Partial and Full PCR-Based Reverse Genetics Strategy for Influenza Viruses

Hongjun Chen, Jianqiang Ye, Kemin Xu, Matthew Angel, Hongxia Shao, Andrea Ferrero, Troy Sutton, Daniel R. Perez*

Virginia-Maryland Regional College of Veterinary Medicine, Department of Veterinary Medicine, University of Maryland, College Park, Maryland, United States of America

Abstract

Since 1999, plasmid-based reverse genetics (RG) systems have revolutionized the way influenza viruses are studied. However, it is not unusual to encounter cloning difficulties for one or more influenza genes while attempting to recover virus de novo. To overcome some of these shortcomings we sought to develop partial or full plasmid-free RG systems. The influenza gene of choice is assembled into a RG competent unit by virtue of overlapping PCR reactions containing a cDNA copy of the viral gene segment under the control of RNA polymerase I promoter (pol1) and termination (t1) signals – herein referred to as Flu PCR amplicons. Transfection of tissue culture cells with either HA or NA Flu PCR amplicons and 7 plasmids encoding the remaining influenza RG units, resulted in efficient virus rescue. Likewise, transfections including both HA and NA Flu PCR amplicons and 6 RG plasmids also resulted in efficient virus rescue. In addition, influenza viruses were recovered from a full set of Flu PCR amplicons without the use of plasmids.

Citation: Chen H, Ye J, Xu K, Angel M, Shao H, et al. (2012) Partial and Full PCR-Based Reverse Genetics Strategy for Influenza Viruses. PLoS ONE 7(9): e46378. doi:10.1371/journal.pone.0046378

Copyright: © 2012 Chen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The research was funded by contract N° HHSN266200700010C from the National Institute of Allergy and Infectious Diseases, National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: dperez1@umd.edu

Introduction

Type A Influenza (Flu) viruses belong to the family Orthomyxoviridae and their genome consist of eight segments of single strand RNA of negative polarity [1–3]. The viral RNA (vRNA) is found in the virion and infected cells in the form of viral ribonucleoprotein particles (vRNPs) associated with three polymerase subunits (PB1, PB2, and PA) and the nucleoprotein (NP) [4,5]. The virus encodes for two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), the proton pump transmembrane protein (M2), the matrix protein (M1), the nuclear export protein (NS2/NEP), the nonstructural protein (NS1) and, in some influenza viruses (from an alternative translation start site in segment 1) the PB1-F2, an apoptosis modulatory protein [6–8]. Additional viral protein products include PB1-N40, derived from the PA mRNA and consists of the N-terminal 191 aa of PA fused to 61 aa that result from +1 frameshifting [9,10].

De novo synthesis of influenza viruses by reverse genetics (RG) requires not only the viral RNA but also the viral protein components [11–14]. Thus, RG systems for influenza rely invariably on a dual promoter concept: One for the synthesis of vRNA segments and another for the synthesis of viral mRNAs [11]. Since the termini of influenza vRNPs are crucial for virus replication, plasmids carrying a RNA polymerase I (pol1) or T7 RNA polymerase promoters have been used to generate vRNPs with the exact 3′ end, whereas a pol1 terminator sequence (t1) or a hepatitis δ ribozyme have been used to generate the exact 5′ end. Plasmids carrying typical RNA polymerase II (pol2) promoters (CMV and/or chicken β-actin promoters) have been utilized for the synthesis of influenza mRNAs [15–19]. Despite the great advantages of this technology, and although variations to the plasmid-based approach have been developed, they inevitably rely on a cloning step [20,21]. A system that does not rely on cloning could speed up studies on the significance of mutations in the viral genome for replication and/or modulation of virulence. In this report, Flu PCR amplicons, instead of plasmids, are an efficient and viable alternative to the plasmid-based RG system.

Results

A Flu reporter PCR amplicon results in reporter activity

Flu GFP PCR amplicons were derived from pHW72-EGFP [21]. In order to determine whether a Flu PCR amplicon could be transfected into cells and be amplified by the influenza polymerase complex, a PCR product was produced encoding the GFP reporter gene in negative orientation flanked by the influenza segment 7 untranslated regions (UTRs) and further flanked by the human pol1 promoter and the mouse t1 termination signal, pol1EGFPt1 (Fig. 1A, Fig. S1A, Table S1). Co-transfection of the pol1EGFPt1 amplicon along with 4 protein expression plasmids encoding the influenza virus polymerase complex (3P) and NP into 293T cells resulted in efficient amplification of the reporter replicon and detection of green fluorescence signal (Fig. 2A). The proportion of green cells observed was comparable to those observed in the positive control cells co-transfected with pHW72-EGFP and the 3P and NP expression plasmids (Fig. 2B). The fluorescence signal of another amplicon, pol1EGFPutr, which lacks the t1 signal, was present in fewer cells compared to the...
**pol1EGFPtr1** amplicon indicating that run off transcription by the RNA pol1 complex may result in vRNA fragments with incorrect and/or incomplete influenza sequences (Fig. 2C). As expected, no fluorescence signal appeared when cells were transfected with a Flu GFP PCR amplicon lacking the pol1 and t1 elements (UTREGFPutr) (Fig. 2D) or by removing the PB1 plasmid in co-transfected cells with either PCR amplicons or pHW72-EGFP plasmid (Fig. 2E and data not shown).

These initial studies were expanded in order to test whether PCR amplicons containing RNA polymerase II (pol2) and polyadenylation sequences flanking an appropriate ORF would result in gene expression (Fig. S1B). Thus, PCR amplicons of the 3P and NP genes were obtained using a set of primers spanning the cytomegalovirus promoter (CMV) and bovine growth hormone (bgh) polyA elements. Co-transfection of the pol2PB2bgh, pol2PB1bgh, pol2PBhgh, and pol2NPbgh, along with pHW72-EGFP, resulted in efficient reporter replicon expression indicating that PCR amplicons with either pol1 or pol2 transcription elements are appropriately transcribed by the corresponding transcription complexes (Fig. 2F).

### Generation of Flu PCR amplicons by overlapping PCR

In order to demonstrate whether Flu PCR amplicons could be used to replace plasmids in the RG system, the strains mouse-adapted A/California/04/2009 (H1N1) [22] and A/chicken/North Sumatra/072/2010 (H5N1) - herein referred to as H1N1pdm and 072, respectively - were used as donors for the HA and NA genes (Fig. 1B and 3). A specific set of internal primers designed within conserved regions of these gene segments were then developed in order to maximize gene amplification from viral cDNA preparations and to assemble the appropriate HA and NA PCR amplicons (Fig. 3, Fig. S 2A and B). The pol1HA_{pdm}t1 PCR amplicon carried a full-length copy of the HA gene from the H1N1pdm strain flanked by the pol1 and t1 signals (Fig. 2A). With respect to the 072 HA gene, the internal primers were designed to delete (Δ) the polybasic amino acid signal sequence (RERRKRRR) and replace it with one carrying a monobasic cleavage site (TETR) (Fig. 3B, Fig. S 2). Similar strategies were followed to create the NA amplicons pol1NA_{pdm}t1 and pol1NA_{072}t1 from viral cDNAs (Fig. 3C and D). Full-length pol1HA_{pdm}t1 and pol1NA_{pdm}t1 PCR amplicons were obtained and confirmed by sequencing (Fig. 2E). In addition, full-length HA and NA PCR amplicons were generated lacking either the t1 sequence or both the pol1 and t1 sequences, which serve as controls for efficiency of virus rescue as described below. Sequencing results confirmed the amplification of an overlapping ΔH5 HA amplicon, pol1HA_{4072}t1, with a deleted polybasic cleavage site and the full-length amplification of the pol1NA_{072}t1 (Fig. 3F, Fig. S 2).

---

**Figure 1. PCR-based reverse genetics.** A) PCR-based Flu reporter replicon encoding GFP: PCR amplification was performed using primers spanning the pol1 to t1 sequences and pHW72EGFP. After agarose gel purification and testing to show that the PCR product is devoid of plasmid DNA contamination, the Flu GFP amplicon is transfected into 293T cells along with four expression plasmids encoding the polymerase complex of influenza virus. Expression of GFP reflects influenza polymerase activity on a vRNA Flu GFP replicon generated from pol1 transcription of the Flu GFP amplicon. Variations to this these are described in the main text and shown in Fig. 2. B) Starting with a influenza virus candidate, vRNA, cDNA and reconstitution of a full-length Flu PCR amplicon (in this case, the HA and NA PCR amplicons are depicted) is performed. Transfection of Flu PCR amplicons along with appropriate complementary RG plasmids into susceptible cells leads to the generation of recombinant influenza viruses with the desired gene constellation. The strategy speeds up the reverse genetics process by obviating a classical cloning step, which is currently part of the plasmid-based RG system.

doi:10.1371/journal.pone.0046378.g001
Efficient influenza virus rescue using Flu PCR amplicons in either "1+7" or "2+6" modes

The pol1HA<sub>pdmt1</sub> or pol1HA<sub>A072t1</sub> HA PCR amplicons (Table 1) were co-transfected into co-cultured 293T/MDCK cells in a "1+7" mode along with 7 RG plasmids encoding the corresponding additional gene segments from the influenza A/Puerto Rico/8/1934 (H1N1) strain (PR8). At 48 h and 72 h post-transfection (hpt) cells co-transfected with the pol1HA<sub>pdmt1</sub> PCR amplicon plus 7 RG PR8 plasmids (H1<sub>pdm</sub>-7PR8) showed typical virus-induced cytopathic effect (CPE). H1<sub>pdm</sub>-7PR8 virus titers at 72 hpt reached 3.16 x 10<sup>4</sup> TCID<sub>50</sub>/ml, which was 5 times lower than the one obtained using the corresponding pH1pdm RG plasmid (pH1<sub>pdm</sub>-7PR8, 1.58 x 10<sup>5</sup> TCID<sub>50</sub>/ml) (Table 1). After a subsequent blind passage in MDCK cells or 9 day-old embryonated chicken eggs, virus titers increased >10<sup>7</sup> TCID<sub>50</sub>/ml with either the pol1HA<sub>pdmt1</sub> PCR amplicon or the whole plasmid-based RG system.

Likewise, the H5N1 virus could be rescued using the pol1HA<sub>pdmt1</sub> HA PCR amplicon and 7 RG PR8 plasmids (H5<sub>A072t</sub>-7PR8, Table 1). At 72 hpt, H5<sub>A072t</sub>-7PR8 virus titer in transfected cells was 1.58 x 10<sup>5</sup> TCID<sub>50</sub>/ml, and increased to 2.32 x 10<sup>6</sup> TCID<sub>50</sub>/ml when passaged in eggs (Table 1). At 72 hpt, the TCID<sub>50</sub> titer of the PCR-based H5<sub>A072t</sub>-7PR8 virus was 10,000 times less than that of pH5Δ<sub>A072t</sub>-7PR8 virus, which was obtained with the bidirectional pRF1437 plasmid (Table 1).

The identity of the 1+7 reassortants was further confirmed by sequencing the HA gene, and by immunofluorescence assay (IFA) (Fig. 4A) and plaque assay (Fig. 4B). As expected, no CPE and no virus was detected after transfection of cells with 7 RG PR8 plasmids (and in which the plasmid encoding the HA segment was omitted, not shown). By plaque assay, the H5Δ<sub>A072t</sub>-7PR8 virus was found to form plaques in agar plates in the presence, but not in the absence, of 1 μg/ml TPKC (Fig. 4C), consistent with the reconstruction of a monobasic cleavage site in the HA gene during PCR amplification. To further confirm that these viruses carry a ΔH5 gene and/or do not present additional mutations introduced during PCR amplification, each of these viruses was plaque-purified and the HA gene was sequenced and analyzed for 48 plaque-purified viruses for each of three RG viruses, H5Δ<sub>A072t</sub>-7PR8, pH5Δ<sub>A072t</sub>-7PR8, and control RG-derived PR8. Every single virus isolate from the H5Δ<sub>A072t</sub>-7PR8 virus group contained a monobasic cleavage site identical to the one designed in the PCR primers. Mutations were identified elsewhere but in only 4 out of the 48 sequences analyzed for the H5Δ<sub>A072t</sub>-7PR8 virus and in each case corresponded to single nucleotide changes (Table S2). The mutations caused amino acids changes in 3 of them; however, these changes did not alter the antigenic make up of these viruses by HI assay (Table S2). No mutations were identified in HA sequences derived from plaques obtained from the RG-derived PR8 or pH5Δ<sub>A072t</sub>-7PR8 viruses (data not shown). These results highlight the high fidelity of the PCR approach.

Because the PR8 virus is a fully laboratory adapted strain and can be recovered very efficiently by RG, studies were performed to test whether the pol1HA<sub>pdmt1</sub> or pol1HA<sub>A072t1</sub> PCR amplicons could be recovered in the background of other RG systems including the A/guinea fowl/Hong Kong/WF10/1999 (H9N2) [23] and the cold-adapted A/Ann Arbor/6/1960 (H2N2) [24] strains, herein referred to as WF10 and AA60<sub>a</sub>, respectively (Table 1). In addition, virus rescue was performed in the context of the H1N1pdm background (Table 1). Regardless of the RG virus background used, virus rescued was possible. This was particularly the case using the WF10 background in which high virus titers (1.58 x 10<sup>6</sup> TCID<sub>50</sub>/ml) in the initial co-transfected cells were obtained with either pol1HA<sub>pdmt1</sub> or pol1HA<sub>A072t1</sub> HA PCR amplicons (H1<sub>pdm</sub>PCR:7WF10 and H5Δ<sub>A072t</sub>-7WF10, respectively, Table 1). When passaged into eggs, virus titers increased significantly, about 1,000 fold for the H5Δ<sub>A072t</sub>-7WF10 virus. Virus titers below or just at the limit of detection were observed in cells co-transfected in the context of 7 RG plasmids from the AA60<sub>a</sub> strain carrying either the pol1H1<sub>pdm</sub> (H1<sub>pdm</sub>:7AA60<sub>a</sub>) or pol1H1<sub>A072t1</sub> (H5Δ<sub>A072t</sub>-7AA60<sub>a</sub>) HA PCR amplicons or the control plasmid pH1<sub>pdm</sub> (pH1<sub>pdm</sub>:7AA60<sub>a</sub>). However, a >1,000 fold increase in virus titers were observed after blind passage in eggs of supernatants of the AA60<sub>a</sub>-based co-transfected cells from these reassortant groups (Table 1).

In the 2+6 mode, efficient virus rescue was also obtained. No statistical differences were observed in rescue efficiency between the Flu HA PCR amplicon plus 7 PR8 RG plasmids and the Fluc reporter PCR amplicon results in reporter activity. 293T cells were co-transfected with A) polIEFPr1 PCR amplicon and 4 plasmids expressing the influenza virus replication complex (3P - PB2, PB1, PA - and NP), B) pHW72-EGFP plasmid and 3P and NP plasmids, C) pol1EGFPrut and 3P and NP plasmids, D) UTREGFPrut (without hpol1 and t1 sequences) and 3P and NP plasmids, E) pHW72-EGFP plasmid and 2P (minus PB1) and NP plasmids, and F) pol2PB2bgh, pol2PB1bgh, pol2PB4bgh, and pol2NPbgh amplicons and pHW72-EGFP plasmid. Pictures obtained at 24 hpt (20x magnification using the Carl Zeiss Axioshot Photomicroscope and UV filter). The amplicons were amplified by the method described in the supplemental information.

Figure 2. A Flu reporter PCR amplicon results in reporter activity.
(H1pdm:7PR8) compared to the HA and NA amplicons and 6 PR8 RG plasmids (H1N1pdm:6PR8). If the WF10 background was used, co-transfection of the HA and NA amplicons resulted in approximately 10 fold less virus (H1N1 pdm:6WF10) in the supernatant of transfected cells compared to using the HA amplicon alone (HApdm:7WF10). A similar trend was observed when the AA60ca background was used (compare reassortants H1N1pdm:6AA60ca with HApdm:7AA60ca).

**D**

(H5N1 viruses were rescued from the 072 strain using the amplicons pol1HA072t1 and pol1NA072t1 co-transfected with either the PR8, WF10, or AA60ca backgrounds with efficiencies similar to those obtained using the HA and NA amplicons from the H1N1pdm strain. It must be noted that reassortant viruses carrying 7 gene segments from either WF10 or AA60ca encode a N2 NA subtype, which may have affected the rescue efficiency of the H1pdm or ΔH5 HA gene segments. Nevertheless, the results showed that either the 1+7 or 2+6 strategies using Flu PCR amplicons is a suitable method to speed up the recovery of influenza viruses by RG.

**Low efficiency of virus rescue using Flu PCR amplicons lacking the t1 signal**

Since the PCR strategy with pol1 and t1 signals was initially used, we wanted to determine whether amplicons lacking the t1 signal could be better substrates for the generation and subsequent amplification of vRNA segments. PCR amplicons were prepared and designated as pol1HAΔt1 or pol1NAΔt1 using the overlapping PCR method mentioned above, and in which the t1 signal was omitted. Similarly, PCR amplicons for HA and NA lacking both the pol1 and t1 signals were prepared and used as controls. Using either the PR8 or H1N1pdm virus backgrounds, virus rescue was possible with HA and NA PCR amplicons lacking the t1 signal, although the rescue efficiency was 100–200 fold lower
than using the same amplicon with the t1 signal. After passage in MDCK cells, virus titers of these reassortants (H1pdm:7PR8, H5pdm:7pdm, H1N1pdm:6PR8, and H1N1pdm:6pdm) were increased although they were 10-30 fold lower than those obtained with the full-length t1 signal-containing amplicons (H1pdm:7PR8, H5pdm:7pdm, H1N1pdm:6PR8, and H1N1pdm:6pdm, Table 1). These results are consistent with the previous observation using the Flu GFP amplicon lacking the t1 signal, which suggests that the presence of the t1 signal helps generate optimal full-length Flu PCR amplicons.

**Plasmid-free reverse genetics using PCR amplicons**

To expand the potential use of PCR amplicons as a suitable surrogate system to recover influenza viruses without the use of plasmids, each virus segment was amplified to generate a full set of Flu PCR amplicons encoding each one of the viral segments (Fig. S 3). The optimal cocktail of eight PCR amplicons, based on the HA and NA genes of the H1N1pdm and 6 other amplicons from the PR8 strain, consisted of polPB1polpb1 (1 μg), polPB2polpb2 (1 μg), polPB3polpb3 (1 μg), polPB3polpb3 (1 μg), polNApolna (0.5 μg), polNApolna (0.5 μg), polNApolna (0.5 μg), and polNApolna (0.3 μg), along with the 3P (1 μg each) and NP (1 μg) expression helper plasmids. The mixture was transfected into co-cultured 293T/MDCK cells with a ratio of 500/1 and at a density of 5×10^5 cells. It resulted in low efficiency virus rescue with a titer of 1.5×10^2 TCID_{50} after 72 hpi with no detectable HA titer (Table 1, 8PCR:3P/NP (PR8) virus). Blind passage in MDCK cells, resulted in virus titers in the order of 1×10^5 TCID_{50}/ml with an HA titer of 128. These studies
H1N1pdm:6PR8 viruses, respectively (X10). D) mAb DPJY01 specific to H5N1 virus has no reaction with PR8-infected cells (X20). E) Positive reaction of VN1203 strain in MDCK cells resulted in virus rescue with titers HA PCR amplicons and 7 k9pol1-driven RG plasmids from the polybasic cleavage site sequences (Fig. S 4). Co-transfections of the were amplified using overlapping PCRs that removed the gene’s promoter (k9pol1) and termination signals (k9t1). Both HA genes were amplified using overlapping PCRs that removed the gene’s polybasic cleavage site sequences (Fig. S 4). Co-transfections of the HA PCR amplicons and 7 k9pol1-driven RG plasmids from the VN1203 strain in MDCK cells resulted in virus rescue with titers of ~10^8 TCID_{50}/ml at 120 hpt and 10^7 TCID_{50}/ml after blind passage in MDCK cells. Variations to this theme in which 4 PCR amplicons were used to replace the corresponding plasmids (Table 1, 4PCR:4PR8 virus), resulted also in efficient virus rescue indicating that the PCR-based strategy is not limited to just the viral surface genes and it could be easily applied to other gene segments that may be reluctant to cloning.

**Virus rescue by PCR amplicons in Vero and MDCK cells**

Because Vero and MDCK cells have been approved for influenza vaccine production, we investigated whether Flu PCR amplicon rescue, either in 1+7 or 2+6 modes, was possible in these cells. Vero cells co-transfected with the Flu HA (alone or in combination with the NA) PCR amplicon from the H1N1pdm strains and 7 (or 6) PR8 RG plasmids resulted in virus rescue that was observed at 120 hpt (~10^8 TCID_{50}/ml) with about 500 fold lower efficiency compared to the whole plasmid-based system (Table 2). Blind passage of supernatants of Vero cells at 72 hpt into MDCK cells resulted in virus titers similar to those obtained using the whole plasmid RG system (around 10^7 TCID_{50}/ml).

Using the 1+7 approach, virus rescue was also possible in MDCK cells with HA PCR amplicons from two H5N1 strains, 072 and A/Viet Nam/1203/2004 (VN1203) (Table 3). In this case, HA PCR amplicons were prepared carrying the canine pol promoter (k9pol1) and termination signals (k9t1). Both HA genes were amplified using overlapping PCRs that removed the gene’s polybasic cleavage site sequences (Fig. S 4). Co-transfections of the HA PCR amplicons and 7 k9pol1-driven RG plasmids from the VN1203 strain in MDCK cells resulted in virus rescue with titers of ~10^8 TCID_{50}/ml at 120 hpt and 10^7 TCID_{50}/ml after blind passage in MDCK cells (Table 3), reassortant viruses HA\textsubscript{072}:7VN1203 and HA\textsubscript{VN1203}:7VN1203. Like in the previous transfection studies, removing the k9t1 signal from the PCR products resulted in impaired virus rescue (reassortant viruses HA\textsubscript{072}utr:7VN1203 and HA\textsubscript{VN1203}utr:7VN1203), and removing both the k9pol1 and k9t1 sequences resulted in no virus rescue.

**Discussion**

In this report, a significant modification was introduced to the plasmid-based reverse genetics system [25,26] for influenza based on PCR amplicons. In order to optimize and maximize amplification of the genes of interest, a strategy involving overlapping PCR fragments for each segment was designed and used in conjunction with a high fidelity polymerase and corresponding buffer, Phusion high-fidelity PCR master mix with GC Buffer (New England Biolabs). This enzyme performed the best in our hands, compared to seven other commercially available DNA polymerases (Supplementary materials and methods S1). The synthesis of full length Flu PCR amplicons implies producing overlapping PCR fragments with distinct differences in GC- versus AT-rich regions. The human and canine pol1 promoters are approximately 75% GC-rich whereas the HA segment is approximately 60% AT-rich (data not shown). The approach succeeded in producing overlapping PCR amplicons for the HA and NA segments from different subtypes, including H1N1pdm, H5N1, or H9N2 (not shown) and from the 6 internal gene segments of the PR8 strain by designing overlapping primers and optimizing the PCR conditions (Fig. S 2). The amount obtained in each reaction for full length Flu PCR amplicons was in the order of 1~5 μg (Fig. 2), which is sufficient for transfection and virus rescue and comparable to the amount of plasmid DNA used for transfection in the conventional plasmid-based RG system (Table 1). For the H5N1 vaccine candidates, the polybasic cleavage site (RERRRKKKR) in highly pathogenic strains was easily removed and replaced by a low pathogenic sequence (TETR) by virtue of adequate set of primers and overlapping PCR (Table S1, Fig. 3 and Fig. S 2). Plaque assays revealed that the rescued H5\textsubscript{072}PCR:7PR8 virus was strictly dependent on the presence of TPCK-trypsin, which strongly suggests that the virus was able to infect MDCK cells at a MOI of 0.001. At 36 hpi, cells were fixed with 4% Paraformaldehyde in PBS solution (Santa Cruz) and viral antigen detected by IFA using subtype specific monoclonal antibodies and FITC-labeled antimouse antibodies (green). Counterstaining was performed with propidium iodide (red). A) mAb 3B2 specific for the HA H1N1pdm virus has no reaction with the HA of PR8 strain (only cell nuclei can be seen in red reacting with mAb 3B2 in cells infected with either the H1N1pdmutr:6PR8 or H1N1pdm:6PR8 viruses, respectively (X10). B) and C) Positive viral antigen green signal detected using mAb 3B2 in cells infected with either the H1N1pdmutr:6PR8 or H1N1pdm:6PR8 viruses form plaques in agarose plates in the presence of 1 μg/mL TPCK-Trypsin but not in its absence.
the presence of the t1 signal at the 3' end of the Flu PCR amplicon. The results show that whether run off pol1 transcription would make a difference in full-length Flu PCR amplicons, it was important to determine would have either a positive or negative effect on the production of original virus isolated compared to the plasmid-based system. Nevertheless, the results clearly show that the PCR-based finding of the small mutation rate among the H5N1 virus rescued with the VN1203 backbone in MDCK cells. Since the 072 virus used in this report has undergone more than one passage in eggs, it is possible that the homogeneity of its population had contributed to the production of virus from transfected 293T or co-cultured 293T/MDCK cells. Transfection of Vero cells in a 1+7 and 2+6 mode also resulted in virus rescue (Table 2). Virus rescue of the H5N1 1+7 virus in MDCK cells, with the attenuated HA PCR amplicons carrying the k9pol1 and k9t1 signals, was also successful (Table 3).

The boundaries of the system were extended by showing that a complete set of 8 Flu PCR amplicons can be effectively recovered by RG in the context of 4 expression plasmids encoding the influenza polymerase complex (Table 1). More importantly, an expression competent PCR version of the 3P and NP was also effective in rescuing the virus in a transfection reaction that contained no plasmids (Table 1). Our studies imply that protein expression studies could be performed without preparation of clones, although, it must be emphasize that systematic studies to evaluate the differences in protein expression of pol2-based PCR amplicons and their corresponding plasmids were not performed.

Although it can be argued that rescue efficiency was lower than using a plasmid-based approach, further optimization of the PCR-based system is likely possible by manipulating the amount and proportion of each amplicon in the transfection. Such analysis is beyond the scope of the present report. It could also be argued that the PCR-based system may produce a more variable virus population than the one that is obtained using the plasmid-based system. Although there is such possibility, it is an inherent nature of influenza viruses to evolve through point mutations and

### Table 2. Virus rescue with H1N1pdm PCR amplicons in Vero cells.

| Mode | Reassortants | PCR amplicons | Backbone/Plasmids (N) | Transfectants (72 hpt) | Blind passage on MDCK (72 hpt) |
|------|--------------|---------------|-----------------------|------------------------|-------------------------------|
| 7+1  | H1pdm:7PR8   | pol1HA/pdmt1  | PR8/7                 | 1.08x10^8              | 1.58x10^7                    |
| 7+1  | pH1pdm:7PR8  | -             | pH1pdm/1, PR8/7       | 5.00x10^6              | 2.32x10^7                    |
| 6+2  | H1N1pdm:6PR8 | pol1HA/pdmt1  | PR8/6                 | 1.58x10^7              |                               |
| 0+8  | 8PCR3P/8NP (PR8) | pol1HA/pdmt1  | PR8/4                 | <1                     | <1                            |
| 0+12 | 12PCR (PR8)  | pol1HA/pdmt1  | PR8/0                 | <1                     | <1                            |

doi:10.1371/journal.pone.0046378.t002

### Table 3. Flu PCR amplicons rescued with the VN1203 backbone in MDCK cells.

| Reassortants | PCR amplicons | Transfectants (72 hpt) | Blind passage (72 hpt) |
|--------------|---------------|------------------------|------------------------|
| H5AN2-7VN1203 | k9pol1HA/pdmt1 | 2.32x10^5             | 1.58x10^5              |
| H5AN2-utr7VN1203 | k9pol1HA/pdmt1 | 1.58x10^5             | 5.00x10^7              |
| UTRH5A-7VN1203 | UTRH5A/pdmt1  | <1                     | <1                     |
| H5AN1203-7VN1203 | k9pol1HA/pdmt1 | 5.00x10^5             | 2.32x10^7              |
| H5AN1203-utr7VN1203 | k9pol1HA/pdmt1 | 1.08x10^5             | 1.08x10^5              |
| UTRH5A-7VN1203 | UTRH5A/pdmt1  | <1                     | <1                     |

doi:10.1371/journal.pone.0046378.t003
therefore no reverse genetics system is error free. However, the sequencing of reassortants produced in this study does not show mutations that would alter the antigenicity of the HA surface proteins. In this regard, for vaccine development, as long as the vaccine seed stock is antigenically identical to the vaccine candidate, other mutations would be irrelevant. In fact, inactivated influenza vaccines prepared by classical reassortment have only two prerequisites: 1) HA surface gene derived from the vaccine candidate, other mutations would be irrelevant. In fact, inactivated vaccine seed stock is antigenically identical to the vaccine.

Materials and Methods

vaccine viruses is not a pre-requisite for approval of the vaccine by the FDA. Overall, the implications of the PCR-based approach for RG development are highly significant. Using a combination of PCR amplicons and plasmids, it would be possible to streamline the study of gene variants for one or more gene segments and determine fitness, pathogenesis or any other biological aspect of the virus. Several mutant viruses with mutations in one or more genes could be produced without having to prepare individual clones. Recovery of viruses entirely from PCR products implies that other viral systems could be amenable to a similar strategy. This could be particularly important for viruses with genomes larger than the influenza virus that are occasionally associated with cloning difficulties or plasmid instability. In fact, PCR amplification for the purpose of virus generation has already been done for coronaviruses, although the PCR product was used as template for in vitro RNA transcription and the resulting RNA was later transfected into cells [28]. In summary, a RG system for influenza was developed that does not require a cloning step for recovery of viruses and has profound implications for vaccine development, pandemic preparedness, and for the study of influenza viruses.

Materials and Methods

Viruses and cells

The mouse-adapted H1N1pdm virus and WF10 viruses have been previously described [22,23]. The highly pathogenic H5N1 virus strain A/Chicken/NorthSumatra/MDn/072/2010 (strain 072) was a gift from Teguh Prajitno (Vaksindo, Indonesia) and sent to us by Ron Fouchier (Erasmus Medical Center, the Netherlands). The VN1203 virus (H5N1 clade 1) was obtained from the Centers for Disease Control and Prevention, Atlanta, GA (CDC). The influenza PR8 strain was grown from a reverse genetics clone obtained from Peter Palese, Mount Sinai School of Medicine, New York, NY (H1N1) [PR8]. Virus stocks were prepared in specific pathogen free 9-day old embryonated chickens eggs following standard techniques for growth of influenza viruses. MDCK and Vero cells were maintained in Modified Eagle’s medium (MEM) (Sigma-Aldrich, St. Louis, MO) containing 5% fetal bovine serum (FBS) (Sigma-Aldrich). Human embryonic kidney cell-line 293T (HEK293T) were cultured in Opti-MEM I (GIBCO, Grand Island, NY) containing 5% fetal bovine serum (FBS).

Plasmids

The RG 8-plasmid system for the PR8 virus was a gift from Dr. Peter Palese, Mount Sinai School of Medicine, New York, NY. The RG system for the Avian Flu cold adapted (ca) virus was produced from the homologous strain provided to us by Ruben Donis, CDC, and cloned into the pDP2002 vector (Sutton et al, unpublished). The RG 8-plasmid systems for the H1N1pdm and WF10 have been previously described [22,29]. The bidirectional pGD2007 RG vector containing the canine pol1 and t1 sequences was a gift from Ruben Donis. The 8 gene segments of VN1203 were subcloned into the pGD2007 vector from clones originally prepared in the pDP2002 vector (data not shown). The plasmids pRF1428 and pRF1437 were provided by Ron Fouchier and encode the wt H5 HA and ΔH5 HA genes, respectively, from the 072 virus. The plasmids pcDNA74PB1, pcDNA762PB2, pcDNA787PA and pcDNA693NP have been previously described [30]. The pH72-EGFP plasmid encoding the influenza EGFP reporter replicon was a gift from Robert Webster, St Jude Children’s Research Hospital. Memphis, TN [21].

Preparation of viral RNA and cDNA

The vRNAs and cDNAs from wild type and blind passage reassortant viruses were prepared as previously described [22,31]. Brieﬂy, total RNAs were extracted by using the RNasy kit (Qiagen, Valencia, CA) following manufacturer’s instructions. Reverse transcription was carried out with the Uni12 primer (5′- AGCAAAAAGCAAGG-3′) and avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI). The cDNAs were stored at −80°C until use.

PCR strategy for Flu EGFP replicon and pol2-driven P and NP expression PCR amplicons

The Flu EGFP replicon was amplified with the primers pT1FragFwd and hpol1Rev using pHW-EGFP plasmid DNA as the template [30]. PCR amplicons from pcDNA762 (PB2), pcDNA774 (PB1), pcDNA787 (PA) or pcDNA693 (NP) were generated with the primers pCMVF and pBGHR, and were designated as pol2PB2hgh, pol2PB1hgh, pol2PAbgh and pol2NP, respectively [21,30,32]. The pol2-based Flu PCR amplicons were flanked by sequences corresponding to the immediate–early human cytomegalovirus (CMV) promoter and bovine growth hormone (bgh) polyadenylation signal. PCR conditions were similar to the overlapping PCR of pol1HAkdmt1 and pol1Nkdmt1 amplicons except for the use of 10 pg of the corresponding plasmid DNA template (see below).

After PCR amplification, two methods were used to demonstrate that PCR products were devoid of spurious plasmid DNA contamination. PCR products were digested with Dpn I (New England Biolabs, Ipswich, MA) for 1 h, and then separated and purified by agarose gel electrophoresis, and subsequently 100 ng of the purified PCR product were used to transform E. coli TOP10 cells (Invitrogen, Carlsbad, CA). Alternatively, the primer pairs, pCMVF and pT1FragRev, pDP2066-2090F and pDP2392-2410R, and pT1-2F and UTR-H1Rev were used to demonstrate lack of plasmid DNA contamination in purified PCR reactions (Table 1). This strategy was also used to demonstrate no plasmid DNA contamination after PCR amplification of the pollen promoter from the PR8 vector pDP2002. In all instances, no plasmid DNA contamination was observed by either method (data not shown).

PCR strategy of overlapping HA and NA gene segments with human pol1 promoter

A schematic representation of the overlapping PCR approach is shown in Fig. 2 using the set of primers described in Table S1. Two overlapping PCR fragments were generated for the HA5im gene: The first fragment spans from the primer set pT1FragFwd, which incorporates the t1 signal, and S1c9451R. The second fragment was amplified with the primer pair S1c9451-732F and polFragRev. The human pollen promoter was PCR amplified using primers polF and hPol1Rev and the pDP2002 plasmid vector as template. Details of amplification conditions for each PCR fragment are provided with the supplementary information (Fig. S2A and B). The 3 PCR products above were purified by agarose gel electrophoresis and combined in equal proportions to generate a full-length HA PCR amplicon using the primer pair pT1FragFwd
and hPol1Rev. The 50 μl PCR reaction mixture contained 10 ng of each PCR product, 25 μl of Master PCR mix, 1.5 μl 100% DMSO, and 50 pmol/μl of each primer. PCR reaction conditions were 98°C for 30 sec, and then 30 cycles at 98°C for 5 s, 56°C for 1 min and 72°C for 3 min, ending with 72°C for 10 min. PCR products were amplified using the Phusion high-fidelity PCR master mix with GC Buffer (New England Biolabs, Ipswich, MA). Alternative HA PCR products without t1 signal sequence or lacking both pol1 and t1 elements were generated to serve as controls for PCR-based reverse genetics (Fig. 2 and Table 2). PCR products were purified by agarose gel electrophoresis and quantitated after gel purification using Nanodrop 1000 (Nanodrop, Wilmington, DE). Overlapping PCR products were produced for the NAvdin gene using the primer pair pTI FragFwd and SwNA-763R and N1-562F and polFragRev, whereas the full length NA PCR amplicon was generated with the primer pair pTI FragFwd and hPol1Rev. Alternative full-length NA amplicons were generated using specific primers as noted in Table 1.

Similar strategies were used to amplify the HAA8V and HAAsV1203 PCR products in which the polybasic cleavage site sequence were removed using overlapping PCR products spanning sequences from the primer pairs pTI FragFwd and IndoH5-ClvR and IndoH5-ClvF and polFragRev whereas the full length HA PCR amplicon as generated with the primer set pTI FragFwd and hPol1Rev. The full length NA gene segment from Ind072 strain was amplified without the generation of internal overlapping fragments using the primer set kTIUni12F and polNIRev rather and then subsequently introduced in a PCR reaction to generate the full-length polN4472t1 PCR amplicon carrying the pol1 promoter (Table S1). To compare the rescue efficiency of PCR amplicons, pRF1437 plasmid was also used as control.

Overlapping PCR for the internal gene segments of PR8

To set up a reverse genetics system using a full set of PCR amplicons, the H1N1plasmid surface gene segments and the PR8 virus 6 internal gene segments were selected. The internal genes were amplified from the total cDNAs prepared from a wildtype PR8 strain, with an initial titer of 1.5×10^7 TCID50/ml in MDCK cells. For the overlapping PR8 PB2 PCR amplicon with pol1 and t1 signal sequences, the pTI FragFwd and PB2-1811R primers were used to produce the N terminal fragment of PB2 (PB2-N), and then the PB2-1643F and polFragRev primers were used to produce the C terminal fragment (PB2-C). PB2-N, PB2-C, and pol1 fragments were mixed (10 ng each), and the overlapping PCR reaction was performed using primers pTI FragFwd and hPol1Rev. The PCR parameters were similar to those of po1HAPI9t1 and pol1NA472t1 amplicons described above. The final product was labeled pol1PB2-fg. A similar strategy was followed for other gene segments fused to pol1 and t1 signal sequences, using the primer pairs: PB1-1240F/PB1-1531R, PA-892F/PA-1314R, HA-760F/HA-1274R, NP-1116F/NP-1441R, NA-743F/NA-905R, M-741F/M-913R, or NS-469F/NS-887R primers respectively. Final PCR amplicons were designated as po1PB1-fg1, po1PA-fg1, po1HAPI9t1, po1NP-fg1, po1NA472t1, po1M1f, and po1NS-fg1, respectively. Additional details are provided with the supplementary information (Fig. S. 3).

Overlapping PCR with canine pol1 promoter

The generation of the HA PCR amplicon from H5N1 containing the k9pol1 promoter was performed as follows: The k9pol1 promoter was amplified from the pGD2007 vector using the primer pair k9pol1F and k9pol1R under these conditions: the initial denaturation at 98°C for 30 sec, 30 cycles of 98°C for 8 sec, 60°C for 30 sec, and 72°C for 1 min and the final extension at 72°C for 10 min (Table S1). Overlapping HA fragments, one containing the k9t1 signal was produced with the primer pair kTIUni12F and IndoH5-ClvR (H5N1) and the second one with the primer pair IndoH5-ClvF (H5N1) and k9polUTRR under the following conditions: pre-PCR treatment at 98°C for 30 sec, 30 cycles of 98°C for 8 sec, 36°C for 30 sec, and 72°C for 1 min and the final extension at 72°C for 10 min (Table S1). The amount of overlapping HA PCR products and the k9pol1 amplicons were calculated and mixed together at a concentration of 10 ng each. A full length HA PCR amplicon was generated with the primer pair kTIUni12F and k9pol1R. The thermal profile was denaturation at 98°C for 30 sec, 30 cycles of 98°C for 8 sec, 36°C for 30 sec, and 72°C for 1 min, and 72°C for 4 min, and then extension at 72°C for 10 min. All the PCR products were amplified using the Phusion high-fidelity PCR master mix with GC Buffer.

Generation of virus by reverse genetics using PCR amplicons

For partial plasmid-free rescue, the plasmid of choice was replaced with the corresponding Flu PCR amplicon and virus rescue performed essentially as described [26] with minor modifications. Briefly, co-cultured 293T/MDCK cells at a ratio of 500:1 (5×10^7 cells per well) was seeded into each well of a 6-well tissue culture plate. The plates were incubated at 37°C overnight. The following day, 1 μl of each plasmid or Flu PCR amplicons was incubated for 45 min with 16 μl of Transit-L1 transfection reagent (Mirus Bio LLC, Madison, WI) and then the transfection allowed to occur overnight before the media was replaced with fresh serum-free Opti-MEM. At 24 h post-transfection (hpt), L-(tosylamide-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin (1 μg/ml) was added to the cell supernatants.

MDCK and Vero cells were grown to 70% confluency in 75-cm² flasks and then trypsinized with trypsin-EDTA (Invitrogen) and resuspended in Opti-MEM I containing 5% FBS. Cell suspensions were seeded into 6-well tissue culture plates and incubated at 37°C overnight before transfection. Transfections and post-transfection steps proceeded as described for the 293T/MDCK co-cultured cells, except that Vero cells were incubated with 2 μg/ml of TPCK-trypsin.

Supernatant of transfected cells were collected at the times indicated in Tables 1, 2 and 3 and blind passage in either or both MDCK cells or 10-day old embryonated chicken eggs to monitor for the presence of rescued viruses. TCID50 titers were determined in MDCK cells by the Reed and Muench method as described [33]. Virus stocks were prepared and frozen at −80°C until use.

Plaque staining and purification

The rescued viruses were examined by plaque assay in MDCK cells (30). Briefly, confluent cell monolayers in 6-well plates were infected with 10-fold dilutions of virus in a total volume of 0.4 ml PBS for 1 h at 37°C. Cells were washed twice with PBS and covered with an overlay of modified Eagle’s medium containing 0.9% agar, 0.02% BSA, 1% glutamine, and, when noted, 1 μg/ml TPCK-treated trypsin. The plates were then incubated at 37°C under 5% CO2. After 3 days of incubation the overlays were removed and the cells were stained with 0.1% crystal violet.

For sequencing the HA gene from plaque-purified viruses, the agarose was plucked from areas of the plate were plaques were observed. Viruses were eluted from the agarose pluck in tissue culture media and later expanded in MDCK cells for 72 h prior to RNA extraction, cDNA synthesis, PCR, and sequencing.
HI assay
Antisera against the ΔH5N1 Indo072 virus were raised in chickens by infecting them with a live attenuated version of the ΔH5N1 Indo072 virus in the background of the WFI01att virus similar to as previously described [34]. Collected sera were treated with receptor-destroying enzyme (Accurate Chemical and Scientific Corp., Westbury, NY) prior to HI assays as described in the WHO Animal Influenza Training Manual (WHO/CDS/CSR/NCS/ 2002.5) (30).

Sequence analysis
Sequencing of overlapping PCR products, viral cDNAs and HA sequences from the purified clones of PR8 or rescued reassortants was performed using a combination of universal primers [31] and custom made primers (available upon request) and the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) on a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Sequence analysis was performed using software available through the Lasergene package (DNASTAR Inc., Madison, WI).

Immunofluorescence assay
Cells grown in 96-well plates were infected with rescued influenza viruses at a dose of 1 TCID₅₀/well. At 36 hpi, the cells were washed in precooled 0.01 M Phosphate Buffered Saline (PBS) buffer and fixed in neutral formaldehyde for 20 min at room temperature. The cells were then incubated with blocking solution (10% normal goat serum in PBS) for 1 h and probed with a primary antibody for 30 min. Two monoclonal antibodies were used to identify the recombinant influenza viruses: mAb 3B2 is specific for the HA protein of H1N1 pdm viruses and which does not react with the HA of PR8 and other subtypes of influenza A viruses [35], mAb DPJY01 is specific for the HA of H5 subtype influenza viruses [36]. The antibody-antigen complexes were further incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (H+L) (Southernwest Biotech Associates Inc, Birmingham, AL) for 30 min at room temperature. The cells were washed three times with PBS after incubation and then counterstained with propidium iodide (PI) and examined under an Axiophot Photomicroscope produced by Carl Zeiss (Em of 522/590 nm for 100 ms).

Supporting Information
Materials and Methods S1 Enzymes and kits used to generate full-length Flu PCR amplicons.

Figure S1 Poll- and pol2-driven PCR amplicons. A) Generation of Flu EGFP replicons from pHW72EGFP. Lane 1, pol1EGFPfr1 amplicon amplified with the primer pair pT1FragFwd hpol1Rev. The Flu EGFP amplicon (1103 bp) contained the Flu EGFP replicon (846 bp) flanked by the human pol1 (222 bp) and mouse t1 (35 bp) sequences. Lane 2, pol1EGFPfr1 (1068 bp, lacking the t1 sequence) produced with the primer pair Bm-M-1F and hpol1Rev. Lane 3, UTREGFPfr1 amplicon (846 bp, lacking the pol and t1 sequences) amplified with the primers Bm-M-1F and Bm-M-1043R. Lane 4, UTREGFPfr1 amplicon (881 bp, lacking pol1 sequence) generated with the primers pT1FragFwd and pol1FragRev. B) Pol2 Flu PCR amplicons produced from pcDNA762 (PB2), pcDNA774 (PB1), pcDNA787 (PA) and pcDNA693 (NP), respectively using the primer pair pCMVp and pBGHR. Each pol2 Flu PCR amplicon contained the cytomegalovirus immediate early promoter sequence (CMV, 659 bp), the bovine growth hormone pol7A signal (BGHpA, 228 bp) and additional non coding regions present within the multiple cloning site of pcDNA3 (Invitrogen). Lane 1, pol2PB2bgh (3,306 bp); lane 2, pol2PB1bgh (3,385 bp); lane 3, pol2PAbgh (3,271 bp); and lane 4 pol2NPbgh (2,603 bp). “M” in panels A and B corresponds to DNA molecular weight markers (GeneRuler™ 1 kb Plus DNA Ladder, Fermentas).

Figure S2 Generation of HA and NA amplicons. cDNAs from the H1N1pdm and H5N1 072 viruses were prepared as described in the main text. M, GeneRuler™ 1 kb plus DNA Ladder. Lane 1, unspcific PCR products obtained using one-step RT-PCR to generate the full length of HApdm gene (1,840 bp) with the primers pTIHF and polHR. Lane 2, the N terminus of HApdm specific PCR product (998 bp) obtained using the primer pair pTI FragFwd, which incorporates the t1 signal, and SwHA-931R. Lane 3, the C terminus of overlapping HApdm specific PCR product (1,022 bp) using the primer pair SwHA-752F and polFragRev. Lane 4, the N terminus of NApdm specific PCR product (799 bp) obtained using the primer pair pTI FragFwd and SwNA-763R. Lane 5, the C terminus of NApdm specific PCR product (924 bp) from N1-562F and polFragRev primer set. Lane 6, the first HAA072 specific PCR fragment (1,090 bp) obtained with the primers pTI FragFwd and IndoH5-cvlR. Lane 7, the second HAA072 specific PCR fragment (762 bp) obtained with the primers IndoH5-cvlF and polFragRev. Lane 8, the full-length HAA072 amplicon (1,460 bp) generated with the primer set hTIN1Fwd and polN1Rev. The 25 µL PCR reaction mixture contained 10 ng of cDNAs, 12.5 µL of Master PCR mix, 0.6 µL 100% DMSO, and 10 pmol/µL of each primer. The PCR reaction conditions were 98°C for 30 sec, and then 30 cycles at 98°C for 8 sec, 56°C for 1 sec and 72°C for 2 min, ending with 72°C for 10 min. PCR products were amplified using the Phusion high-fidelity PCR master mix with GC Buffer.

Figure S3 Full-length PCR amplicons from PR8 virus gene segments. A) The PR8 virus gene segments were amplified as two overlapping PCR fragments, which were performed as follows: Lane 1, amplification of the N terminal fragment of PB2PR8 (1,846 bp) with primer pair pTI FragFwd and PB2-1811R. Lane 2, amplification of the C terminal fragment of PB2PR8 (741 bp, yellow arrow) with primer pair PB2-1643F and polFragRev. Lane 3, amplification of the N terminal fragment of PB1PR8 (1,566 bp) with primer pair pTI FragFwd and PB1-1531R. Lane 4, amplification of the C terminal fragment of PB1PR8 (1,128 bp) with primer pair PB1-1240F and polFragRev. Lane 5, amplification of the N terminal fragment of PAPR8 (1,349 bp) with primer pair polT FragFwd and PA-392F and polFragRev. Lane 7, amplification of the N terminal fragment of HApdm (1,309 bp) with primer pair pTI FragFwd and HA1274R. Lane 8, amplification of the C terminal fragment of HApdm (1,042 bp) with primer pair HA-760F and polFragRev. Lane 9, amplification of the N terminal fragment of NA1PR8 (1,476 bp, yellow arrow) with primer pair pTI FragFwd and NP-1441R. Lane 10, amplification of the C terminal fragment of NA1PR8 (1,476 bp) with primer pair NP-1116F and polFragRev. Lane 11, amplification of the N terminal fragment of NA2PR8 (940 bp) with primer pair pTI FragFwd and NA 905R. Lane 12, amplification of the C terminal fragment of NA2PR8 (657 bp) with primer pair NA 743F and polFragRev. Lane 13, amplification of the N terminal fragment of MPR8 (950 bp) with primer pair pTI FragFwd and M-915F. Lane 14, amplification of the C.
terminal fragment of $M_{PR8}$ (313 bp) with primer pair M-741F and polFragRev. Lane 13, amplification of the N terminal fragment of $N_{PR8}$ (923 bp) with primer pair pT1FragFwd and NS-80rlR. Lane 16 amplification of the C terminal fragment of $N_{PR8}$ (468 bp) with primer pair NS-469F and polFragRev. PCR conditions were similar to those described in SFig 2. B) Full-length PR8 PCR amplimers. Overlapping PCR products generated in A) were mixed at a concentration of 10 ng (each product) and amplified with the forward primer pT1FragFwd and the reverse primer hpol1Rev as described in the main text. The thermal profile was: denaturation at 98°C for 2 min, 30 cycles of 98°C for 30 sec, 56°C for 1 min, and 72°C for 4 min, and then extension at 72°C for 10 min. All the PCR products were amplified using the Phusion high-fidelity PCR master mix with GC Buffer. The final overlapping PCR amplimers were designated as pol1PB2frag1 (2,598 bp, lane 1, yellow arrow), pol1PB1frag1 (2,490 bp, lane 2, yellow arrow), pol1NAfrag1 (1,822 bp, lane 3), pol1NAfrag2 (1,670 bp, lane 4), pol1MPfrag1 (1,284 bp, lane 5), and pol1NSfrag1 (1,147 bp, lane 6), pol1PB1frag1 (2,598 bp, lane 7), and pol1HAfrag1 (2,032 bp, lane 8). M, GeneRuler™ 1 kb Plus DNA Ladder.

Figure S4 HA PCR amplicons flanked with kpol1 promoter. Two produce overlapping PCR products for $H_{A1N1203}$ and $H_{A1N1203}$ gene segments. PCR conditions used were similar to those described in SFig 2 and in the main text. Lane 1, the kpol1 promoter (351 bp) was amplified from the pGD2007 vector using the primer pair kpol1F1 and kpol1R1. Lane 2, PCR fragment containing the N-terminus of $H_{A1N1203}$ and the k9t1 signal (36 bp) was produced with the primer pair kTIUni12F and IndoH5-chR with a size of 1,091 bp. Lane 3, PCR fragment containing the C-terminus of $H_{A1N1203}$ (760 bp) produced with the primer pair IndoH5-chF and kpolUTRR. Lane 4, PCR fragment containing the N-terminus of $H_{A1N1203}$ (1,091 bp) amplified as in lane 2. Lane 5 PCR fragment containing the C-terminus of $H_{A1N1203}$ (760 bp) produced as in lane 3, Lane 6, the two overlapping $H_{A1N1203}$ PCR products and the k9pol1 PCR fragment were mixed at a concentration of 10 ng (each product) to generate the full length of k9pol1HA072t1 PCR amplimer (2,144 bp) using the primer pair kTIUni12F and kpol1R1. Lane 7, the two overlapping $H_{A1N1203}$ PCR products and the k9pol1 PCR fragment were mixed at a concentration of 10 ng each and amplified to generate the full length k9pol1HA072t1 amplimer (2,144 bp) using the primer pair kTIUni12F and kpol1R1. Lane 8, same as in lane 6, except that k9pol1HA072t1t (2,109 bp) lacks the k9t1 signal after amplification with the primer pair Bn-HA-1F and kpol1R1. Lane 9, same as in lane 7, except that k9pol1HA072t1t (2,109 bp) lacks the k9t1 signal after amplification.

Table S1 Primer set for production of overlapping Flu PCR amplicons.

Table S2 HA sequences from H5A072 PCR: 7PR8 plaque-purified viruses.

Acknowledgments

We would like to express our gratitude to Qiong Chen and Johanna Lavigne for their technical assistance.

Author Contributions

Conceived and designed the experiments: DRP. Performed the experiments: HC, JY, KX, MA, HS. Analyzed the data: HC, TS, DRP. Contributed reagents/materials/analysis tools: AF, DRP. Wrote the paper: HC, TS, DRP.

References

1. Weiby RJ, Webster RG, Richt JA (2007) Influenza viruses in animal wildlife populations. Curr Top Microbiol Immunol 315: 67–83.
2. Yamanaka K, Ogasawara N, Yoshikawa H, Ishihama A, Nagata K (1991) Influenza A virus polymerase: a novel mitochondrial DNA polymerase that uses a viral nucleoside triphosphate as the sole detectable nucleotide triphosphate. Cell 64: 333–344.
3. Lopez-Turiso JA, Martinez C, Tanaka T, Ortin J (1990) The synthesis of viral RNA in the nuclear matrix fraction. Virus Res 16: 325–337.
4. Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, et al. (1999) Generation of influenza A viruses in avian cells. J Virol 73: 1151–1156.
5. Honda A, Ishihama A (1997) The molecular anatomy of influenza virus RNA polymerase. Bio Chem 378: 483–488.
6. Arias CF, Escalera-Zamudio M, Soto-Del Rio Mde L, Cobian-Guemes AG, Isa P, et al. (2009) Molecular anatomy of 2009 influenza virus A (H1N1). Arch Med Res 40: 643–654.
7. Zell R, Krumholz A, Wutulr P (2006) Influenza virus A PB1-F2 gene. Emerg Infect Dis 12: 1606–1608; author reply 1608–1609.
8. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, et al. (2001) A novel influenza A virus mitochondrial protein that induces cell death. Nat Med 7: 1306–1312.
9. Jagger BW, Wise HM, Kash JC, Walters KA, Wills NM, et al. (2012) An overlapping protein-coding region in influenza A virus segment 3 modulates the influenza A virus mitochondrial protein that induces cell death. Nat Med 7: 1306–1312.
10. Yewdell JW, Ince WJ (2012) Virology. Frameshifting to PAX-influenza. Science 337: 164–165.
11. Kawaguchi A, Nagata K (2007) De novo replication of the influenza virus RNA genome is regulated by DNA replicative helicase, MCM. Embryo 26: 4566–4575.
12. Vrede FT, Brownlee GG (2007) Influenza virion-derived viral ribonucleoprotein synthesizes both mRNA and eRNA in vitro. J Virol 81: 2196–2204.
13. Deng T, Vrede FT, Brownlee GG (2006) Different de novo initiation strategies are used by influenza virus RNA polymerase on its mRNA and viral RNA promoters during viral RNA replication. J Virol 80: 2337–2348.
25. Fodor E, Devenish L, Engelhardt OG, Palese P, Brownlee GG, et al. (1999) Rescue of influenza A virus from recombinant DNA. J Virol 73: 9679–9682.
26. Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG (2000) A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A 97: 6108–6113.
27. Watanabe Y, Ibrahim MS, Ellakany HF, Kawashita N, Minakre R, et al. (2011) Acquisition of human-type receptor binding specificity by new H5N1 influenza virus sublineages during their emergence in birds in Egypt. PLoS Pathog 7: e1002068.
28. Yount B, Curtis KM, Fritz EA, Henley LE, Jahrling PB, et al. (2003) Reverse genetics with a full-length infectious cDNA of severe acute respiratory syndrome coronavirus. Proc Natl Acad Sci U S A 100: 12993–13000.
29. Wan H, Sorell EM, Song H, Hossain MJ, Ramirez-Nieto G, et al. (2008) Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS One 3: e2923.
30. Perez DR, Donis RO (1998) The matrix 1 protein of influenza A virus inhibits the transcriptase activity of a model influenza reporter genome in vivo. Virology 249: 52–61.
31. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR (2001) Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol 146: 2275–2289.
32. Perez DR, Donis RO (2001) Functional analysis of PA binding by influenza a virus PB1: effects on polymerase activity and viral infectivity. J Virol 75: 8127–8136.
33. Reed LJ, Muench H (1938) A simple method for estimating fifty percent endpoints. Am J Hyg 27: 493–497.
34. Song H, Nieto GR, Perez DR (2007) A new generation of modified live-attenuated avian influenza viruses using a two-strategy combination as potential vaccine candidates. Journal of virology 81: 9238–9248.
35. Shao H, Ye J, Vincent AL, Edworthy N, Ferrero A, et al. (2011) A novel monoclonal antibody effective against lethal challenge with swine-lineage and 2009 pandemic H1N1 influenza viruses in mice. Virology.
36. Ye J, Shao H, Hickman D, Angel M, Xu K, et al. (2010) Intranasal delivery of an IgA monoclonal antibody effective against sublethal H5N1 influenza virus infection in mice. Clin Vaccine Immunol 17: 1363–1370.