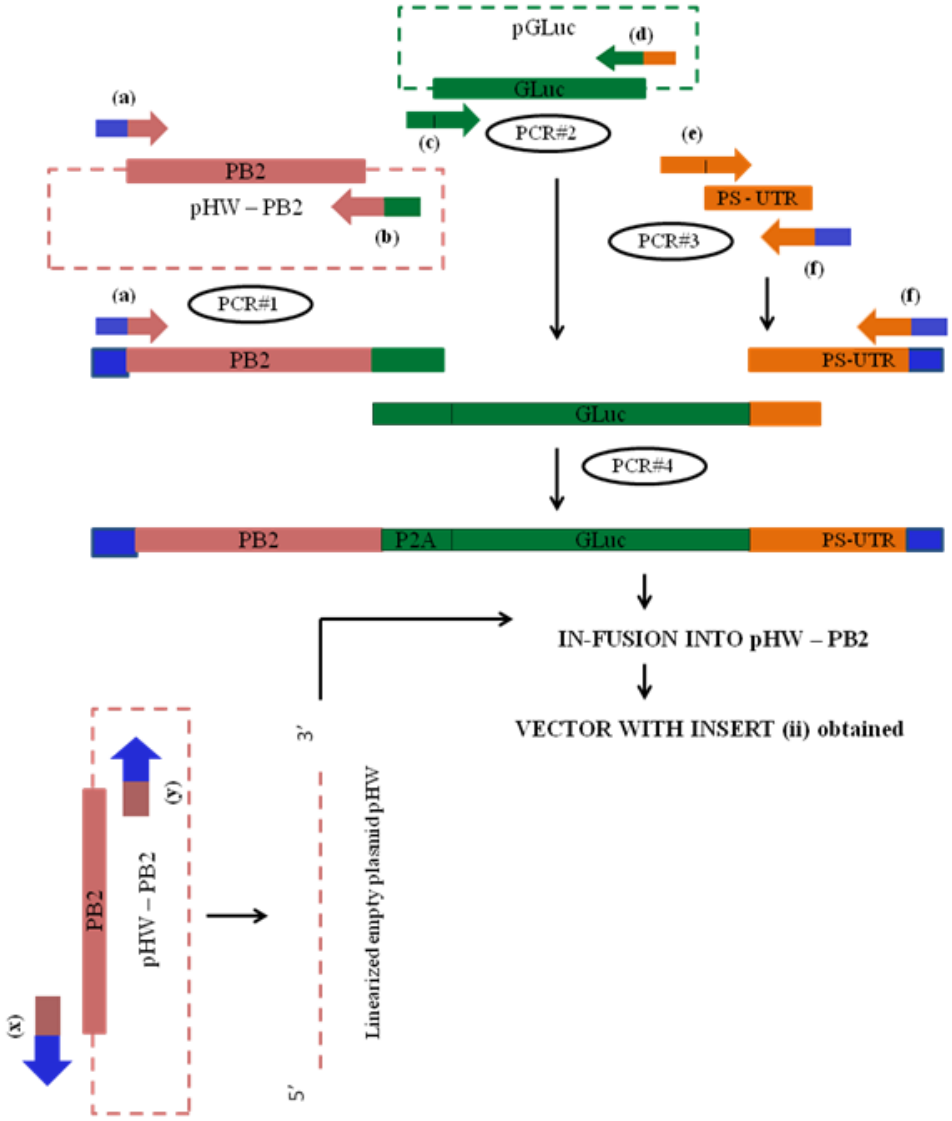

Figure 3.3: Designing the recombinant plasmid pHW-PB2-GLuc. The color of each primer - primer (a) to primer (f), and (x) and (y), is only illustrative and is matched with the color of the template DNA it binds to.

the 3' end of wt PB2 from PCR#1.

- PCR #3: the sequence for the 3' packaging signal of segment 1 (PS) was fused he 3'UTR of wt PB2 using the primers (e) and (f). Result: amplicons that had the sequence for 3' end of PS-UTR of PB2 gene. These amplicons had a 3' end that overlaps with the 5' end of linearized empty vector pHW to be made in the second PCR reaction of step 2; and a 5' end that overlaps with the 3' end of GLuc amplicons made in PCR#2.

Step 2:

- The amplicons from these three PCR reactions had overhangs that could act as separate primers in the overlapping PCR reaction, PCR #4 to produce the whole of insert (ii) as shown in Fig. 3.2.

- Simultaneously, the backbone of plasmid pHW-PB2 (i.e. pHW) was amplified in another PCR reaction using the primers (x) and (y) to produce a linear empty vector (pHW). The amplicons from this PCR reaction and from PCR#4 had overlapping ends (15-25 bases on each end). These two products were then fused together in an In-fusion reaction (Clonetech labs). The reaction product from In-fusion was treated with DpnI to digest old templates (pHW-PB2). Thus, the recombinant PB2 plasmid with insert (ii) viz. – pHW-PB2-GLuc was obtained which was amplified in *E.coli* after transforming it into competent bacterial cells before further use.

3.3.3 Designing the plasmid pHW-PB2-GLuc-KDEL

The vector obtained in the previous step was subjected (pHW-PB2-GLuc) to site-directed mutagenesis in order to add a small piece of DNA encoding the KDEL peptide to the 3' end of GLuc.

As shown in Fig. 3.4, primers (h) and (i) were used as forward and reverse

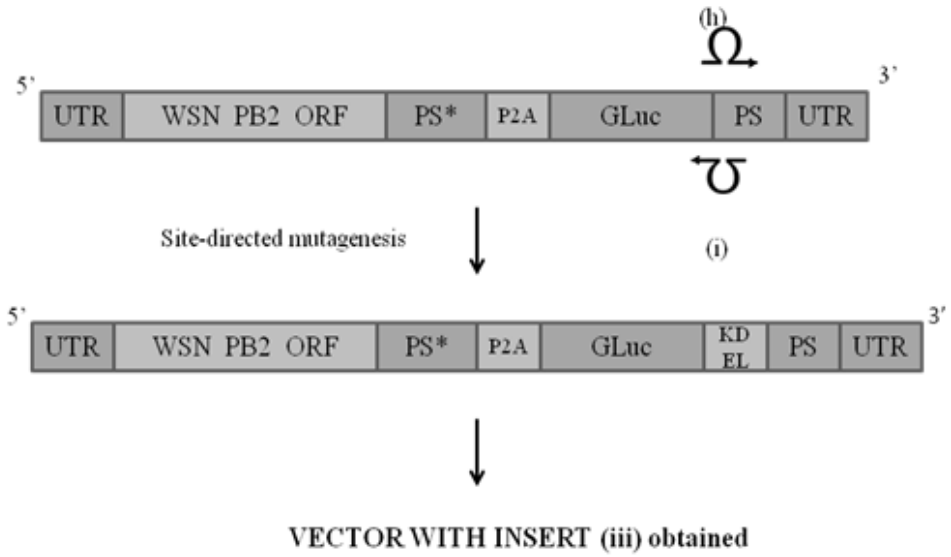

Figure 3.4: Designing the recombinant plasmid pHW-PB2-GLuc-KDEL. Inserts (ii) and (iii) are shown independently of the backbone plasmid pHW for the sake of simplicity. Plasmid pHW-PB2-GLuc from the previous step was subjected to insertional mutation using QuikChange II XL kit (Agilent Technologies) and KOD HotStart DNA Polymerase kit (EMD Millipore) to obtain the vector with insert (iii), that is, pHW-PB2-GLuc-KDEL.

primers for this PCR reaction respectively. Primer (h) binds exactly at the intersection of 3' end of GLuc gene and 5' of PS on the coding strand, while primer (i) binds at the intersection of 3' end of GLuc gene and 5' of PS on the non-coding strand. The reaction products were then treated with DpnI restriction digestion enzyme to eliminate the old template. The DpnI digest then had enriched concentrations of the required vector i.e., recombinant PB2 plasmid for intracellular GLuc (pHW-PB2-GLuc-KDEL) which was amplified in E.coli after transforming it into competent bacterial cells. Section 4.4.1 provides the sequence for each primer used in this reaction.

CHAPTER 4

Materials and Methods

4.1 Cells, plasmids, reagents and instruments

- Madin Darby canine kidney (MDCK) cells (ATCC# CRL-6253)
- 293 T cells (ATCC# CRL-3216)
- 1× Dulbecco's modified Eagle medium (Invitrogen)
- Fetal bovine serum (FBS), heat inactivated (Hyclone, Invitrogen)
- 100× penicillin/streptomycin solution (Cellgro)
- RNA polI-polII bidirectional plasmids for the eight segments of wild type Influenza A/WSN/33 (the pHW system for WSN)
- Plasmid encoding Gaussia luciferase – pGLuc
- Polyethynylimine (PEI), sterile (Sigma-Aldrich)
- 1× phosphate-buffered saline (PBS), sterile (Invitrogen)
- 15-cm, 6-well and 24-well tissue culture treated dishes
- Water-jacketed, 37^0C, 5% CO_2 humidified incubator
- QuikChange II XL site-directed mutagenesis kit (Agilent Technologies)
- HotStart DNA Polymerase kit (EMD Millipore)

- Renilla Luciferase Assay System (Promega)
- In-fusion HD Cloning Kit (Clontech)
- Luminometer (Bacterial System^{TM}, BG1^{TM})

4.2 Process flow charts

The various steps in making and characterizing the two recombinant WSN viruses are summarized in Fig. 4.1, Fig. 4.2 and Fig 4.3.

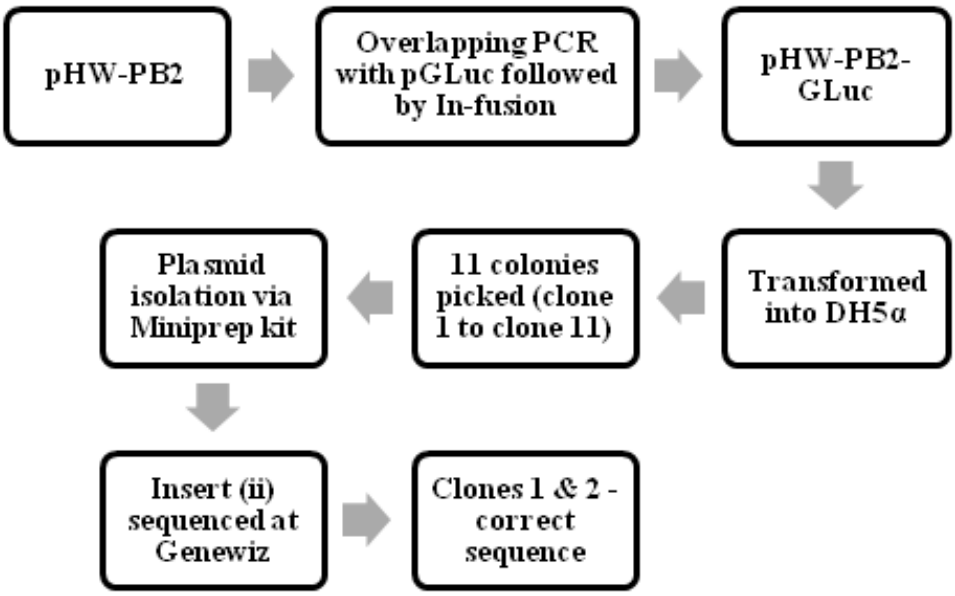

Figure 4.1: The process of making pHW-PB2-GLuc and cloning it into E.coli cells. The wt WSN plasmid pHW-PB2 was used to make the mutant plasmid pHW-PB2-GLuc as described in section 3.3.2. Competent bacterial cells (DH5α E.coli cells) were transformed with this plasmid. 11 colonies were observed on the AmpR containing agar plates, labelled as clones 1 through 11; their plasmids were isolated by a Miniprep and sent for sequencing the insert (ii) as described in Fig. 3.2. The clones 1 and 2 had the correct sequence for making PB2-GLuc fusion gene and hence were used for further experiments.

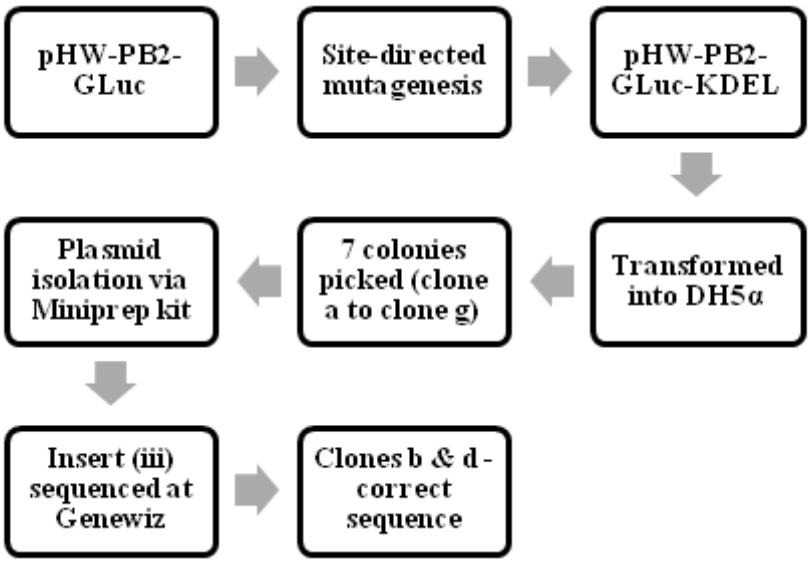

Figure 4.2: The process of making pHW-PB2-GLuc-KDEL and cloning it into E.coli cells. Site-drected mutagenesis was used to insert the sequence for KDEL peptide into the mutant plasmid pHW-PB2-GLuc to obtain the mutant plasmid pHW-PB2-GLuc-KDEL as described in section 3.3.3. Like pHW-PB2-GLuc, the new plasmid was cloned into DH5α cells. 7 colonies were picked 16h after incubation, labelled clone a through clone g and their plasmids were extracted using Miniprep kit (Invitrogen). Insert (iii) on these plasmids, as depicted in Fig. 3.2 were sequenced at Genewiz. Only clones b and d had the right sequence for making PB2-GLuc-KDEL fusion gene, and were used in further experiments.

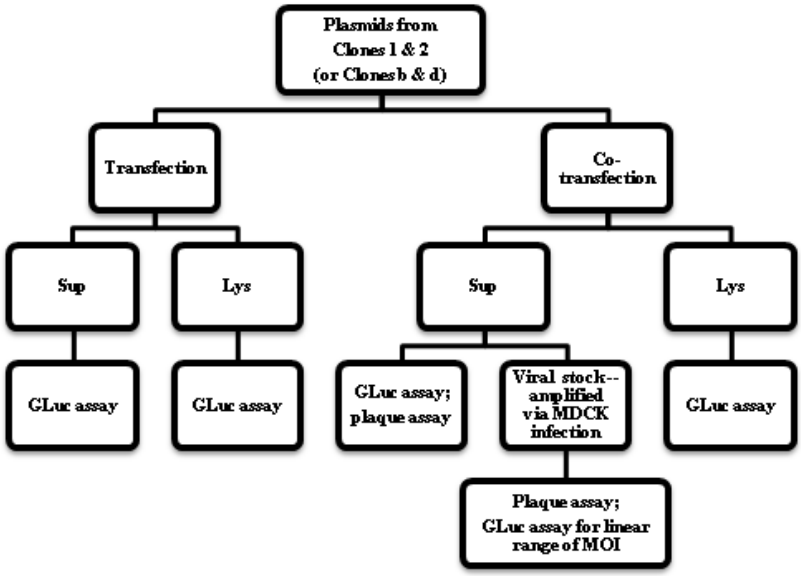

Figure 4.3: The process of testing the GLuc activity for pHW-PB2-GLuc and pHW-PB2-GLuc-KDEL, and characterizing the recWSN strains with either plasmid. The type of experiments performed to test the GLuc activity after transfecting clones 1 & 2 of pHW-PB2-GLuc into 293T cells were very similar to that performed for clones b & d of pHW-PB2-GLuc-KDEL. Each plasmid was singly transfected and also co-transfected with the 7 wt WSN plasmids to rescue the corresponding recWSN virus. The supernatant (sup) and cell lysate (lys) from each well transfected separately with different mutant plasmids were tested for GLuc activity. The sup and lys from each co-transfected with a mutant plasmid + 7 wt WSN plasmids were also tested for GLuc activity. The sup from each co-transfected 293T cell culture was titrated using plaque assay, then amplified separately by infecting a confluent layer of MDCK cells in different 10cm plates at MOI=0.01, the supernatants from which were then titrated to find the viral concentrations in the amplified viral stock; this was followed by a GLuc assay for the amplified virus to find the range of MOI at which it expresses GLuc enzyme in a linear fashion.

As the steps carried out to make both the strains of recWSN were very similar, for the sake of brevity only the methods for obtaining the recWSN virus with the plasmid pHW-PB2-GLuc-KDEL are detailed in this chapter.

4.3 Primers for making pHW-PB2-GLuc

The sequences for all the primers used in the PCR reactions (as described in Fig. 3.3) are listed below:

PB2_fwd = primer (a)

5' AGCGAAAGCAGGTCAATTATATTCAATATGGAAAG 3'

PB2-P2A_rev = primer (b)

5'CGTCTCCTGCTTGCTTTAACAGAGAGAAGTTCGTGGCGTTAATAGCCATTCTGATTCTTTTGGTC GCTGTCTGGCTGTC 3'

P2A_gLuc_fwd = primer (c)

5'CTCTGTTAAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCCATGGGAGTCAAAGTTCTGTT TGCCCT 3'

gLuc_PS_rev = primer (d)

5'- CCTTTCGCAAGGTTGCTCAGTTCATTTTAGTCACCACCGGCCCCCT 3'

PS_fwd = primer (e)

5' AATGAACTGAGCAACCTTGCGAAAGG 3'

PS_rev = primer (f)

5' AGTAGAAACAAGGTCGTTTTTAAACTATTCGACAC 3'

pHWvec_fwd = primer (x)

5' GACCTTGTTTCTACTAATAACCCGGCGG 3'

pHWvec_rev = primer (y)

5' TGACCTGCTTTCGCTCCCCCCCA 3'

4.4 Site-directed mutagenesis

The nucleotides for KDEL ER retention sequence were inserted at the 3' end of GLuc gene in the recombinant PB2 plasmid for intracellular GLuc using a combi-

nation of QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) and KOD HotStart DNA Polymerase kit (EMD Millipore).

4.4.1 Primers used for site-directed mutagenesis

GLuc-KDEL-fwd was the forward primer and was denoted as **primer (h)** in Fig. 3.4. 5'AAGGGGGCCGGTGGTGACaaagatgaactgTAAAATGAACTGAGCAACCTTGC 3'

GLuc-KDEL-rev was the reverse primer and was denoted as **primer (i)** in Fig. 3.4. 5'GCAAGGTTGCTCAGTTCATTTTAcagttcatctttGTCACCACCGGCCCCCTT 3'

4.4.2 The PCR reaction conditions for site-directed mutagenesis

Unlike standard PCR, this reaction amplifies the template DNA in a linear fashion, not exponentially. This is due to the circular nature of the template. The reaction products were immediately subject to *Dpn*I restriction enzyme digestion for 1h at 37^0C to eliminate the old template (pHW-PB2-GLuc). *Dpn*I digests only methylated DNA; hence the new PCR products could be used for transformation.

4.5 Transformation

The new plasmids were cloned into *E.coli* cells by transformation of DH5α E.coli cells using the following protocol:

- Thaw DH5α on ice 5 min; about 50% of the frozen stock must be melted.
- 1 tube for each sample to transform.
- Add 1uL of plasmid to each (1.5mL-2mL) – don't pipette up & down too much.
- Keep on ice for at least 25min (up to 2 hours).

Constituent	Sample (in μL)	Negative control (in μL)
Forward primer	1.25 (100ng/uL)	0.0
Reverse primer	1.25 (100ng/uL)	0.0
KOD HotStart DNA Polymerase	1.0 (1U/uL)	1.0
QuikSolution	3.0	3.0
Template DNA (pHW-PB2-GLuc)	2.0	2.0
dNTP (0.2 mM final concentration)	5.0	5.0
$MgSO_4$ (1 mM final concentration)	2.0	2.0
10X KOD HotStart DNA Polymerase reaction buffer	5.0	5.0
Distilled water	29.5	32.0
Total	50	50

Table 4.1: PCR reaction for site-directed mutagenesis

Constituent	Sample	Negative control
DH5α cells(OD600=1.0)	40 μL	40 μL
Dpn I digest from previous step	1.0 μL	1.0 μL

Table 4.2: Ratio of competent bacteria to DpnI digest for transformation

- Heat shock for 1 min at 42^0C.
- Keep on ice for 2 min, remove put on rack.
- Add 1mL of LB media, near flame, to each tube.
- Put tubes on 15mL falcon tube (with its cap opened) and tape it.
- Put it in shaker at 37 ^0C for 1 h.
- Prewarm AmpR containing agar plates in 37^0C incubator with lid off.
- Spin down for 1 min at max speed in a benchtop Eppendorf centrifuge.
- Remove 900uL of LB from each tube.
- Put back in incubator with lid off for 4 min.
- Put lid back on & flip over. Incubate overnight at 37^0C.

After 12-16h, pick colonies.

- Get autoclaved flasks, add 40mL LB + 40uL AMP (4^0C).
- Pick colonies and inoculate each flask with a different clone.
- Incubate at 37^0C for 12-16h. The clones are now amplified.

16h after incubation, seven colonies were observed in the sample plate but no bacterial growth was seen in the negative control plate. The seven clones were labelled as clone a through clone g and each of them were inoculated in a 250mL conical flask containing 40mL LB media and 40uL ampicillin and incubated in a bacterial shaker incubator for 12-16h as detailed in the protocol above. The suspension cultures of the seven different bacterial clones were pelleted at 5000g for 10min at 4^0C and their plasmids were isolated using the PureLink Quick Plasmid Miniprep kit (Invitrogen). The plasmids were then sequenced at Genewiz.inc.

Only two of the seven clones (clones b and d) had the correct sequence insert (iii) as represented in Fig.3.2.

4.6 Transfection

The mutant plasmids were transfected into a 60-70% confluent monoloyer of 293T cells in two ways mutant plasmid alone or co-transfection with the 7 wt WSN plasmids.

4.6.1 Single transfection of each clone of pHW-PB2-GLuc or pHW-PB2-GLuc-KDEL

293T cells were grown in 24-well plates. Six different transfection mixtures were prepared as follows:

- Plain DMEM = 25uL
- Plasmid* = 250ng
- PEI = 1uL

Plasmid* added was pHW-PB2-GLuc (clone 1), pHW-PB2-GLuc (clone 2), pHW-PB2-GLuc-KDEL (clone b) and pHW-PB2-GLuc-KDEL (clone d) in separate mixtures.

Negative control only received DMEM and PEI (no plasmids). The mixture for positive control received pGLuc. Each of these mixtures was added to different wells in the 24-well plate containing 293T cells in DMEM (with 10%FBS and 1% P/S). The plates were incubated for 48h before the supernatant and cell lysate from each well were taken.

4.6.2 Co-transfection of each clone of pHW-PB2-GLuc or pHW-PB2-GLuc-KDEL with the seven wt WSN plasmids

293T cells were grown in 6-well plates. Six different transfection mixtures were prepared as follows:

- Plain DMEM = 120uL
- Plasmid* = 250ng
- PEI = 4uL

Plasmid* refers to each of the eight plasmids to rescue WSN virus where seven of them were wild type and the eighth plasmid was either pHW-PB2-GLuc (clone 1), pHW-PB2-GLuc (clone 2), pHW-PB2-GLuc-KDEL (clone b) and pHW-PB2-GLuc-KDEL (clone d). Negative control only received DMEM and PEI (no plasmids). The mixture for positive control received all eight wt WSN plasmids. Each of these mixtures was added to different wells in the 6-well plate containing 293T cells in DMEM (with 10%FBS and 1% P/S). The plates were incubated for 48h before the supernatant and/or cell lysate from each well were taken.

4.7 Luciferase assay

All luciferase assays were done using the E2820 Renilla Luciferase Assay System (Promega) and the luminometer (Bacterial System ^{TM}, BG1^{TM}) with the following settings:

- Dispenser - 30uL RLuc substrate (diluted 200 times in substrate dilution buffer)
- Shake - 2 seconds

Sample	Description
Negative control	No plasmid, only media
Positive control	All 8 plasmids for wt WSN virus
Clone 1	Sup from co-transfection of 7 wt WSN plasmids + pHW-PB2-GLuc (clone 1)
Clone 2	Sup from co-transfection of 7 wt WSN plasmids + pHW-PB2-GLuc (clone 2)
Clone b	Sup from co-transfection of 7 wt WSN plasmids + pHW-PB2-GLuc-KDEL (clone b)
Clone d	Sup from co-transfection of 7 wt WSN plasmids + pHW-PB2-GLuc (clone d)

Table 4.3: Plaque assay for supernatant (sup) of different co-transfection mixtures.

- Integration time - 10 seconds

Supernatant from each well was treated with 2X lysis buffer in 1:1 ratio for 15min at room temperature before performing the assay. The cells in 24-well plate (or 6-well plate) were lysed with 100uL (or 200uL) 1X lysis buffer per well, incubated for 15min at room temperature and then the assay was performed. 30uL substrate was added to 10uL of sample (sup or lys) for each mutant plasmid (with or without the other 7 wtWSN plasmids).

4.8 Plaque assay

The viral titres for the supernatant of each sample with co-transfection of each mutant plasmid with the 7 wt WSN plasmids were calculated using a plaque assay as follows:

- Six 6-well tissue culture plates were seeded with MDCK cells (˜$0.8*10^6$ cells/well). 2mL/well of DMEM supplemented with 10% FBS and 1% P/S was used for culturing MDCK cells.
- About 10-12 h after seeding, the wells reached ¿95% confluency
- The media was aspirated from each well, washed with 1X PBS once.
- The cells were infected with serial dilutions of the viral stock (six dilutions from 10-3 to 10-8 in viral dilution buffer). Incubated at 37^0C for 1h to allow viruses to attach to MDCK cells.
- 1hpi the media was aspirated, cells washed with 1X PBS twice and 2mL of fresh molten agar overlay media, cooled to 37^0C was added to each well.
- The 6-well plates were transferred to 37^0C incubator once the agar solidified. Incubated for 48h.
- 48hpi plaques were observed in all the plates except the negative control plate.
- The agar overlay media was gently aspirated from each well, and the cells were stained with 0.5% crystal violet in 25% EtOH and 4% PFA for 20min then washed with distilled water.
- Plaques were counted and viral titres were calculated for each sample.

4.9 Amplification of recWSN viruses

A confluent layer of MDCK cells in 10cm plate was infected with the supernatant from the co-transfection step (section 4.6) at an MOI=0.01. The cells were incubated at 37^0C for two days. 48hpi severe cytopathic effect was observed in each

plate. Cells were pelleted at 1500g for 4min and the supernatant was stored in aliquots at -80^{0}C for further experiments.

4.10 Plaque assay for amplified recWSN viruses.

The recWSN viruses amplified by infecting MDCK cells, were titrated using a plaque assay similar to that explained in section 4.8.

4.11 Luciferase assay to estimate the range of MOI over which GLuc activity varies linearly

Six two-fold dilutions of the recWSN viruses (clone 1 or clone d) were used to infect MDCK cells at MOI=1.0 to 0.0. The basic protocol described in section 4.7 was used to assay GLuc activity for supernatant of MDCK cell culture infected with recWSN-PB2-GLuc at 1hpi, 4hpi, 10hpi and 24hpi; and for supernatant and cell lysates of MDCK cell culture infected with infected with recWSN-PB2-GLuc at 5hpi, 10hpi and 26hpi.

CHAPTER 5

Results and Discussion

5.1 To test the luciferase activity of the recombinant plasmids pHW-PB2-GLuc and pHW-PB2-GLuc-KDEL

To check the luciferase activity of the integrated GLuc gene, different wells in a 24-well plate with a monolayer of 293T cells were transfected as follows:

- Clone 1 or clone 2 of pHW-PB2-GLuc alone
- Clone b or clone d of pHW-PB2-GLuc-KDEL alone

The results of these luciferase assays are shown in Figs. 5.1 and 5.2.

To test for the luciferase activity of the recombinant plasmids in the presence of the seven other plasmids (wild type) in the WSN reverse genetics system, different wells in a 6-well plate with a monolayer of 293T cells were co-transfected with the wild type plasmids for segment 2 to segment 8 in the WSN reverse genetics system, as follows:

- Clone 1 or clone 2 of pHW-PB2-GLuc
- Clone b or clone d of pHW-PB2-GLuc-KDEL

The results of these luciferase assays are shown in Figs. 5.3 and 5.4.

On average, when the same amount of plasmids were transfected into 293T cells (single plasmid transfection), the supernatant from pPB2-GLuc exhibited only 2.8

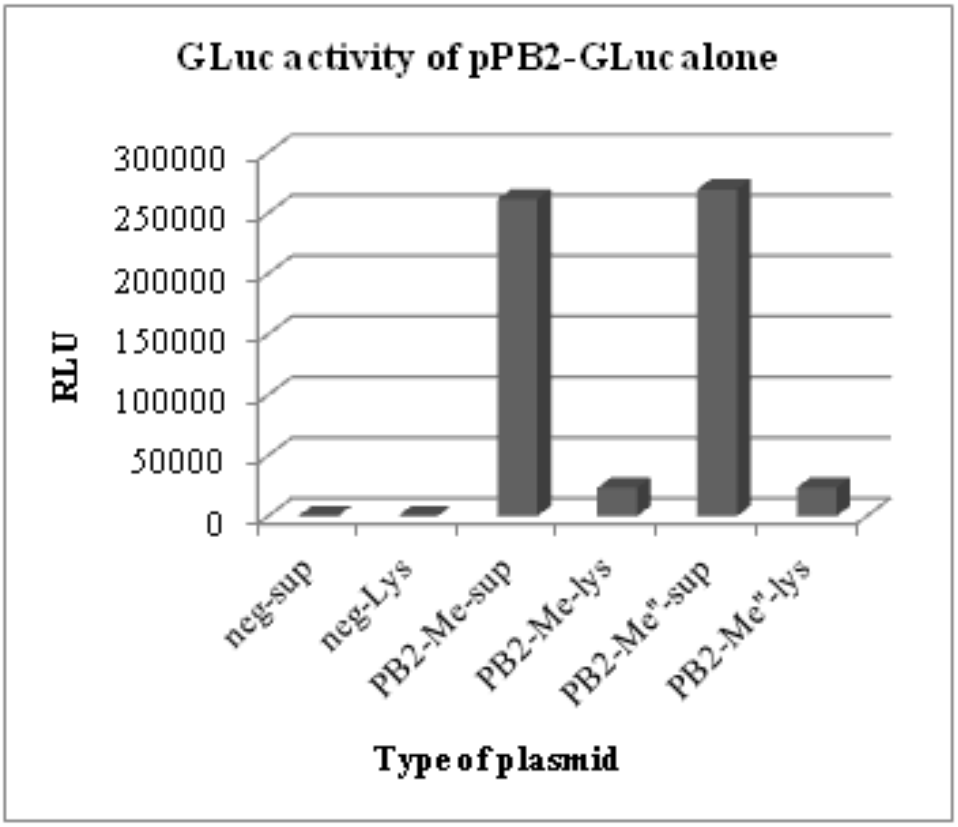

Figure 5.1: GLuc activity of pHW-PB2-GLuc alone. The supernatant and cell lysate from negative control (neg-sup & neg-Lys resp.) gave signals that were very close to the background signal (as expected). PB2-Me refers to clone 1 of WSN plasmid pHW-PB2-GLuc with extracellular GLuc activity, PB2-Me" = clone 2 of pHW-PB2-GLuc with extracellular GLuc activity. (These axis labels were used for brevity's sake). The supernatant of cell culture transfected with either clone 1 or clone 2 of this recombinant plasmid pHW-PB2-GLuc each gave a strong luminescence (50,000 times the background) and was over 11 fold greater than the luminescence from the corresponding cell lysates.

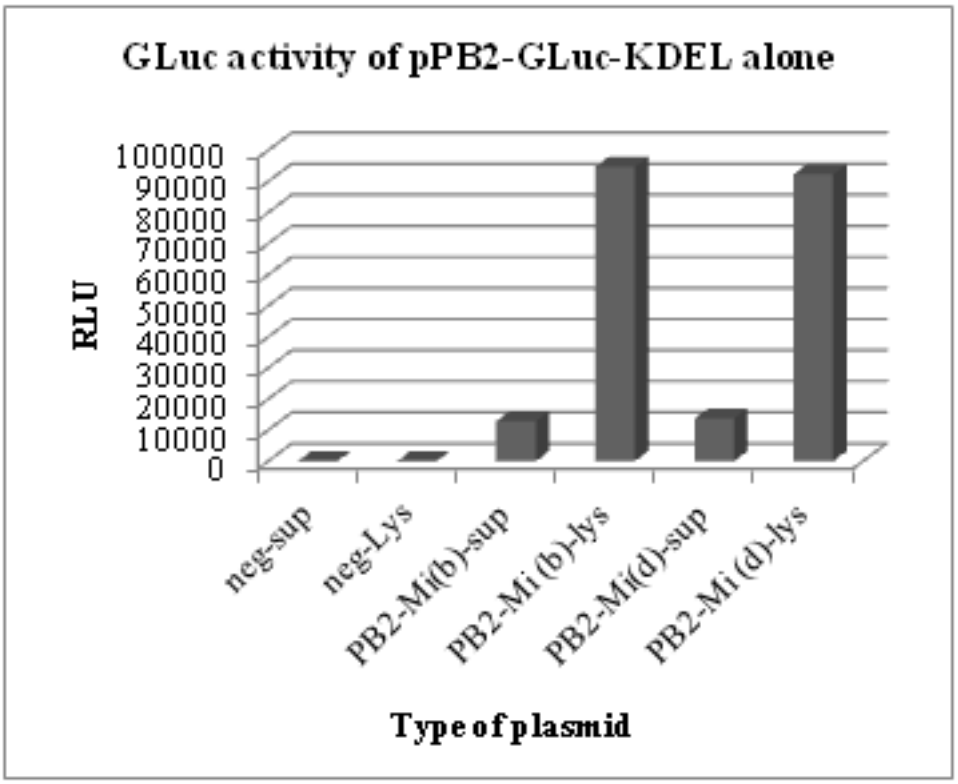

Figure 5.2: GLuc activity of pHW-PB2-GLuc-KDEL alone. The cell lysate and supernatant from negative control (neg-Lys & neg-sup resp.) gave signals that were very close to background. PB2-Mi(b) refers to the clone b of WSN plasmid pHW-PB2-GLuc-KDEL with intracellular GLuc activity, PB2-Mi(d) = clone d of pHW-PB2-GLuc-KDEL with intracellular GLuc activity. (These axis labels were used for brevity's sake). The cell lysate of cell culture transfected with either clone b or clone d of this recombinant plasmid pHW-PB2-GLuc-KDEL each gave a strong luminescence (19,000 times the background) and was 8 times more than that from the corresponding supernatants.

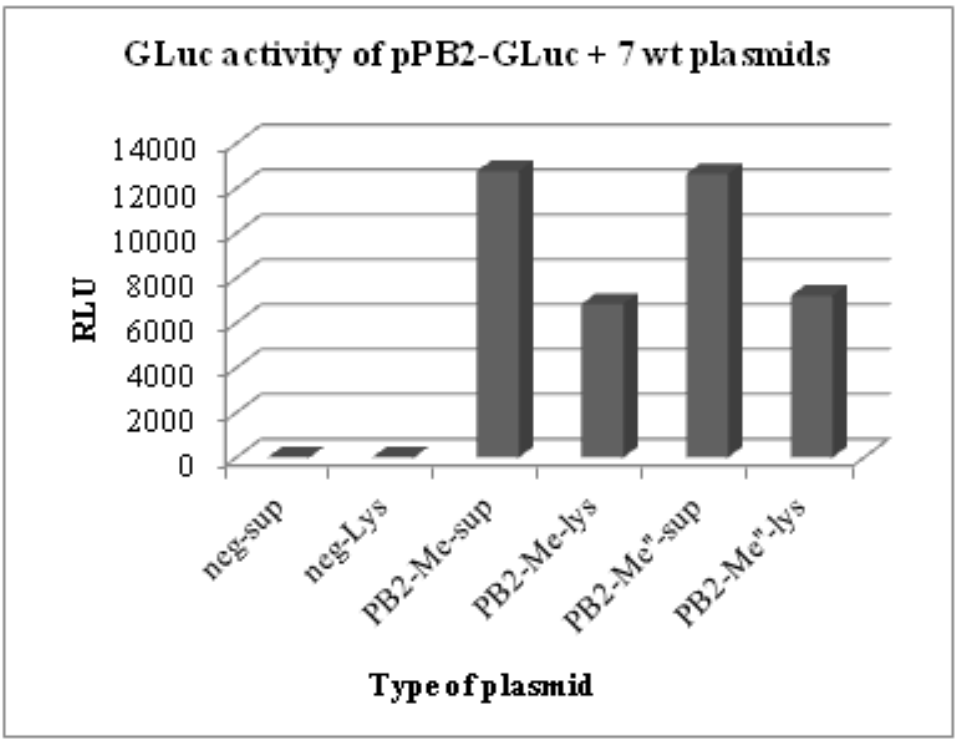

Figure 5.3: GLuc activity of pHW-PB2-GLuc + 7 wt plasmids. The supernatant from cell culture (with clone 1, PB2-Me-sup or clone 2, PB2-Me-sup) still showed a luminescence of over 2500 fold compared to that from the background (i.e. from negative control, neg-sup). In this co-transfection, the signal from supernatant was 1.9 fold more than that from the corresponding cell lysate.

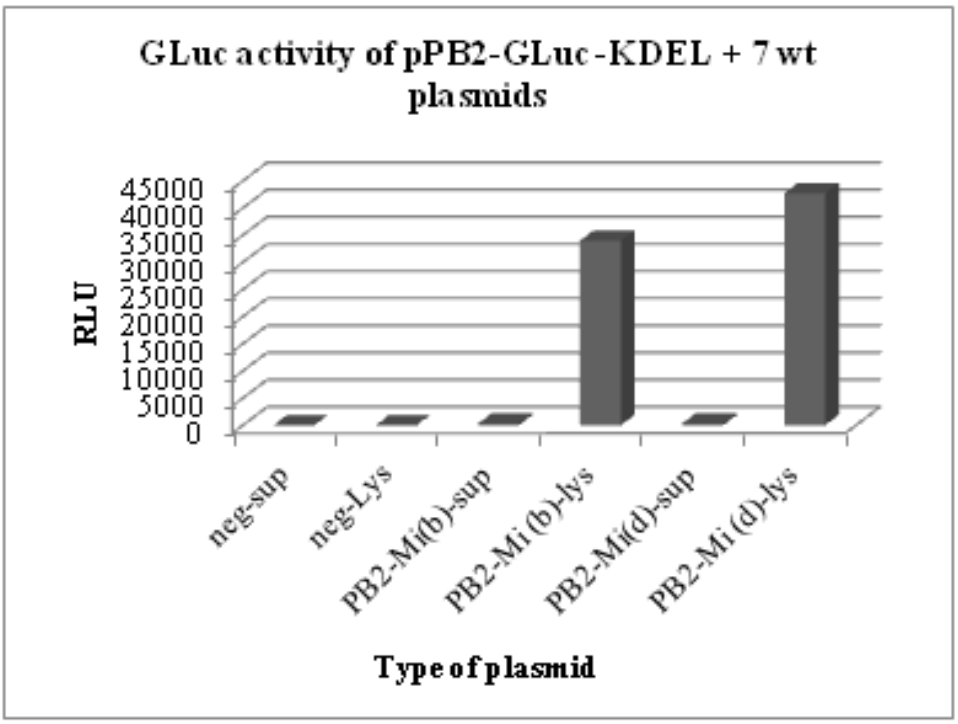

Figure 5.4: GLuc activity of pHW-PB2-GLuc-KDEL + 7 wt plasmids. The cell lysates from cell culture with clone b (PB2-Mi(b)-Lys) and clone d (PB2-Mi(d)-Lys) still showed a luminescence of over 6,800 fold and 8,500 fold greater than that from the background respectively. In this co-transfection, the signal from cell lysate for each clone b and clone d was 130 fold greater than that from the corresponding supernatant. Thus, most of the GLuc enzyme was retained within the ER of 293T cells.

fold greater luciferase activity than the cell lysate from pHW-PB2-GLuc-KDEL. This indicates that there is a very small reduction in luciferase activity from the plasmid encoding extracellular GLuc to the plasmid encoding intracellular GLuc when they are expressed alone.

In comparison, when the same amount of plasmids were transfected into 293T cells (co-transfection with 7 wt plasmids), the opposite trend was observed. On average, the cell lysate from pHW-PB2-GLuc-KDEL showed 3.1 fold greater luciferase activity than supernatant from pHW-PB2-GLuc. This indicates that there is a small increase in luciferase activity from the plasmid encoding intracellular GLuc to the plasmid encoding extracellular GLuc when they are expressed with the seven wt plasmids.

In sum, it is clear that both the constructs exhibit strong luciferase activity from the integrated gene for GLuc over the background with or without the seven wt plasmids in cell culture.

5.2 Plaque assay

Viral titres were calculated using plaque assay. The supernatant from each well containing 293T cells, transfected with the 7 wt plasmids + 1 recombinant plasmid was collected, a part of which was used for luciferase assay (as described in section 5.1 and fig. 4.3), another part was used for plaque assay and the remaining sup was stored for amplification in MDCK cells. The positive control used was wt WSN virus (known titre: 4×10^7 pfu/mL) and the negative control received only media (no virus).

A part of each supernatant was used to infect MDCK cells (at MOI=0.01) in four different 10cm plates. Strong CPE (cytopathic effect) was observed 48hpi in each plate indicating the amplification of the recombinant viruses. Plaque assay was done for the amplified virus stocks as well.

Before amplification in MDCK	
Sample	pfu/mL
Clone 1	1.45×10^4
Clone 2	1.24×10^4
Clone b	1.97×10^4
Clone d	2.00×10^4
Positive control (wt WSN)	3.80×10^7
Negative control (only media)	0.00

Table 5.1: Viral titres for supernatants collected from co-transfection of different recombinant plasmids with the 7 wt plasmids.

After amplification in MDCK	
Sample	pfu/mL
Clone 1	8.10×10^6
Clone 2	7.46×10^6
Clone b	1.28×10^7
Clone d	5.25×10^7
Positive control (wt WSN)	3.75×10^7
Negative control (only media)	0.00

Table 5.2: Viral titres for supernatants collected from amplification of different recombinant viruses.

From the viral titres, it is clear that the recombinant WSN virus with plasmid pHW-PB2-GLuc (clones 1 and 2) as well as the recombinant WSN virus with plasmid pHW-PB2-GLuc-KDEL (clones b and d) replicate as efficiently as wild type WSN virus. The recombinant WSN virus with pHW-PB2-GLuc (clone 1) and the recombinant WSN virus with pHW-PB2-GLuc-KDEL (clone d) were arbitrarily chosen for the remaining experiments but were simply called recWSN-PB2-GLuc and recWSN-PB2-GLuc-KDEL respectively.

5.3 To estimate the linear range of luciferase activity for the recWSN-PB2-GLuc virus

To establish the range of MOI at which the luciferase activity varies linearly at a particular time point post infection, MDCK cells were infected with serial dilutions of the recWSN-PB2-GLuc virus with known viral titre obtained from amplification in MDCK cells in the previous step (as described in section 5.2). Luciferase assay was done on the supernatant collected from each well with different MOI at different time points.

Zero hour virus stock: One hour after the cells were infected with serial dilutions of the virus, the supernatant from each well was collected, the cells were gently washed with 1X PBS, fresh media was added and continued with incubation. The supernatant taken at this time point was called the zero hour virus stock, as it represents the pre-existing Gaussia luciferase protein in the virus stock. Then the supernatant from each well was collected at different time points (1hpi immediately after adding fresh media, 4hpi, 10hpi and 24hpi). The results of the luciferase assay were as in Figs. 5.5 throuh 5.9.

The recWSN-PB2-GLuc virus (recombinant IAV with mutant plasmid encoding extracellular GLuc) showed good growth characteristics and it expresses luciferase in a linear fashion over a wide range of MOI at 10 hpi. Thus, the virus

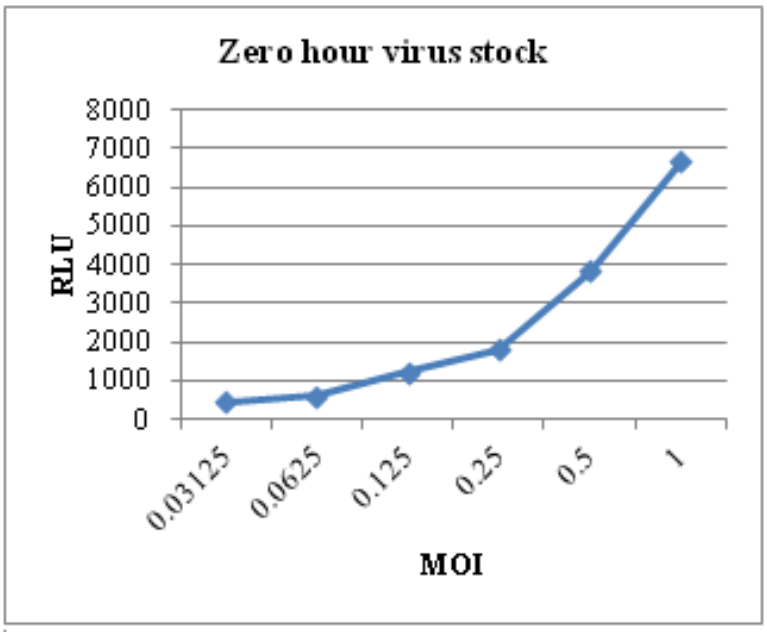

Figure 5.5: Zero hour virus stock. The supernatant collected 0-1hpi exhibits a significant amount of luciferase activity. This can be explained by the fact that this recombinant virus has the plasmid that produces extracellular GLuc enzyme which was already present in the supernatant of the previous cell culture infected with the virus. The luminescence increases linearly with increasing MOI. Hence, the cells were washed with 1X PBS 1hpi after collecting this sample and fresh DMEM was added.

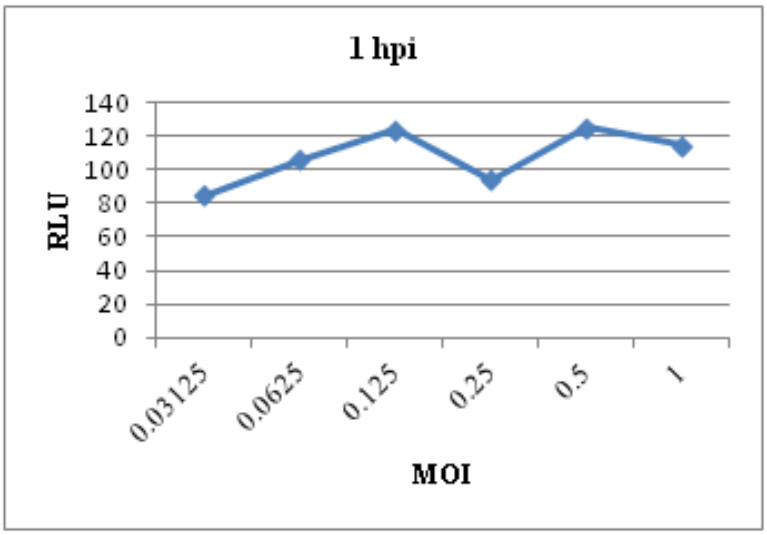

Figure 5.6: The reading at 1hpi essentially represents the background luminescence.

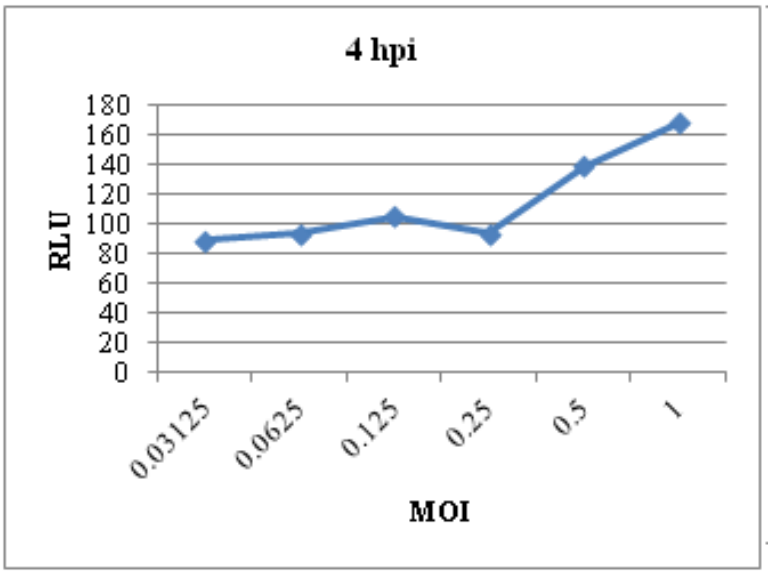

Figure 5.7: Even at 4hpi the luciferase activity is close to background indicating that the virus is yet to translate the mRNA for its polymerase complex (including PB2).

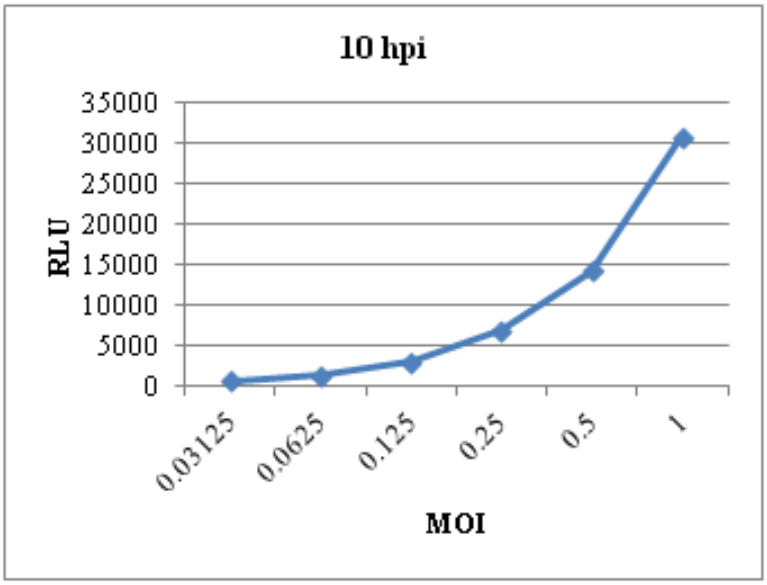

Figure 5.8: At 10hpi the luminescence from the supernatant in the linear range was about 100 times that of the background. The luminescence increased linearly over a broad range of MOI (0.125 to 1).

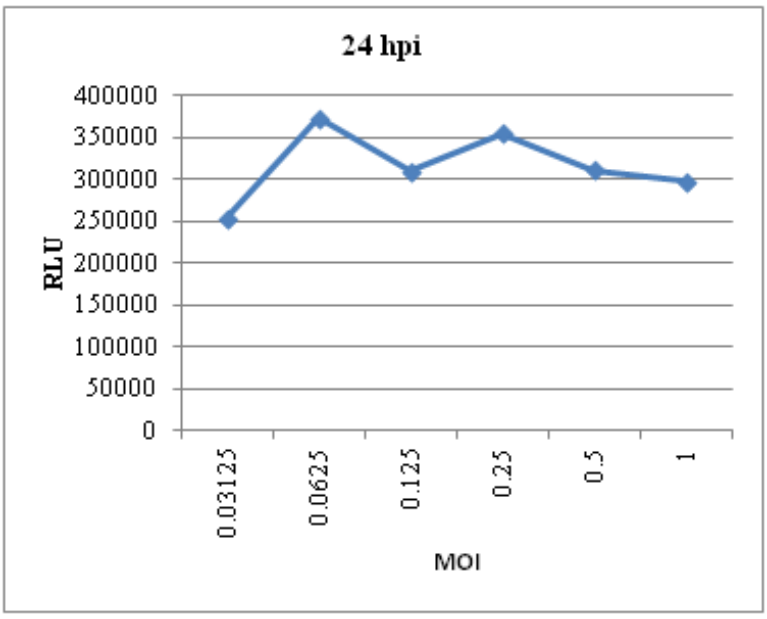

Figure 5.9: At 24hpi, the GLuc activity reached saturation.

can be used for in vitro studies (e.g. to test efficacy of novel drug candidates) over a wide range of MOI at this time point.

From figs. 5.6 and 5.7, it can be inferred that little to no luciferase is secreted before 4 hours of infection i.e. viral proteins including PB2 are not expressed before 4 hours of infection of cells.

It is important to note there are enough Gaussia luciferase proteins in the medium in which the original viral stock is suspended, to interfere with the luciferase assay results. Hence, once the infecting virions have attached to the host cells at about 1hpi the pre-existing Gaussia luciferase proteins must be washed off and replaced with fresh media before collecting samples for luciferase assay.

5.4 To estimate the linear range of luciferase activity for the recWSN-PB2-GLuc-KDEL virus

A luciferase assay similar to the one for the recWSN-PB2-GLuc virus was done for this virus, with a few changes. MDCK cells were infected with serial dilutions of the viral stock and the cell lysate and the supernatant from each well was collected at different time points and used for luciferase assay. As this virus had the plasmid for intracellular Gaussia luciferase proteins no zero hour virus stock reading was taken for this assay. Also, a blank well for each time point with no virus added was used as the blank reading for that time point. The results of the luciferase assay are shown in figs. 5.10 to 5.12.

At 5hpi, cell lysates for only MOIs greater than 0.5 showed a significant amount of luminescence. This could be indicative of the beginning of secretion the newly translated viral proteins.

Most of the GLuc enzyme from this virus was retained within the ER of MDCK cells as desired. For the linear range of MOI at 10hpi, the strong signal from cell

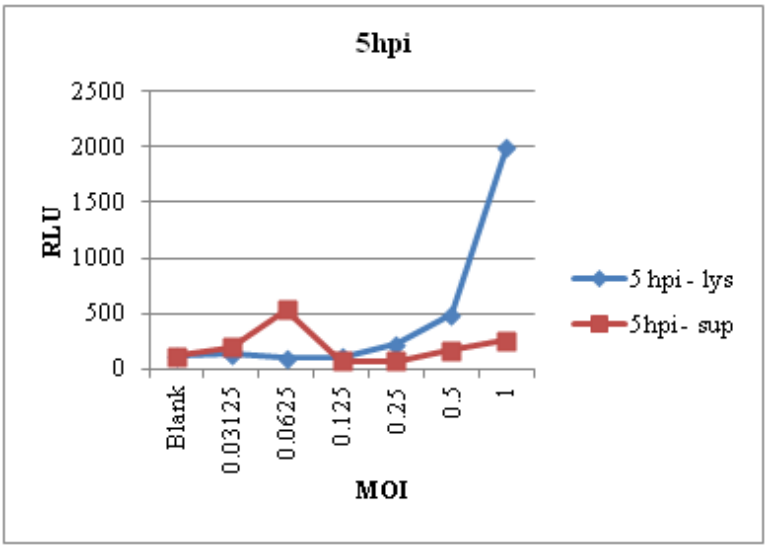

Figure 5.10: Very low signal was observed in the cell lysates for most dilutions of the virus at 5hpi. As was expected, the luminescence from the supernatant was close to background luminescence for all MOIs at this time point.

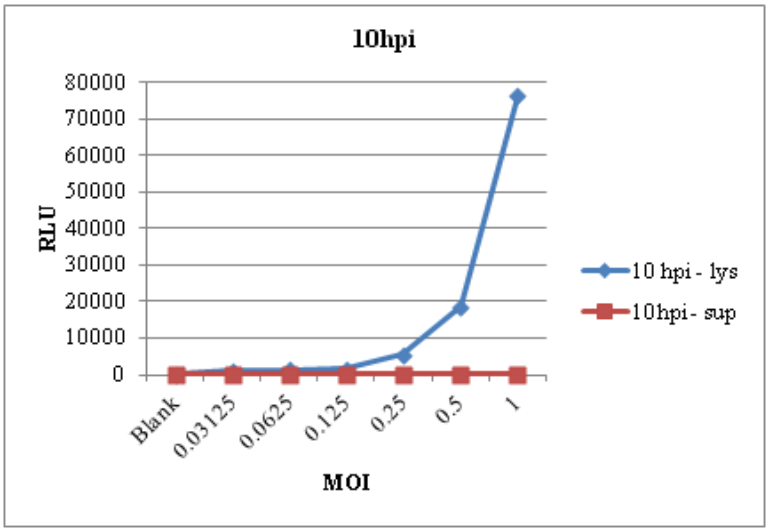

Figure 5.11: The luminescence from cell lysate increased linearly from an MOI of 0.125 to MOI of 0.5, this can be the most reliable range of MOI for this virus to study its pathogenesis. For the linear range of MOI, the signal from the cell lysate is over 50-90 fold greater than that from the supernatant.

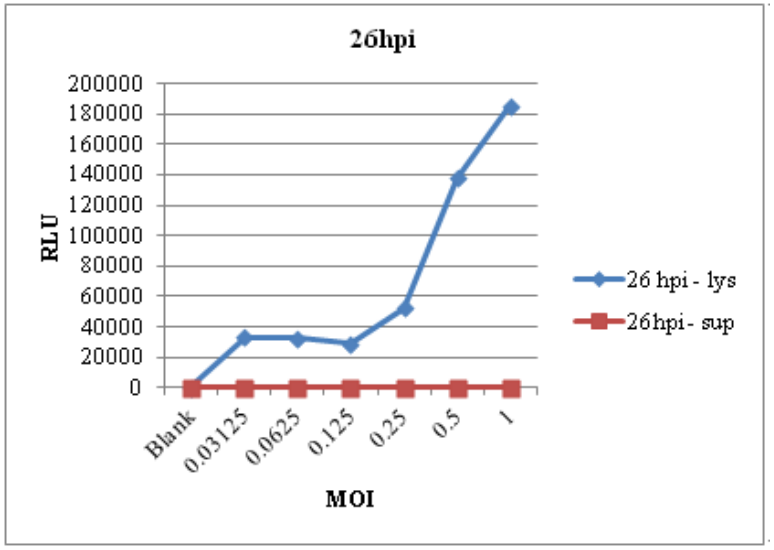

Figure 5.12: The signal from cell lysate for each MOI was still several folds greater than that from the corresponding supernatant. Surprisingly, the luminescence from cell lysates did not seem to saturate even at 26hpi. Further tests may be done to find the saturation point for this virus.

lysate (about 200 times that of background) was 50-90 fold greater than that from the corresponding supernatant. Thus, this virus has a great potential for use in non-invasive *in vivo* imaging.

Additional tests can be done in order to establish the stability of both the strains of recombinant WSN virus. Once the growth kinetics has been well established for each strain they will have multiple applications in vitro and in vivo studies of Influenza A virus.

CHAPTER 6

Conclusion

Influenza A virus causes significant loss to humans each year due to its high mutability and frequent genetic reassortment capability. There is a continuing need to better understand the pathogenesis of the virus. Novel tools designed in this direction are invaluable. Whole-body bioluminescent imaging is one such tool that is becoming increasingly popular in the study of different diseases [21].

In this work, the wild type Influenza A/WSN/33(H1N1) was successfully engineered for use in *in vivo* bioluminescent imaging. The strategy employed to engineer the wild type lab strain of WSN/33 virus resulted in two recombinant WSN viruses with an integrated gene encoding the Gaussia luciferase enzyme. Both the viruses were shown to be replication-competent *in vitro* and to exhibit strong bioluminescent properties. The first mutant virus (recWSN-PB2-GLuc virus) can be used for high-throughput viral quantification, drug screening, antibody characterization and other studies *in vitro* for better understanding the life cycle of Influenza A virus. Apart from being useful in vitro drug screening and antiviral vaccine evaluation, The second virus carrying an extra insertion of the nucleotide sequence for KDEL peptide (recWSN-PB2-GLuc-KDEL virus) can be used for real time, non-invasive *in vivo* imaging.

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