Improved and simplified recombineering approach for influenza virus reverse genetics

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ABSTRACT

Typical reverse genetics systems for generating influenza viruses require the insertion of each genome segments by DNA ligation into vectors for genome synthesis and expression. Herein is described the construction and use of a novel pair of plasmid vectors for cloning all eight genome segments of influenza A virus by homologous recombination for influenza virus reverse genetics. Plasmids, pLLBA and pLLBG, were constructed to possess opposing RNA polymerase I and RNA polymerase II transcription units for generating influenza genomic and messenger RNAs, respectively. In addition these promoters flanked a recombination cassette which comprised the conserved 5' (13bp) and 3' (12bp) terminal promoters of influenza virus. These vectors differed due to the presence of an A or a G (plus sense) to correspond to differences at nucleotide position 4 among negative-sense influenza virus promoters. The cloning approach involved homologous recombination of each influenza gene segment and the appropriate linearized pLLBA or pLLBG vectors in *E. coli*. Direct cloning by recombination was simpler and faster than conventional restriction digestion and ligation methods. This new vector system was successfully used to clone and rescue various influenza viruses and thus has the potential to promote the rapid analysis and vaccine development of novel influenza strains.

KEYWORDS: Influenza A virus, reverse genetics, vector construction, homologous recombination

INTRODUCTION

Reverse genetics methods have become essential for the genetic manipulation of influenza viral genomes in both basic and applied research (Neumann and Kawaoka, 2001; Subbarao and Katz, 2004; Marsh and Tannock, 2005; Kash et al 2006, Palese, 2006; Horimoto and Kawaoka 2006). Previously described reverse genetics systems for the generation of influenza virus from cloned genes include the 8-plasmid, 12-plasmid, and recently a 3-plamid system (Neumann et al, 1999; Neumann and Kawaoka,

2001; Neumann et al, 2005) with the 8- and 12-plasmid systems being the most widely used. The 8-plasmid system consists of each of the 8 genome segments under control of the RNA polymerase I promoter and terminator transcription units that function to generate genomic sets of negative sense viral RNA. This transcription unit is further nested within an opposing RNA polymerase II promoter and a polyadenylation signal sequence, that simultaneously functions to generate synthetic influenza mRNA from the same cDNA templates. Translation of viral proteins functions to produce infectious virus from

the viral genome (Neumann and Kawaoka, 2001). In the traditional reverse genetics systems, restriction endonuclease enzyme cleavage and DNA ligation are used to clone each influenza virus genome segment into expression vectors. This cloning process is not only time-consuming but is also prone to complications due to the presence of restriction endonuclease cleavage sites within the genome segments of some influenza strains that prevents the cloning of full length copies of genome segments. Cloning such genome segments that possess restriction sites used for cloning necessitates the use of alternative cloning and/or mutagenesis strategies.

Homologous recombination involves a process of breakage and reunion in regions of identical DNA sequences between 2 DNA molecules to result in new combinations of genetic material (Watt et al, 1985). The process of genetic engineering by recombination is increasingly being applied to high-throughput cloning applications and has been termed recombineering (Copeland et al, 2001; Sawitzke et al, 2007). We now describe the construction of a new pair of vectors for reverse genetics of influenza virus that are designed for homologous recombination with influenza virus genome segments. The pLLBA and pLLBG plasmid vectors were constructed using component DNA fragments amplified by PCR that were recombined by using homologous recombination throughout the construction process. This approach avoided the requirement for unique restriction sites between plasmid components for restriction enzyme digestion and ligation and both simplified and shortened the construction process.

The new reverse genetics vectors consist of three nested transcription units composed of opposing RNA polymerase I and II, DNA depended RNA polymerase promoters and terminators, for expressing viral and mRNA surrounding an influenza virus recombination cassette. The influenza recombination cassette consisted of both plus and minus strand influenza virus RNA dependent RNA polymerase promoters, separated by a *Stu*I restriction site that was nested between the RNA polymerase I and II promoters. These vectors provided a means of cloning of all 8 influenza virus genome segments via recombination in *E. coli*, and that functioned to provide the synthesis of the negative-sense viral RNA and positive-sense mRNA from the same cDNA template in 293T cells. Using this system, the genomes of three influenza virus subtypes were cloned into the newly constructed vectors by recombination and were subsequently used to generate recombinant and re-assortant influenza virus strains.

MATERIALS AND METHODS

Cells, viruses and RT-PCR

293T human embryonic kidney cells and Madin-Darby canine kidney cells (MDCK) were maintained in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), Penicillin (100U/ml), and Streptomycin (100μg/ml). All cells were maintained at 37°C in a 5% (v/v) CO_2 atmosphere. Influenza viruses A/Duck/Northern China/61/07 (H5N1), and A/Duck/Memphis/546/74

(H11N9) were propagated in 10-day old embryonated eggs. The A/WSN/33 (H1N1) genome was obtained from pHH21 plasmids, which were maintained in the laboratory of Dr Earl Brown. Viral RNA was extracted from 200 μl volumes of allantoic fluid using the Promega viral RNA mini kit (USA). Influenza cDNA were subsequently prepared using a universal plus sense primer mixture U12 5'GGGGAGCAAAAGCAGG plus 5'GGGGAGCGAAA GCAGG, and reverse transcription was performed in a reaction mixture containing 14μl RNA, 40pM primer, 20mM of each dNTP, 40U RNasin, 200U Murine Leukemia reverse transcriptase (Promega) in a total volume of 20μl, then incubate at 37°C for 1hr. Each influenza virus segment was amplified from the cDNA mixture for each of the 3 virus subtypes by PCR using the appropriate segment specific primers (Table 1). The PCR reactions were carried out in a final volume of 25μl that contained 4μl cDNA, 0.25mM dNTP, 2.5μl 10x PCR buffer, 2.5U *Pfu* DNA polymerase (Promega) and 10pM of each oligonucleotide primer. For PCR the samples were heated for 4min at 94°C, followed by 30 cycles of 94°C for 30sec, annealing at 55°C for 30sec and elongation at 72°C for 6min, followed by single 10min incubation at 72°C to extend incomplete templates. PCR products were purified by agarose gel electrophoresis and eluted into water using the QIAgen Gel Elution kit (Qiagen).

Construction of a pair of influenza reverse genetics vectors by recombinational cloning

Construction of a minimal protein expression plasmid
Firstly, a protein express vector was constructed by recombinational cloning. Two pairs of primers were designed to amplify the necessary regions from pCDNA3.0 (Invitrogen) and pIRES-EGFP (Invitrogen) for a complete but minimal protein expression vector. The primer set A (Primer A-plus 5'<u>GAATTCTGCAGATATC CTCGAG</u>CATGCATCTAG3'; Primer A-negative 5'<u>TGTT CTTTCCTGCGCCGCTACA</u>GGGCGCGTGG3') was used for amplifying the ampicillin resistance gene, the pBR322 *E. coli* origin of replication and the human cytomegalovirus (CMV) immediate early promoter of the pCDNA3.0 vector (Invitrogen). Primer set B (Primer B-plus 5'<u>TGTAGCGGCGCAGGAAAGAACA</u>TGTGAGCA AA3'; Primer B-negative 5'<u>CTCGAGGATATCTGCA GAATTC</u>CAGCACAC3') was designed to amplify the bovine growth hormone (BGH) and the polyadenylation signal site from the pIRES-EGFP vector (Invitrogen). To promote site-directed recombination, the plus sense primer A and negative sense primer B had 22bp extensions of identical sequence at their 5' ends (regions of homology are underlined). Similarly 22bp homology regions were designed into the termini of the negative sense A and plus sense primer B.

PCR of plasmid DNA employed 2.5U of *Pfu* DNA polymerase (Promega), 10ng of pCDNA3.0 or pIRES-EGFP, 2.5μl 10xPCR buffer, 5mM dNTPS, 10pM of each oligonucleotide primer in a 25μl reaction. The PCR reaction mixture was heated for 5min at 94°C, followed by 30 cycles of 94°C for 30sec, annealing at 55°C for 30sec and elongation at 72°C for 4min, followed by one 10min

Table 1. Specific PCR primers for each genome segments of A/Duck/Northern China/61/07(H5N1), A/WSN/1933 (H1N1), and A/Duck/Memphis/546/74 (H11N9).

Genome segment/virus	[a]UF/G- and [b]UF/A- forward primer sequences	[c]UR- reverse primer sequence
PB2/H5N1 PB2/H1N1 PB2/H11N9	UF/G-TCAAATATATTC UF/G-TCAATTATATTC UF/G-TCAAATATATTC	UR-TCGTTTTTAAAC UR-TCGTTTTTAAAC UR-TCGTTTTTAAAC
PB1/H5N1 PB1/H1N1 PB1/H11N9	UF/G-CAAACCATTTGA UF/G-CAAACCATTTGA UF/G-CAAACCATTTGA	UR-CATTTTTTCAC UR-CATTTTTTC UR-CATTTTTTC
PA/H5N1 PA/H1N1 PA/H11N9	UF/G-TACTGATCC UF/G-TACTGATCC UF/G-TACTGATCC	UR-TACTTTTTTGG UR-TACTTTTTTGG UR-TACTTTTTTG
HA/H5N1 HA/H1N1 HA/H11N9	UF/A-GGTTCAATC UF/A-GGAAAAT UF/A-GGAAATATC	UR-GTGTTTTTAAC UR-GTGTTTTTC UR-GTGTTTTTACTGCA
NP/H5N1 NP/H1N1 NP/H11N9	UF/A-GTAGATAATC UF/A-GTAGATAATC UF/A-GTAGATAATC	UR-GTATTTTTC UR-GTATTTTTC UR-GTATTTTTC
NA/H5N1 NA/H1N1 NA/H11N9	UF/A-AGTTCAAAATG UF/A-AGTTTAAAATG UF/A-GTCAAGATGA	UR-AGTTTTTTG UR-AGTTTTTTG UR-GTCTTTTTTC
M1,2/H5N1 M1,2/H1N1 M1,2/H11N9	UF/A-TAGATGTTG UF/A-TAGATATTG UF/A-TAGATGTTG	UR-TAGTTTTTTAC UR-TAGTTTTTTAC UR-TAGTTTTTTAC
NS 1,2/H5N1 NS 1,2/H1N1 NS 1,2/H11N9	UF/A- GTGACAAAAAC UF/A-GTGACAAA UF/A-GTGACAAA	UR-GTGTTTTTTATC UR-GTGTTTTTTATC UR-GTGTTTTTTAGT

Notes: (a) Universal forward (G): UF/G = GGGGAGCGAAAGCAGG
(b) Universal forward (A): UF/A = GGGGAGCAAAAGCAGG
(c) Universal reverse: UR = GGTTATTAGTAGAAACAAGG

incubation at 72°C to extend incomplete templates. PCR products were treated with 10U of *Dpn*I for 1hr to digest the template DNA and purified by gel electrophoresis and elution into water using the QIAgen Gel Elution kit (Qiagen).

To perform recombinational cloning, the BGH-polyA gene and the Ampicillin gene-pBR322 origin-CMV promoter PCR products were mixed together (200ng:50ng) and transformed into competent DHF5α *E. coli* cells where the two sequences recombined via site directed homologous recombination into an intact circular vector (Figure 1). The sequence of an isolated ampicillin resistant colony was determined to be a recombinant plasmid, designated pHP and shown to be free of unwanted mutations after sequencing using a commercial sequencing service (ShangHai Sangon Ltd).

Production of bidirectional influenza reverse genetics plasmids
The RNA polymerase I promoter and terminator elements and influenza recombination cassettes were derived from

pHH21A and pHH21G plamids (maintained in the laboratory of Dr EG Brown) and inserted between the RNA polymerase II promoter and BGH-polyA signal site of the pHP plasmid using the recombinational strategy described above. Therefore two primer sets, C and D, were designed for amplifying the complete pHP and the RNA polymerase I plus influenza recombination units, respectively. The following sequences were synthesized for primer sets C and D: Primer C-plus 5'ACCCAAG CTTCCGGAGTACTGGTCGACCTCC3'; primer C-negative: 5'ATGCTCGAGGCCGGGAGGGCGTCCCCG GC3'; primer D-plus 5'AGTACTCCGGAAGCTTGG GTCTCCCTATAG3'; and primer D-negative 5'GCCCTC CCGGCCTCGAGCATCATCTAGAGG3'. The termini of the two pairs of primers had complementary sequences for insertion via recombination (regions of homology are underlined).

The RNA polymerase I promoter and terminator elements flanking the influenza minus and plus stranded influenza promoter sets, that possessed either an A or G at position 4 of the minus sense promoter, were cloned into the
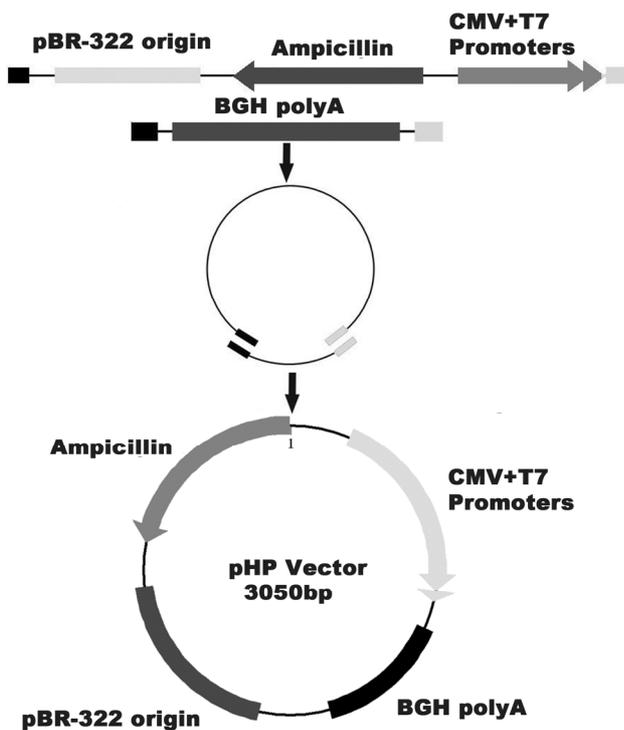
Figure 1. The construction strategy of the minimal protein expressing plasmid, pHP vector, using homologous recombination. The BGH-polyA signal was recombined with the homologous termini of DNA that possessed the ampicillin gene-pBR322 origin of replication-CMV promoter into a single vector by homologous recombination after transfection into *E. coli*.

previously described protein expressing plasmid, pHP vector. This was achieved by transformation of competent DHF5α with a mixture of linear PCR products derived from the pHP and pHH21A or pHH21G plasmids to generate plasmids pLLBA and pLLBG, respectively. For use in recombinational cloning the pLLBA and pLLBG plasmids were linearized by digestion with *Stu*I using 10μg of plasmid DNA and 50U of *Stu*I in a 100μl reaction volume with incubation at 37°C for 2 hr before agarose gel purification using the QIAquick Gel Elution kit (Qiagen).

Recombinational cloning of influenza genomes
Each of the 8 genome segments of three influenza viruses, A/Duck/Memphis/546/74 (H11N9), A/WSN/33 (H1N1) and A/Duck/Northern China/61/07 (H5N1) were amplified by PCR using appropriate primers (see Table 1) such that they possessed terminal complementarity with the linearized pLLBA and pLLBG vectors. Thus PCR products of all 8 genome segments of each virus were cloned into the appropriate vector by recombinational cloning in *E. coli*. According to the corresponding identities of the 4th base pair from the terminus of the negative sense influenza virus promoter the PB2, PB1, and PA segments were cloned into the pLLBG vector, and the HA, NA, NP, M1/M2, NS1/NS2 segments were cloned into pLLBA, where the two plasmids possessed a G or A, respectively. Insertion of influenza virus genome segments into these plasmid vectors was achieved by

using a mixture of 200ng of cDNA and 50ng linearized vector for transformation into competent DHF5α cells. Colony PCR was used to screen the ampicillin resistant colonies using appropriate primers (Table 1). PCR positive clones were sequenced to assure that plasmids were inserted in the correct orientation and without unwanted mutations.

Generation of recombinant influenza virus from plasmids
To rescue virus from plasmids, 293T cells were transfected with genomic sets of 8 plasmids using cationic lipid transfection reagent, Lipofectin 2000 (LP2000), according to the manufacturer's instructions. Briefly, genomic sets of 8 DNA plasmids (1μg of each segment except PA for which 0.1μg was used) and LP2000 were mixed in Opti-MEM, incubated at room temperature for 20min, and then added to the cells, 6hr later, the DNA-LP2000 mixture was replaced by Opti-MEM (Gibco) containing 2.5μg/ml trypsin. Forty-eight hours post-transfection, the culture supernatant was inoculated into 10-day-old eggs to amplify the rescued viruses.

To compare the efficiency of the simplified eight plasmid versus the 12 plasmid reverse genetics systems, virus rescue was also compared for a mixture of the 8 pLLB-WSN-PB2, -PB1, -PA, -HA, -NP, -NA, -M, -NS plasmids versus the 8 corresponding pHH21 plasmids plus the 4 polymerase expressing plasmids (pCDNA-WSN-PB2, PB1, PA, NP) in 293T cells. Virus yields were determined at 48hr post-transfection, by titration of the 50% tissue-culture infectious dose (TCID50) in MDCK cells as previously described (Brown, 1990).

RESULTS AND DISCUSSION

Homologous recombination plays important roles in DNA replication and repair, and the promotion of genetic diversity in eukaryotic and prokaryotic organisms (Muyrers et al, 1999 and 2000; Yu et al, 2000; Ellis et al, 2001). The principles of homologous recombination have also been widely used in gene therapy and genetic engineering, referred to as recombineering (Yu et al, 2003; Amos et al, 2004; Datta et al 2006; Berrow et al, 2007; Chan et al, 2007). Here, we used recombinational cloning in influenza virus reverse genetics by constructing a pair of vectors that could recombine with influenza virus genomic cDNA strands in *E. coli*. Recombination was mediated by short terminal homologies engineered into each DNA molecule (15-19bp) that were then joined in transformed *E. coli*. Cloning via recombination in *E. coli* constitutes a simple method of engineering DNA that removes the need for restriction and ligation steps and thus both simplifies and shortens the cloning process relative to traditional cloning methods. Importantly, recombination method avoids the need for modified cloning strategies for influenza genome segments that possess internal restriction enzyme cleavage sites that prevents their cloning using traditional restriction enzyme digestion and ligation procedures.

The pHP, pLLBA and pLLBG plasmids were constructed by recombination as described in Materials and Methods using the strategy outlined in Figures 1, 2 and 3. The pLLBA and pLLBG vectors possessed an influenza virus
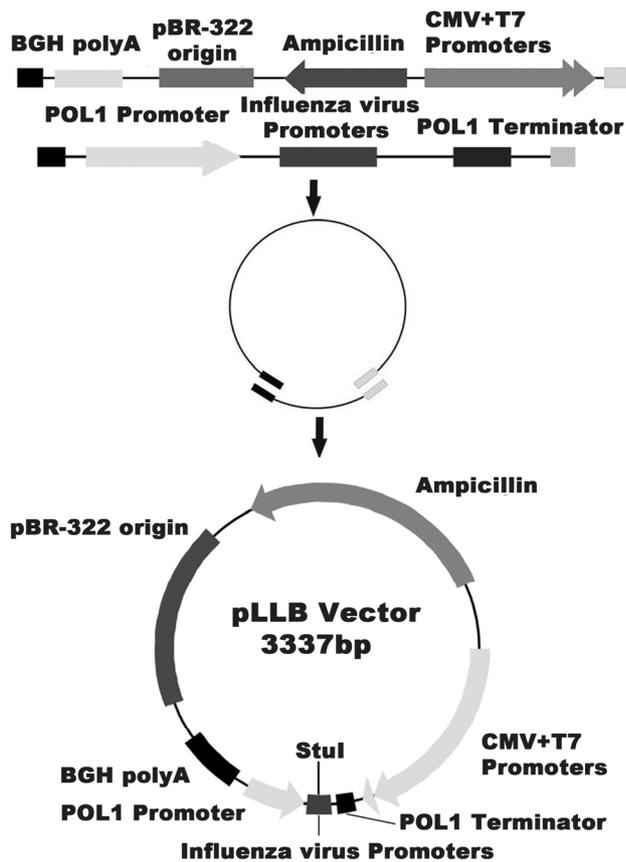
Figure 2. Construction strategy for generating the pLLBA and pLLBG vectors by homologous recombination. The RNA polymerase I (A/G) transcription units that flanked the influenza recombination cassettes composed of influenza virus promoters, were cloned between the RNA polymerase II promoter and the terminator of the linearized pHP vector, respectively, by homologous recombination.

Figure 3. Influenza virus genome cloning strategy by using the recombineering pLLBA and pLLBG plasmid vectors. The location and nature of the promoter components of the influenza recombination cassette is shown for the pLLBA and pLLBG vectors that differ by a single nucleotide at position 4 of the plus strand indicated for pLLBA and shown in parenthesis for the pLLBG sequence. The plasmids were linearized with *Stu*I, and mixed with the appropriate cDNA before transformation of competent *E. coli*. The cDNA recombined into circular plasmid within the regions of terminal complementarity to introduce virus genome segments between the start and stop sites of the RNA polymerase I promoter and terminator. The opposing RNA polymerase II promoter acts to generate plus sense mRNA form each genome segment.

recombination cassette nested between RNA polymerase I promoters and terminators that were further nested within an opposing RNA polymerase II promoter and polyadenylation signal sequence. The RNA polymerase I transcription units of the pLLBA and pLLBG plasmids were derived from the pHH21A and pHH21G vectors that possessed a further nested set of influenza virus plus and minus sense promoters for RNA dependent viral replication of the viral complementary RNA strand and viral genomic RNA strands respectively (that constituted the influenza recombination cassette). This cassette consisted of the minus sense influenza virus promoter; nucleotides 1-13, AGTAGAAACAAGG, and both of the viral plus strand promoters (plus sense nucleotides1-12: AGCAAAAGCAGG or AGCGAAAGCAGG); inserted between the human RNA polymerase I promoter and mouse polymerase I terminators (Figures 2 and 3). Thus the resulting pLLBA and pLLBG plasmids were 3337bp in length and possessed influenza recombination cassettes that differed at the 4th position of the constituent negative sense influenza virus promoter; possessing an A or G respectively (plus sense nucleotides). The junction of the two influenza virus promoters formed a "natural" *Stu*I cleavage site, AGGCCT (Figure 3). The plasmids were

linearized by digestion with *Stu*I to cleave at the junction point of the 2 promoters, and gel purified in order to generate vectors for recombination with each viral PCR products. Insertion of influenza genome segments was achieved by recombination with the terminal influenza promoters of linearized pLLBA and pLLBG vectors. The cloning of each influenza genome segments into these vectors allowed the synthesis of both the negative-sense viral RNA and positive-sense mRNA from the same cDNA template when introduced into 293T cells.

The primers sets listed in Table 1 were effective at amplifying individual genome segments of the three influenza viruses H1N1, H11N9, H5N1. The PCR products of each genome segment were cloned into the recombinational vectors by homologous recombination in competent DHF5α cells. The complete genomes of three influenza viruses were cloned into the appropriate pLLBA and pLLBG vectors with high cloning efficiency that ranged from 20-100% for each genome segment (Table 2).

Table 2. Cloning Efficiency of viral genome segments of A/Duck/Northern China/61/07 (H5N1), A/WSN/1933 (H1N1), and A/Duck/Memphis/546/74 (H11N9) into pLLBA and pLLBG vectors.

Segments	Positive clones/All screened clones		
	H1N1	**H5N1**	**H11N9**
PB2	4/5 (80 %)	2/10 (20 %)	1/5 (20 %)
PB1	5/5 (100 %)	2/10(20 %)	2/5 (40 %)
PA	4/5 (80 %)	3/10(30 %)	4/5 (80 %)
HA	4/5 (80 %)	5/10(50 %)	3/5 (60 %)
NP	4/5 (80 %)	6/10(60 %)	2/5 (40 %)
NA	3/5 (60 %)	4/10(40 %)	3/5 (60 %)
M	5/5 (100 %)	8/10(80 %)	4/5 (80 %)
NS	2/5 (40 %)	5/10(50 %)	2/5 (40%)

In addition, the genomes of other avian H9N2, swine H3N2, and swine H1N1 viruses were cloned into the pLLB vectors with high efficiency in our laboratories (data not shown). Our data suggests that influenza virus reverse genetics method was simpler, faster and more efficient than conventional approaches. High cloning efficiency required the use of sufficient amounts of PCR product at the appropriate ratio to linearized-vector (~200ng:50ng). All genes that were inserted in the pLLBA or pLLBG vectors were sequenced to show that all viral genome segments were inserted in the correct orientation without any mutations relative to the parental sequences, and thus were ready for use in virus rescue. The vector and primer sets were also compatible with the design features of the commercial In-Fusion^{TM} DNA cloning system (Clontech Laboratories Inc, CA) that can be used to further increase cloning efficiency and that would allow smaller amounts of plasmids and inserts to be used for cloning by recombination (Berrow et al, 2007).

A number of recombinant viruses, including A/Duck/Memphis/546/74 (H11N9), A/WSN/33(H1N1) and A/Duck/Northern China/61/07 (H5N1), as well as several reassortant viruses, including H5N9, H1N9, and H11N1, were successfully rescued using genome segments cloned into the pLLBA and pLLBG vectors for recombination. The subtypes of each virus and reassortants were determined by HI and NI assay using subtype-specific antiserum (Liu et al, 2005). The 8-plasmid system was less complex than the 12-plasmid system as virus rescue only required the transfection of cells with 8 instead of 12 plasmids. We compared the efficiency of virus rescue using the previously constructed A/WSN/33 12 plasmid system and the newly constructed 8-plasmid A/WSN/33 system. The yield of infectious WSN/33 virus rescued at 48hr post-transfection using the 8WSN- pLLB-PB2, PB1, PA, HA, NP, NA, M, NS, plasmids was 104.3±0.3 TCID50/ml that was significantly higher than the titer of WSN/33 virus obtained in experiments in which cells were transfected with the 12-plasmid system. The 12-plasmid system consisted of eight RNA polymerase I-driven plasmids for the transcription of the WSN influenza vRNAs, four protein expressing plasmids for the synthesis of NP and three RNA polymerase subunits that yielded 103.15±0.35 TCID50/ml. These data showed that the pLLBA/G 8-plasmid system was >10 fold more efficient than the 12-plasmid system for generating the same recombinant A/WSN/33 virus in 293T cells. This may be due to the increased efficiency of introducing 8 as compared to 12 plasmids into individual cells.

In the present study, recombinational cloning was also used to construct all three vectors, pHP, pLLBA and pLLBG. The vectors were designed to be minimal constructs of small size and thus are expected to be more genetically stable that larger plasmids described for use in other conventional influenza reverse genetics plasmid systems. Moreover, we constructed various other kinds of vectors by this recombinational cloning method, and have found it to be a robust approach (data not shown). Although, designed for reverse genetics, the influenza genome segments cloned into pLLB plasmids could be used to express individual influenza viral RNA molecules, or for performing *in vitro* replication assays of mutant influenza RNA polymerases in primate cells. The pLLB plasmids can also be used to produce proteins *in vitro* in conjunction with T7 RNA polymerase or RNA polymerase II -driven systems.

In summary, we describe the construction and use of a pair of plasmid DNA vectors, pLLBA and pLLBG, that were designed for recombinational cloning of any influenza genome segment without the need for enzymatic cloning steps aside from RT-PCR synthesis of the viral cDNA. The pLLBA and pLLBG vectors were also generated by recombination demonstrating the general utility of recombination in general genetic modification. The general recombineering approach taken in this influenza virus reverse genetics system is applicable to the design of

reverse genetics systems for other single stranded negative sense RNA viruses. For example, the pLLB plasmids could be modified for the expression of other segmented or non-segmented RNA viruses by replacing the influenza cloning cassette with the appropriate terminal promoter sites separated by restriction enzyme sites for linearization. Additional proteins required for viral replication of some single-stranded RNA viruses could be introduced, as needed, into the pHP or similar protein expressing plasmids. The use of the currently described recombinational vectors for 8-plasmid influenza virus rescue will facilitate rapid analysis and vaccine production of novel influenza strains through the application of reverse genetics.

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COMPETING INTERESTS

None declared.

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