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Development of a swine RNA polymerase I driven Influenza reverse genetics system for the rescue of type A and B Influenza viruses

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Abstract

Influenza viruses are among the most significant pathogens of humans and animals. Reverse genetics allows for the study of molecular attributes that modulate virus host range, virulence and transmission. The most common reverse genetics methods use bi-directional vectors containing a host RNA polymerase (pol) I promoter to produce virus-like RNAs and a host RNA pol II promoter to direct the synthesis of viral proteins. Given the species-dependency of the pol I promoter and virus-host interactions that influence replication of animal-origin influenza viruses in human-derived cells, we explored the potential of using the swine RNA pol I promoter (*spol1*) in a bi-directional vector for rescuing type A and B influenza viruses (IAV and IBV, respectively) in swine and human cells. The *spol1*-based bi-directional plasmid vector led to efficient rescue of IAVs of different origins (human, swine, and avian) as well as IBV in both swine- and human-origin tissue culture cells. In addition, virus rescue was successful using a recombinant bacmid containing all eight segments of a swine origin IAV. In conclusion, the *spol1*-based reverse genetics system is a new platform to study influenza viruses and produce swine influenza vaccines with increased transfection efficiency.

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CRediT authorship contribution statement

Brittany Seibert: Methodology, Investigation, Data curation, Validation, Writing - original draft. **Matthew Angel:** Conceptualization, Methodology, Formal analysis, Validation, Writing - review & editing. **C Joaquin Caceres:** Supervision, Methodology, Writing - review & editing. **Troy Sutton:** Methodology, Writing - review & editing. **Ayush Kumar:** Methodology. **Lucas Ferreri:** Supervision, Methodology, Writing - review & editing. **Stivalis Cardenas Garcia:** Supervision, Methodology, Writing - review & editing. **Ginger Geiger:** Methodology. **Daniela Rajao:** Supervision, Data curation, Writing - review & editing. **Daniel R. Perez:** Conceptualization, Methodology, Supervision, Resources, Data curation, writing - review & editing, Funding acquisition, Project administration.

Declaration of Competing Interest

The work described in this report contains data under patent applications filed by DRP and MA.

Keywords

Influenza virus; Reverse genetics; Swine RNA polymerase I; Swine cells; Human cells

1. Introduction

Influenza viruses are considered major pathogens of humans and animals. In the swine production industry, type A influenza (IAV) infections rank among the top three major health problems in the industry (Vincent et al., 2017). Swine are often referred to as the "mixing vessel" of IAVs favoring reassortment between strains of human and animal origin, which may lead to zoonotic and/or pandemic influenza strains. In addition, about 38 % of sampled domestic swine herds in the U.S. Midwest region showed antibodies against type B influenza viruses (IBV) (Ran et al., 2015).

Reverse genetics, a technology that generates genetically defined RNA viruses from a cDNA copy, serves as the cornerstone for modern studies investigating influenza replication, pathogenesis and transmission (Perez et al., 2020). Plasmid-based reverse genetic systems for influenza viruses require two sources of RNA: 1) negative sense, genomic viral RNA copies of each segment (vRNA), and 2) messenger RNA copies of the most relevant structural components of the virus, particularly the RNA-dependent RNA polymerase complex (RdRp) (Perez et al., 2020). It has been noted that the number of plasmids required to recover virus could be consolidated by arranging the promoters for viral mRNA and vRNA transcription in opposite orientations around the cloned cDNA, which would then serve as template for both RNA species (Hoffmann et al., 2000). Exact vRNA copies can be produced by an RNA polymerase I (pol I) promoter and terminator (t1) sequences (Chen et al., 2012). Conversely, viral mRNAs can be synthetized from suitable RNA polymerase II (pol II) promoters like cytomegalovirus promoter (pCMV) (Chen et al., 2012; Perez et al., 2017a, b). Previous reports have described species specificity of the RNA pol I promoter, which could potentially affect virus rescue efficiency (Eberhard and Grummt, 1996; Heix and Grummt, 1995). The use of the swine pol I promoter (*spol1*) to produce a strain of swine-origin IAV was recently published; however, details on the boundaries of the promoter as well as its potential to serve as a universal platform for rescue of IAV and IBV strains was not reported (Moncorge et al., 2013; Wang et al., 2017). In this report, we demonstrate that a *spol1*-based bi-directional vector is efficient for de novo rescue of IAVs of human, swine, and avian origin as well as IBV in cells of swine and human origin. Further, we explored the possibility of producing an all-in-one *spol1*-driven reverse genetics system using a recombinant bacmid containing all eight segments of swine origin IAV. In addition to increasing the efficiency of viral rescues, all-in-one approaches also allow for the development of alternative vaccination platforms as we previously described for a similar human *hpol*1-driven system (Chen et al., 2014).

2. Materials and methods

2.1. Cells

Human embryonic kidney 293 T (HEK293 T) (ATCC® CRL-3216™, Manassas, VA, USA), pig kidney (PK(15)) ((ATCC® CCL-33™, Manassas, VA, USA) and Madin-Darby canine kidney (MDCK) (ATCC® CRL-2935™, Manassas, VA, USA) cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10 % fetal bovine serum (FBS) (Sigma, St. Louis, MO), 2 mM of L-glutamine (Sigma, St. Louis, MO) and 1 % of antibiotic/antimycotic solution containing 100 units/mL penicillin G, 0.1 mg/mL streptomycin sulfate, and 0.25 ug/mL amphotericin B (VWR, Radnor, PA) (DMEM/FBS). The cells were cultured at 37 °C under 5 % CO_2. Opti-MEM (Life Technologies, Carlsbad, CA)) supplemented with 1 % of antibiotic/antimycotic solution (Opti-MEM/AB) was used during transfections and virus rescue as indicated below.

2.2. Plasmids

The pCMV-SEAP plasmid contains the secreted alkaline phosphatase gene under the control of the pCMV promoter and was used to normalize transfection efficiency as described previously (Santos et al., 2017). The plasmid pDZ_IAV(PR8)_NS1_mCherry/NEP was a kind gift from Dr. Luis Martinez Sobrido (University of Rochester, NY, USA) and utilized to analyze transfection efficiency. The pDP-Gluc(NS) plasmid encodes an influenza reporter replicon under the control of the human pol I promoter (*hpol1*) harboring the Gaussia luciferase gene (Gluc) that is flanked by the non-coding regions (NCR) of segment 8 (NS) from the influenza A/guinea fowl/Hong Kong/WF10/1999 (H9N2) strain as previously described (Fig. 1A) (Pena et al., 2011). To generate the pPIG-vGluc(NS) plasmid, the *hpol1* promoter of pDP-Gluc(NS) was replaced with a minimal *spol1* promoter corresponding to nucleotide positions 372–539 of *Sus scrofa* 45S ribosomal RNA promoter region (Fig. 1B). Subsequently, the *spol1*-vGluc(NS) cassette was subcloned between a pCMV promoter and a bovine growth hormone polyadenylation signal (aBGH) of pDP2002 to produce the bidirectional vector pPIG2012_Gluc(NS) (Perez et al., 2020). To produce the reverse genetics vector pPIG2012, the pPIG2012_Gluc(NS) plasmid was amplified by inverse PCR with the primer set 5′-ATATCGTCTCGTCCCCCCCAACTTCGGAGGTCG-3′ and 5′-TATTCGTCTCGATCTACCTGGTGACAGAAAAGG-3′ and digested with BsmBI (NEB, Ipswich, MA) to remove the vGluc(NS) cassette. Following, the digested PCR product was ligated to a small double stranded oligonucleotide insert produced by annealing the oligonucleotide set 5′Phos-GGGACGAGACGATATGAATTCTATTCGTC TCG-3′ and 5′Phos-AGATCG AGACGAATAGAATTCATATCGTCTCG-3 (Fig. 1C). The reverse genetics systems for influenza A/California/04/2009 (H1N1) (CA04), A/turkey/Ohio/313053/2004 (H3N2) (Ty04), A/green-winged teal/SGML/CIP1 02_RG SCG01/2013 (H7N3) (SGML) and influenza B/Brisbane/60/2008 (B/Bris) were cloned into *hpol1*-driven pDP2002 vector as previously described (Baker et al., 2014; Chen et al., 2014, 2012; Hoffmann et al., 2001; Jacobs et al., 2019; Perez et al., 2020; Santos et al., 2017; Song et al., 2007). The eight segments of each virus were then subcloned into pPIG2012 using appropriate restriction sites to produce *spol1* driven versions of such systems (Tables 1 and 2). To generate the pΔFast-P8 (Ty04) construct, gene cassettes comprising the pCMV promoter, t1 terminator, viral cDNA copies, *spol1*, and aBGH were amplified from the

individual reverse genetics plasmids encoding the Ty04 system with CMV Fwd ([RS]TGCCAAGTACGCCCCCTATTG) and BGH Rev ([RS] TGGCCGATTCATTAATGCAGCTG) where [RS] represents one of the restriction enzymes used to clone into pΔFast essentially as described previously with minor modifications (Chen et al., 2014). To generate the recombinant bacmid encoding the complete reverse genetic system for Ty04 (Bcmd-P8 Ty04), the pΔFast-P8 (Ty04) plasmid was transfected into DH10Bac, which contained the shuttle bacmid vector together with the components required for Tn7 integration. Bacmids containing the Tn7 insert from pΔFast-P8 (Ty04) were selected for on kanamycin and gentamycin LB agar plates.

2.3. Minigenome assays

The minigenome assays were performed in PK(15) and HEK293 T cells by seeding 300,000 cells/well in 24 well plates in DMEM/FBS. The following day, cells at 70–90 % confluency were co-transfected with 1 μg of pCMV-SEAP, 1 μg of pPIG-vGluc(NS) (*spol1*) or pDP-Gluc(NS) (*hpol1*), and with 1 μg of each of the plasmids encoding the polymerase complex (PB1, PB2, PA, and NP) from either CA04, Ty04, or SGML under the control of the *spol1* (pPIG2012) or *hpol1* (pDP2002) promoters. Transfections were performed using TransIT-LT1 transfection reagent (2 μL/μg DNA; Mirus, Madison, WI) according to the manufacturer's instructions. SEAP expression was analyzed at 24 h post transfection (hpt). Background signal was accounted for by including a well with cells transfected with the same amount of total DNA (6 μg) encoding the NP gene. As a control for IAV amplification, transfections were performed with or without the addition of the plasmid expressing PB1. Cell culture supernatants were collected at 12, 24, 48, 72, and 96 hpt. Fresh media was added after every supernatant collection time-point in order to keep the volume constant. Gluc activity was measured using the Pierce Gaussia Luciferase Glow Assay (ThermoFisher, Waltham, MA) and SEAP activity was measured using the Phospha-Light SEAP reporter gene assay system (Life Technologies, Carlsbad, CA). Gluc and SEAP signals were measured using the Victor X3 multilabel plate reader (PerkinElmer, Waltham, MA). Relative polymerase activity was calculated as a ratio of Gluc activity minus cell background divided by SEAP activity. Experiments were performed at least twice, each time in triplicate.

2.4. Transfection efficiency of swine- vs. human-origin cells

To evaluate the transfection efficiency differences between the swine-origin PK(15) and human-origin HEK293 T cells, 300,000 cells/well were seeded in DMEM/FBS in 24 well plates. The cells were transfected the next day with the mCherry expression plasmid (pDZ_IAV (PR8)_NS1_mCherry/NEP) containing pCMV using TransIT-LT1 transfection reagent (2 μL/μg of DNA) following manufacturer instructions. Fluorescent microscopy imaging using an Olympus 1 × 51 fluorescent microscope was performed at 24, 48, and 72 hpt using a wavelength between 560–630 nm and Nuance software version 3.02 (Perkin Elmer, Waltham, MA). For quantitative analysis of differences in transfection efficiency between the two cell lines, the concentration of SEAP in tissue culture supernatants was measured at 24 hpt (from minigenome assays).

2.5. Generation of influenza A and B viruses by reverse genetics

Viruses were rescued by reverse genetics using a co-culture of PK (15)/MDCK (5:2; total = 700,000 cells/well) or HEK293 T/MDCK (3:1; total = 800,000 cells/well) (Perez et al., 2017a; Perez et al., 2017b; Perez et al., 2020). Cells were seeded in Opti-MEM/AB and transfected with 1 μg of eight of the corresponding plasmids for either CA04, Ty04, or SGML in pDP2002 or in pPIG2012 vectors (Perez et al., 2017a; Perez et al., 2017b; Perez et al., 2020). Transfections were performed using TransIT-LT1 transfection reagent (2 μL/μg of DNA) according to the manufacturer's instructions. For the CA04, Ty04 and SGML virus rescue systems, transfections proceeded overnight (14 h) at 37 °C in a 5 % CO_2 chamber. Subsequently, the media was replaced with 2 mL of Opti-MEM/AB containing 1 μg/mL tosylsulfonyl phenylalanyl chloromethyl keton (TPCK)-trypsin (Worthington Biochemicals, Lakewood, NJ). Additional 1 μg/mLTPCK-trypsin (2 mL/well) was added at 48 h post media replacement. At 12, 24, 48, 72 and 96 h post-addition of TPCK-trypsin, 100 μL of cell culture supernatant was collected and then virus titers measured by 50 % tissue culture infectious dose ($TCID_{50}$) using the Reed Muench method as described previously (Reed LJ and H, 1938).

For IBV rescue, transfection proceeded similarly as described above with changes: Co-culture cells were incubated with the transfection mix for 14 h at 35 °C in a 5 % CO2 chamber. Subsequently, the transfection mix was removed and replaced with 1 mL of Opti-MEM/AB. At 30 hpt, 1 mL of Opti-MEM/AB containing 1 μg/mL TPCK-trypsin was added (2 mL total/well). At 72 hpt, additional TPCK-trypsin was added to a final concentration of 1 μg/mL. Cell supernatants were collected at 120 hpt and 50 μL was used for hemagglutination (HA) assay as previously described (Killian, 2014). Samples positive by HA assay were titrated as described above. If no HA activity was observed at 120 hpt, a blind passage was performed by inoculating MDCK (350,000 cells/well in a 6-well plate) cells with 500 μL of cell supernatant for 1 h at 35 °C, 5 % CO_2. The cell supernatant was then removed and replaced with 2 mL of Opti-MEM/AB and 1 μg/mL TPCK. At 48 hpt, 50 μL of collected cell supernatant was used to perform HA assay and the remaining was stored at −80 °C. Virus titers were measured by $TCID_{50}$ using the Reed Muench method as described previously (Reed and H, M., 1938).

For Bacmid-P8 Ty04 transfections, the DNA copy numbers of Bcmd-P8 Ty04 were normalized to that of pPIG2012-PB2 (Ty04), which contains 1.76×10^{11} copies/μg. This corresponded to 31.5 μg of Bcmd-P8 Ty04. As a single bacmid contains the complete complement of gene cassettes required to rescue influenza virus, we reasoned that transfection of Bcmd-P8 Ty04 and subsequent virus rescue would be more efficient than the standard eight-plasmid transfection scheme at low DNA concentrations. To do this, transfections were performed with 2.8×10^{10} copies of either Bcmd-P8 Ty04 or each of the eight pPIG2012 plasmids encoding Ty04. This represents a 6.4x reduction in the amount of DNA typically used in plasmid-based reverse genetics. For the bacmid, this transfection consists of 5 μg total DNA while the individual plasmids range from 159 ng (pPIG2012-PB2) to 115 ng (pPIG2012-NS) depending on the length of the influenza gene insert. Virus titers were measured by $TCID_{50}$ using the Reed Muench method as described previously

(Reed and H, M., 1938). Experiments were carried out independently at least twice, each time in triplicate.

2.6. Sequencing

Plasmids were sequenced and analyzed as follows. Maxipreps were cleaned by 0.45x of Agencourt AMPure XP Magnetic Beads (Beckman Coulter, Pasadena, CA) according to manufacturer's protocol and eluted in 30 μL of Hyclone molecular biology water (Genesee Scientific, San Diego, CA). Concentration of the eluate was measured using the Qubit dsDNA HS Assay kit (ThermoFisher, Waltham, MA) on the Qubit 3.0 fluorometer (ThermoFisher, Waltham, MA). Samples were normalized to 0.2 ng/μL. Adapters were added by tagmentation using Nextera XT DNA library preparation kit (Illumina, San Diego, CA). The reaction was set up using 40 % of the suggested final volume. Libraries were purified using 0.7X Agencourt AMPure XP Magnetic Beads, and fragment size distribution was analyzed on the Agilent Bioanalyzer using the High Sensitivity DNA kit (Agilent, Santa Clara, CA). Next, samples were normalized to 4 nM and pooled. The loading concentration of the pooled libraries was 15 pM. Libraries were sequenced using the MiSeq v2, 300 cycle reagent kit (Illumina, San Diego, CA) in a paired-end fashion (150 × 2). MiSeq output was mapped back to reference sequences using Burrows-Wheeler Alignment (BWA) (Li and Durbin, 2009) and further analyzed through the Lasergene package (DNASTAR Inc, Madison, WI). Sequencing of the bacmid was performed as previously described (Chen et al., 2014) using universal primers (Hoffmann et al., 2001), custom primers, and the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) on a 3500 Genetic Analyzer according to the manufacturer's instructions. Further sequence analysis was performed using software available through Lasergene package (DNASTAR Inc, Madison, WI).

2.7. Statistical analysis

All data analyses were performed using GraphPad Prism software, version 8 (GraphPad Software Inc., San Diego, CA). For multiple comparisons performed in the luciferase assays, two-way analysis of variance (ANOVA) for multiple comparisons with post hoc Bonferroni test was executed. Regarding two group comparisons performed in the rescue kinetics and SEAP value comparison between PK(15) and HEK293 T cells, multiple t-tests with correction for multiple comparisons using Holm-Sidak method was implemented. A p-value below 0.05 was considered significant.

3. Results

3.1. Activity and species specificity of spol1 in cells of swine and human origin

The unidirectional *spol1*-driven plasmid was created to produce an influenza-like vRNA reporter replicon encoding the Gaussia luciferase gene flanked by the NCR regions of IAV's segment 8 (NS) (Fig. 1) (Pena et al., 2011). To examine the ability of the *spol1* to drive vRNA production and the extent of its species specificity, we transfected the reporter replicon vector under the control of either the *spol1* or *hpol1* promoters along the corresponding bi-directional promoter plasmids encoding the IAV viral ribonucleoprotein (vRNP) complex composed of PB2, PB1, PA, and NP in either PK(15) (swine) or HEK293

T (human) cells. Viral complexes of CA04, Ty04, or SGML PB2, PB1, PA, and NP were tested in the plasmid vector pDP2002 (*hpol1*) (Chen et al., 2014, 2012; Jacobs et al., 2019; Santos et al., 2017; Song et al., 2007) and pPIG2012 (*spol1*). It must be noted that in this minigenome assay system, Gluc expression reflects not only the levels of amplification of the replicon itself, but also the activity of the pol I promoter to stimulate expression of the remaining components of the vRNP leading to self-amplification of the virus polymerase complex. We routinely co-transfected the RNA pol II driven pCMV-SEAP reporter plasmid to establish and normalize transfection efficiencies. Following transfection, cell supernatant was harvested at 12, 24, 48, 72 and 96 hpt for measurement of SEAP and Gluc reporter activities. Gluc reporter activity was normalized by cell background and SEAP enzyme activity. We selected the SEAP activity at 24 hpt to normalize Gluc activity measured at any other time point. Marginal Gluc activity was detected in the negative controls, as expected (Fig. 2). Significantly higher levels of Gluc reporter activity were observed when CA04 and SGML polymerase complex plasmids under the *spol1* were evaluated in PK(15) in comparison with the *hpol1* dependent plasmids at 48 hpt ($p < 0.005$(CA04), $p = 0.0076$ (SGML); ANOVA), 72 hpt ($p < 0.005$(CA04), $p = 0.022$ (SGML); ANOVA) and 96 hpt ($p < 0.005$(CA04), $p = 0.0357$ (SGML); ANOVA) (Fig. 2A and C). Interestingly, Ty04 polymerase complex controlled by the *spol1* had similar Gluc reporter activity as the *hpol1* at all time points in swine PK(15) cells (Fig. 2B) ($p > 0.05$; ANOVA). In addition, the *hpol1* displayed significant activity in PK(15) cells compared to the negative controls at 24, 48, 72 and 96 hpt ($p < 0.05$; ANOVA) (Fig. 2A–C). We also evaluated the Gluc expression using the same systems in human HEK293 T cells. The results from HEK293 T cells showed that the polymerase complex driven by the *spol1* was active but significantly lower in comparison with the *hpol1*, independent of the polymerase complex evaluated (Fig. 2D–F). Significant differences between pPIG2012 (*spol1*) and pDP2002 (*hpol1*) polymerase activity for all three polymerase complexes were observed at 48 hpt ($p < 0.0001$ (CA04), $p = 0.0025$ (Ty04), $p < 0.0001$ (SGML); ANOVA), 72 hpt ($p < 0.0001$ (CA04)(Ty04) (SGML); ANOVA) and 96 hpt ($p < 0.0001$ (CA04)(Ty04)(SGML); ANOVA) in HEK293 T cells (Fig. 2D–F). This was expected considering the RNA pol I species specificity (Eberhard and Grummt, 1996; Heix and Grummt, 1995), thus explaining why the *spol1* functioned more efficiently than the *hpol1* in swine cells and vice-versa in human cells; however, the difference in activity among the *hpol1* and *spol1* is much larger in HEK293 T than in PK(15) cells.

Although swine PK(15) cells can support influenza virus replication; they have been shown to have lower transfection efficiency than HEK293 T cells (Chen et al., 2014). We wanted to confirm whether the differences observed between PK(15) and HEK293 T in the Gluc assays (Fig. 2A, B,C vs D, E, F) could be explained in part by differences in transfection efficiency. We transfected PK(15) and HEK293 T cells with a reporter plasmid expressing mCherry and fluorescent images were captured at 24, 48, and 72 hpt following previously described methods (Fig. 2G) (Nogales et al., 2015). A negative control without the mCherry reporter plasmid was included. Results showed that mCherry expression was not present in the negative control as expected (data not shown). Conversely, mCherry expression was detected in both PK(15) (Fig. 2G, left panel) and HEK293 T (Fig. 2G, right panel) cells from 24 hpt to 72 hpt with a greater overall expression in the latter. To further emphasize

these differences, we measured the levels of SEAP concentrations in PK(15) (Fig. 2H, white bars) and HEK293 T (Fig. 2H, black bars) cells at 24 hpt. Results showed significantly higher SEAP activity in HEK293 T cells correlating with the mCherry assays ($p < 0.005$; t-test) (Fig. 2H). Taken all together, we demonstrate that transfection efficiency between the two cell lines is in part responsible for differences of Gluc activity between *hpol1* and *spol1* in HEK293 T cells compared to the two groups in PK(15) cells. More importantly, we show decreased activity of the *spol1* in human cells and of the *hpol1* in swine cells.

3.2. The pPIG2012 bi-directional vector can rescue IAVs of human, avian and swine origin in swine and human cells

To determine if the *spol1*-based pPIG2012 bi-directional vector is suitable for reverse genetics of IAVs in cells of swine and human origin, reverse genetics ready clones of IAV strains CA04, Ty04, and SGML were subcloned into pPIG2012 and then tested in PK(15)/MDCK and in HEK293 T/MDCK co-culture cells (Table 1) (Hoffmann et al., 2000). Rescue efficiencies were compared to isogenic virus clones under the *hpol1*-based pDP2002 vector. Rescued viruses in cell supernatants were collected at 12, 24, 48, 72, and 96 h post-addition of TPCK-trypsin and then titrated by $TCID_{50}$. Results showed that the IAV segments in the pPIG2012 backbone were able to generate human-, avian- and swine-origin IAV in swine PK(15) cells (Fig. 3A–C). The pPIG2012 backbone functioned significantly better than the pDP2002 backbone in swine cells at early stages of infection with CA04 at the 12 and 24 h time points ($p < 0.0005$; t-test, Fig. 3A) although significant differences were not observed for the other two backbones (Ty04 and SGML) (Fig. 3B,C). Virus titers from the *spol1*- and *hpol1*-based vectors were similar at the 48, 72, and 96 h time points for all three virus strains consistent with virus replication and spread in MDCK cells ($p > 0.05$; t-test) (Fig. 3A–C). We also sought to evaluate the ability of the *spol1* plasmids to produce virus in human HEK293 T cells. Not surprisingly, HEK293 T cells transfected with the virus clones in pDP2002 backbone had higher titers for CA04 (Fig. 3B, left panel) and SGML (Fig. 3B, right panel) at the 12 h ($p < 0.0005$ (CA04), $p < 0.005$ (SGML); t-test), 24 h ($p < 0.0005$ (CA04 and SGML); t-test) and 48 h ($p < 0.005$ (SGML); t-test) time points compared to the pPIG2012-based clones (Fig. 3D,F) (Eberhard and Grummt, 1996; Heix and Grummt, 1995). However, no such differences were observed for the rescue of Ty04 with the pPIG2012-based backbone showing the same efficiency as the pDP2002-based backbone in HEK293 T cells ($p > 0.05$; t-test) (Fig. 3E). Taken all together, these results indicate that the *spol1*-based pPIG2012 vector is suitable for the rescue of IAVs of human-, avian,- and swine-origin in tissue culture cells of swine and human origin.

3.3. The pPIG2012 bi-directional vector can be used to rescue influenza B virus in swine and human cells

To further expand the utility of the pPIG2012 reverse genetics vector, we attempted to rescue influenza B/Bris in swine and human cells. After transfection and addition of TPCK-trypsin, cell culture supernatants were collected at 120 h post-transfection. Amount of virus in the supernatants was evaluated by HA assay and $TCID_{50}$. The results showed the presence of IBV by HA assay in units between 64–256 and an average $TCID_{50}$/mL (log10) titer of 6.394 in PK(15)/MDCK co-culture cells (Table 3) after transfection of the pPIG2012-based B/Bris plasmid set (Table 3). IBV was also rescued from PK(15)/MDCK co-culture cells following

transfection of the pDP2002-based B/Bris plasmid set (HA units between 8–128 and average $TCID_{50}$/mL (log10) titer of 7.23 at 120 h) (Table 3). Following, we compared the IBV rescue efficiency of both systems in HEK293 T/MDCK co-culture cells. The results showed effective IBV production using the pDP2002-based backbone at 120 h (HA units between 128–256 and average $TCID_{50}$ /mL (Log10) titer of 6.42) (Table 3). In contrast, cells transfected with the pPIG2012-based IBV produced significantly less virus ($p < 0.0005$; t-test) at 120 h showing negligible HA units and average $TCID_{50}$ (Log10) titer of 2.93 (blind passage of this supernatant further confirmed the presence of IBV; after 48 hpt, CPE was observed, supernatants with HA units between 128–256 and average $TCID_{50}$ /mL (Log10) titer of 5.73). The delayed observation in IBV rescue using *spol1* in HEK293 T/MDCK recapitulates the differences between *spol1* and *hpol1* previously observed with the IAV clones (Fig. 3). Collectively, these results establish that the *spol1* can be used to rescue IBV in cells of swine and human origin.

3.4. All-in-one Spol1-driven bacmid-based reverse genetics system

We previously showed the potential of an all-in-one *hpol1*-driven bacmid reverse genetics system to rescue influenza viruses (Chen et al., 2014). We tested if a similar approach could be used with a *spol1*-driven system. As a proof-of-principle we used the pPIG2012-based Ty04 reverse genetics system (Fig. 4). The resulting pΔFast-P8 (Ty04) plasmid was about 26 kbp in size, and efficiently rescued influenza virus after transfection into PK(15) cells (not shown). Subsequently, the pΔFast-P8 (Ty04) was used to transform DH10Bac cells to produce the recombinant bacmid Bcmd-P8 Ty04. PCR of the bacmid indicated that each influenza gene from Ty04 was present in the transposed bacmid (Fig. 5A). In addition, each gene was found to be present in the bacmid by sequencing devoid of high confidence mutations compared to the initial pPIG2012 vectors (not shown). The Bcmd-P8 Ty04 was transfected into HEK293 T/MDCK co-culture cells to determine whether the reverse genetics cassettes were functional for virus rescue. Similar peak titers were observed for both Bcmd-P8 Ty04 and the pPIG2012 Ty04 transfections (Fig. 5B). These results suggest that while the bacmid contributes to a complete reverse genetics system, the gain in efficiency in vitro is largely minimal and may be modulated by other factors such as lower transfection efficiency of such a large DNA molecule.

4. Discussion

A minimal *spol1* promoter corresponding to nucleotides 372–539 of the swine 45S ribosomal RNA promoter was engineered as an alternative RNA pol I promoter for the rescue of influenza viruses by reverse genetics. The pPIG2012 bi-directional vector was active in the rescue of IAVs and IBV in cells of swine and human origin. As PK(15) cells have been previously utilized for vaccine production (Wang et al., 2017; Whiford and Fairbank, 2011), the *spol1* reverse genetics system opens a wide range of applicability regarding animal vaccines.

We evaluated the *spol1* system using three different IAVs differing in their subtype and origin of animal species. These specific strains were chosen because Ty04 is a triple reassortment H3N2 IAV that circulates within the swine population, SGML is composed of

multiple segments synthesized from avian strains isolated in green and blue-winged teal, and Ca04 is a pandemic H1N1 prototypical strain isolated from a pediatric patient (Maines et al., 2009; Pena et al., 2012; Ye et al., 2010). Using a minigenome assay composed of expression units of IAV vRNPs and a virus-like reporter replicon under control of the *spol1* promoter, we analyzed the relative levels of IAV polymerase activity in swine and human origin cell lines and compared to similar constructs under the *hpol1* promoter. As expected, the *spol1* was more active in swine cells than in human cells and the *hpol1* showed significantly higher activity in human cells than in swine cells (Fig. 2). Based on relative reporter activities, it appears that the *hpol1* promoter was more active in swine cells than the *spol1* in human cells; however, the self-amplifying characteristics of the mini-genome system and substantial differences in transfection efficiency between the two cell lines preclude further conclusions. Of note, there were substantially different levels of replicon activity comparing the three different IAV vRNP polymerase complexes. The CA04 polymerase complex was more active than Ty04 and the latter was more active than the SGML polymerase complex (Fig. 2). Further studies are needed to better dissect the different polymerase activity phenotypes and their potential influence on host pathogenicity. Our studies are consistent with those of a previous report showing lower firefly luciferase replicon activity (controlled by an alternative *spol1* promoter) in newborn pig trachea (NPTr) cells from viral polymerase complexes derived from four avian-origin and two human-origin IAV strains (Moncorge et al., 2013).

Differences in virus yields at the early stages of transfection/infection was consistent with the species preference of the pol I promoter (Fig. 3). The generation of virus in the transfectable cell lines, such as HEK293 T and PK-15 cells, was important for early stages of transfection; however, virus titers were similar among the *hpolI* and *spolI* at later time points post transfection after viable virus was rescued and infected MDCK cells, which are efficient at propagating virus. In addition, by adjusting the relative number of cells in the PK(15)/MDCK co-culture (5:2) compared to HEK293 T/MDCK co-culture (3:1), we observed similar virus yields for both systems when the pol I promoter was matched to the corresponding cells. Thus, at 12 and 24 hpt, *spol1*-driven production of CA04 in PK(15)/MDCK cells were similar to the *hpol1*-driven system in HEK293 T/MDCK cells (Fig. 3A). Similar conclusions can be inferred from the Ty04 and SGML virus rescues. This result is remarkable taking into account the relatively poor transfection efficiency of PK(15) cells compared to HEK293 T cells. Early studies showed that mRNAs from segment 7 undergo over-splicing in HEK293 T cells infected with influenza virus (Lamb, 1989). Thus, it is possible that PK(15) cells are more efficient at generating fully infectious influenza virus particles than HEK293 T cells. Unfortunately, attempts to rescue viruses from either PK(15) cells or HEK293 T cells alone gave inconclusive results mostly due to quick detachment of HEK293 T cells during transfection in the absence of MDCK cells. Interestingly, differences of virus titers at early time points of transfection were not observed in Ty04 for HEK293 T/MDCK and PK(15)/MDCK cells while large differences were observed in the polymerase assay. The differences observed among Figs. 2 and 3 might be explained by other aspects involved in viral replication apart from RNA production, particularly contributing to the efficiency of this virus; however, those aspects are beyond the scope of the manuscript. A previous report described a different *spol1* reverse genetics vector for the rescue of a swine-

origin H3N2 subtype IAV in HEK293 T/PK(15) co-culture cells but not in either PK(15)/MDCK or HEK293 T/MDCK co-culture cells (Wang et al., 2017). Therefore, virus titer results from the HEK293 T/PK(15) system and ours cannot be directly compared.

While reverse genetics helps expedite the generation of influenza virus vaccine candidates, these candidates may not be well adapted to grow in non-natural substrates. Forcing the candidate vaccine virus to grow in non-natural substrates, like embryonated eggs, can result in egg-adapted changes that often affect the antigenic profile of the virus (Rajao and Perez, 2018). Producing IAV vaccine candidates (particularly live attenuated influenza virus vaccines) in swine cells could potentially limit the number of unwanted adaptative mutations. A previous report showed that the N-glycosylation patterns of the HA viral protein changed significantly when the A/Puerto Rico/8/34 (H1N1) strain was switched from growing in MDCK to Vero cells (Roedig et al., 2011).

5. Conclusions

In summary, we described a new *spol1*-based reverse genetics strategy with the ability to rescue type A and B influenza viruses in swine and human cells. Utilizing a *spol1*-based reverse genetics system in swine cells could be potentially advantageous in transfection efficiency for the generation and/or expansion of influenza viruses by limiting potential substrate-adapted changes for vaccine purposes in swine. In addition to serving as an alternative reverse-genetics platform, the *spol1*-based system could be used for in vivo influenza virus reverse genetics (Chen et al., 2014) in swine, particularly using a system similar to the Bcmd-P8 Ty04 system. However, such potential is beyond the scope of this report.

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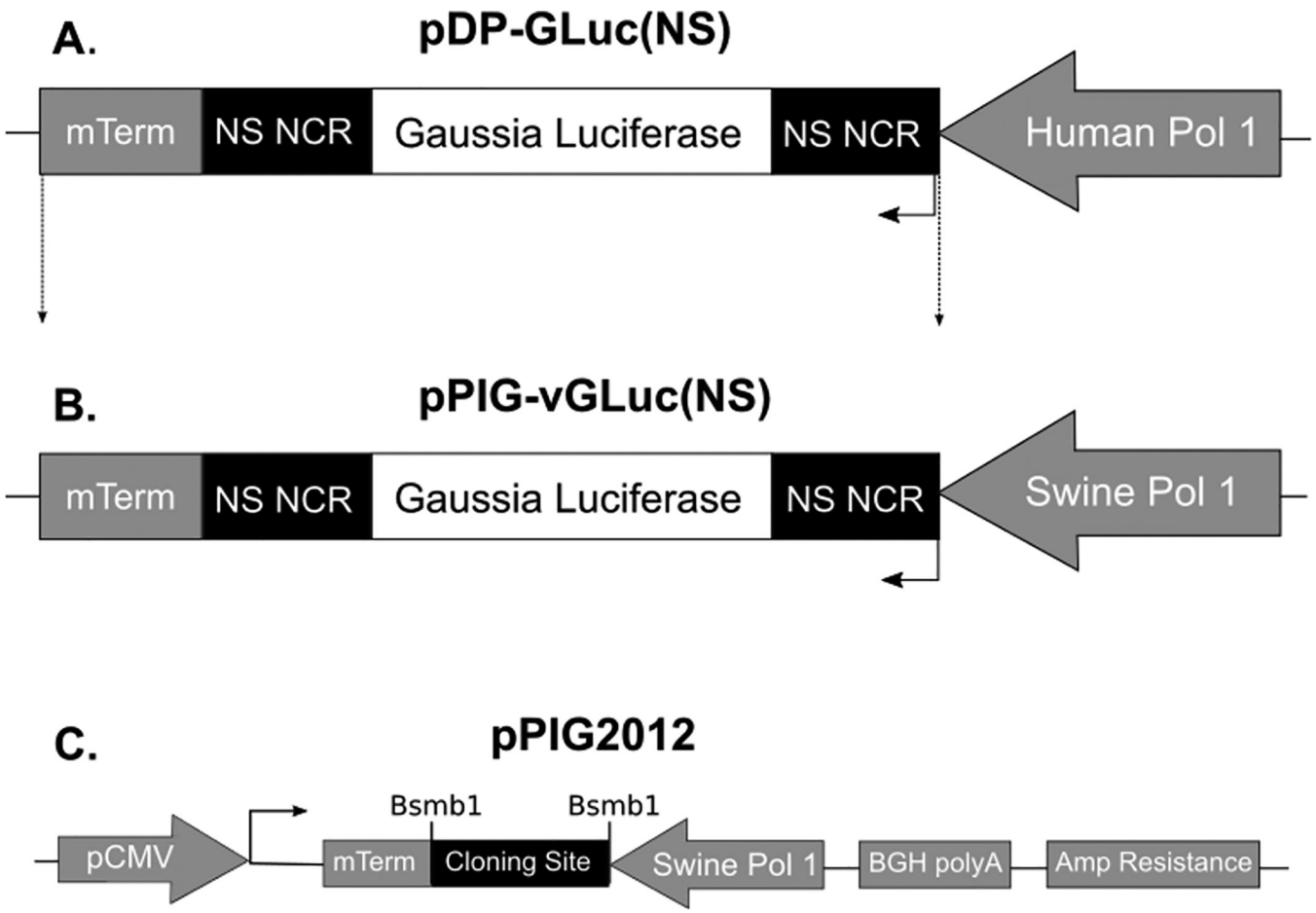

Fig. 1. Schematic representation of the swine pol I-based influenza reverse genetic system.

The murine pol I termination signal (mTerm) and a Gaussia luciferease reporter flanked by NS non-coding regions (NS NCR) was subcloned from the (A) human pol I vector pDP-GLuc(NS) and placed in front of the (B) swine pol I promoter to construct a viral RNA expression vector pPIG-vGLuc(NS). (C) The cassette was then subcloned between the RNA pol II promoter (pCMV) and the bovine growth hormone polyadenylation signal (BGH polyA) to construct a bidirectional vector pPIG2012(BsmbI) containing 2 BsmbI restriction enzyme sites flanking the cloning site.

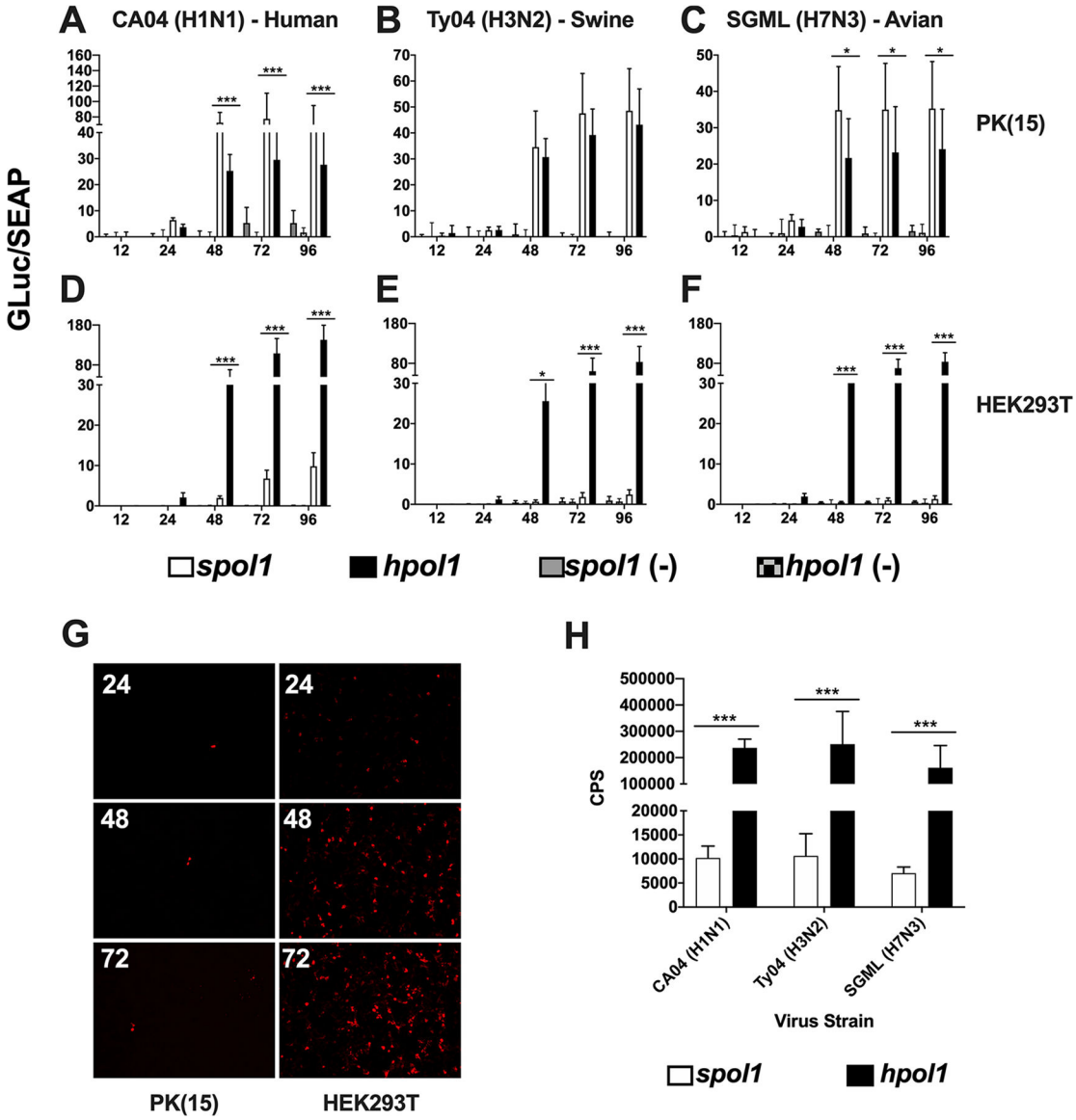

Fig. 2. Virus polymerase activity in *spol1* vs *hpol1* driven influenza replicon systems tested in swine PK(15) cells and human HEK293 T cells.

PK-15 cells or HEK293 T (300,000 cells /well) were transfected with plasmids encoding either the CA04 (A, D), the Ty04 (B, E), or the SGML (C, F) polymerase complex (PB1, PB2, PA, NP) and the influenza replicon reporter encoding Gaussia luciferase in the pPig2012 (*spol1*-driven promoter, white bars) or pDP2002 (*hpol1*-driven promoter, black bars) vectors. A plasmid encoding the secreted alkaline phosphatase (SEAP) controlled by the cytomegalovirus promoter was co-transfected to normalize transfection efficiency. SEAP concentrations were analyzed at 24 h post transfection. Negative controls included the above plasmids except PB1. Cell supernatant was collected at 12, 24, 48, 72 and 96 h post transfection. Relative polymerase activity was calculated as a ratio of luciferase activity minus cell background divided by SEAP. Plotted data represents means +/− standard errors. Two-way ANOVA with post-hoc Bonferroni correction was performed to calculate p-values. * <0.05 ** <0.05 *** <0.0005. G) Transfection efficiency of PK(15) cells vs HEK293 T:

300,000 cells /well were transfected with a reporter plasmid expressing mCherry and its expression analyzed by fluorescence microscopy at 24, 48 and 72 h post-transfection. Data show increased transfection efficiency in HEK293 T cells vs PK(15) cells. H) Total SEAP activity at 24 h post-transfection in PK(15) cells vs HEK293 T cells resulting from co-transfection with different minireplicon systems as in A–F. Plotted data represents means +/− standard errors. Multiple t tests with correction for multiple comparisons using Holm-Sidak method was used for comparisons. * <0.05,*** <0.0005.

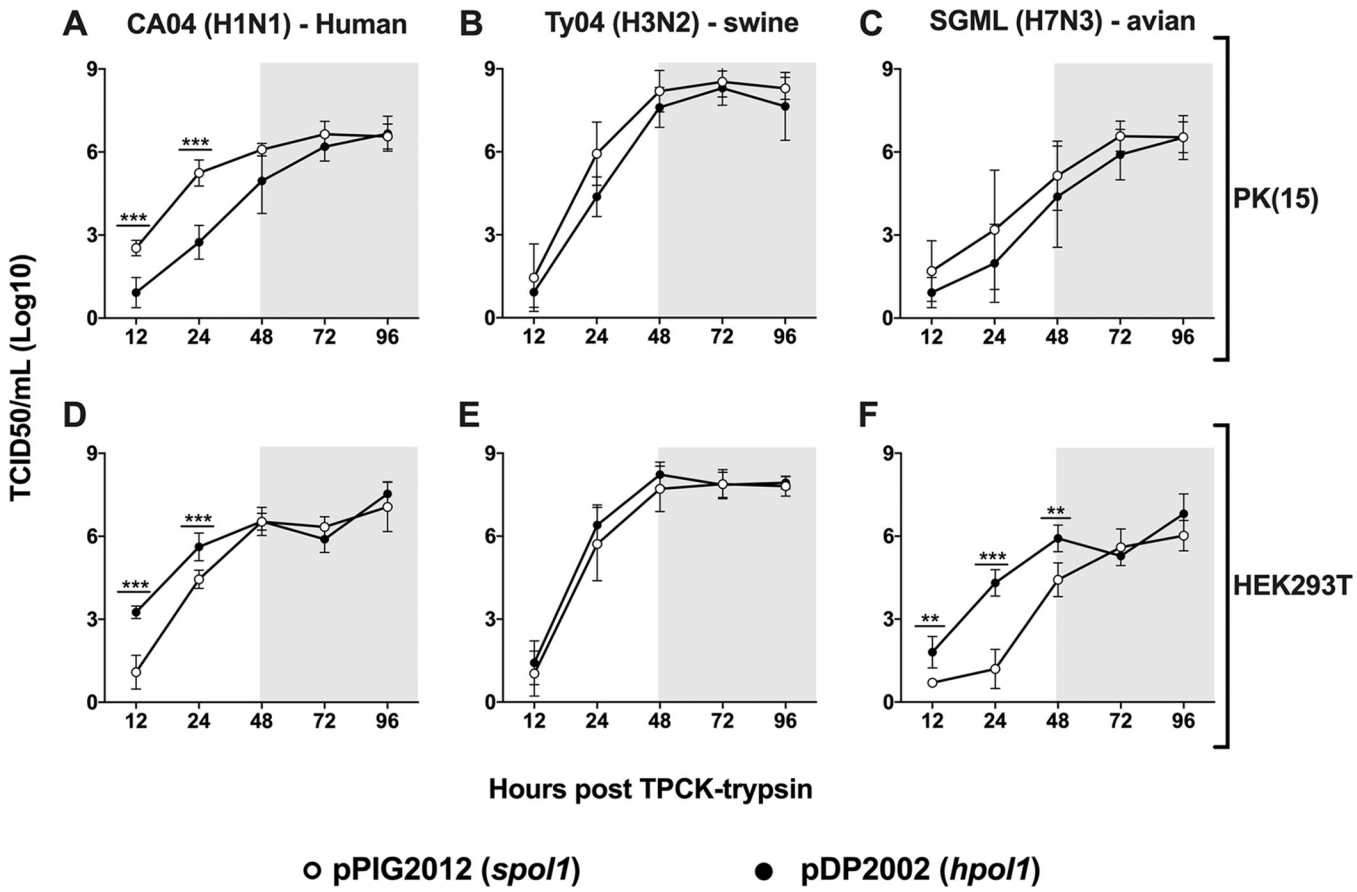

Fig. 3. Rescue of IAV using pPIG2012 and pDP2002 in PK-15/MDCK and HEK293 T/MDCK Co-culture.

Co-culture of PK-15/MDCK (5:2)(A, B, C) or HEK293 T/MDCK (3:1)(D, E, F) were transfected with plasmids encoding CA04 (A, D), Ty04 (B, E), or SGML (C, F) PB2, PB1, PA, HA, NP, NA, M and NS in pPig2012 (white circles) or pDP2002 (black circles) vector overnight (14 h) using TransIT transfection reagent. The media was replaced and supplemented with TPCK-trypsin. Cell culture supernatants were collected at 12, 24, 48, 72, and 96 h after addition of TPCK-trypsin for the quantification of virus titers by TCID50 assays using the Reed-Muench method. Plotted data represents means ± standard errors. Multiple t tests with correction for multiple comparisons using Holm-Sidak method was performed to calculate P values. ** <0.005,*** <0.0005.

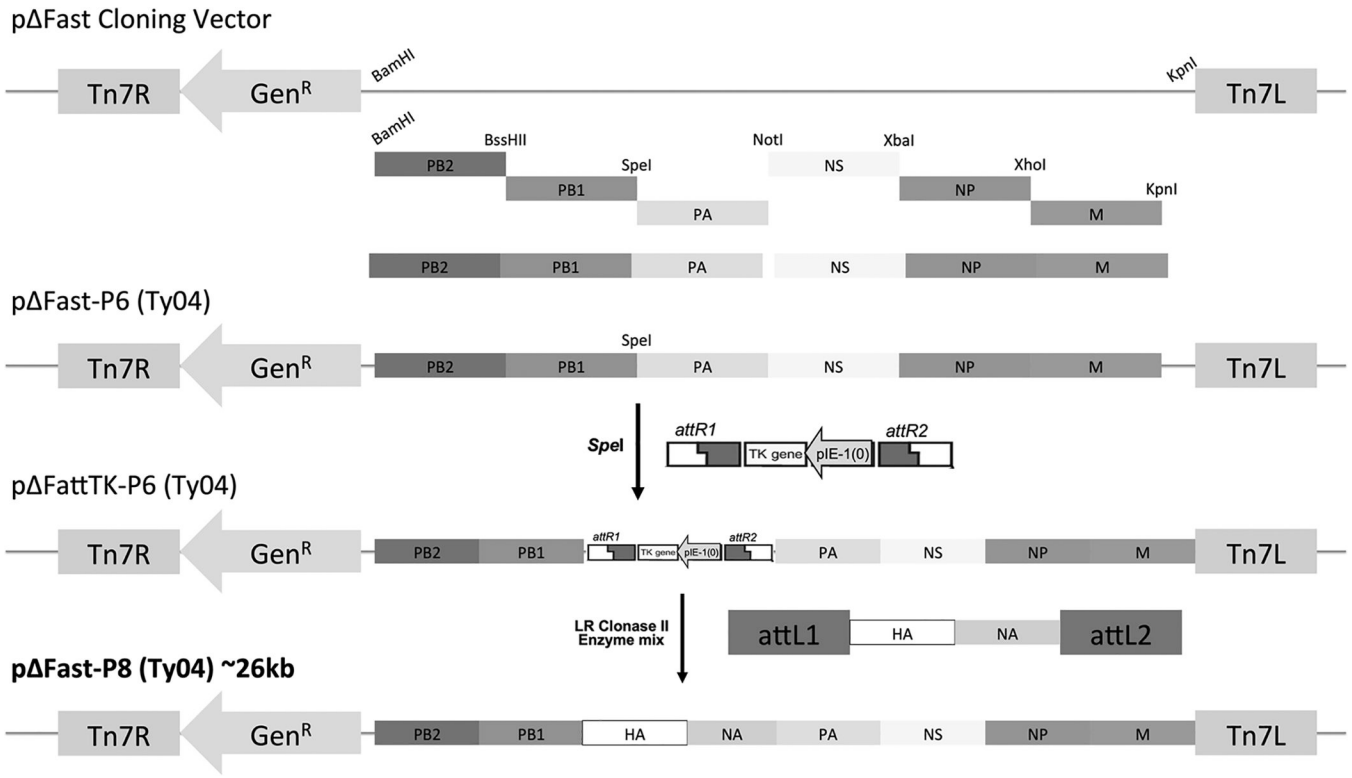

Fig. 4. Influenza cassette assembly strategy.

The pΔFast cloning vector was modified by inserting the reverse genetic cassettes for the polymerase (PB2, PB1, PA and NP), M (M1 and M2), and NS (NS1 and NEP) segments tandemly between the BamHI and KpnI restriction sites. pΔFast-P6 was further modified by the insertion of a constitutively expressed thymidine kinase gene flanked by lambda phage attR1 and attR2 sites. The final pΔFast-P8 construct was constructed by performing a Gateway reaction with HA and NA reverse genetic cassettes cloned into pENTR-1A.

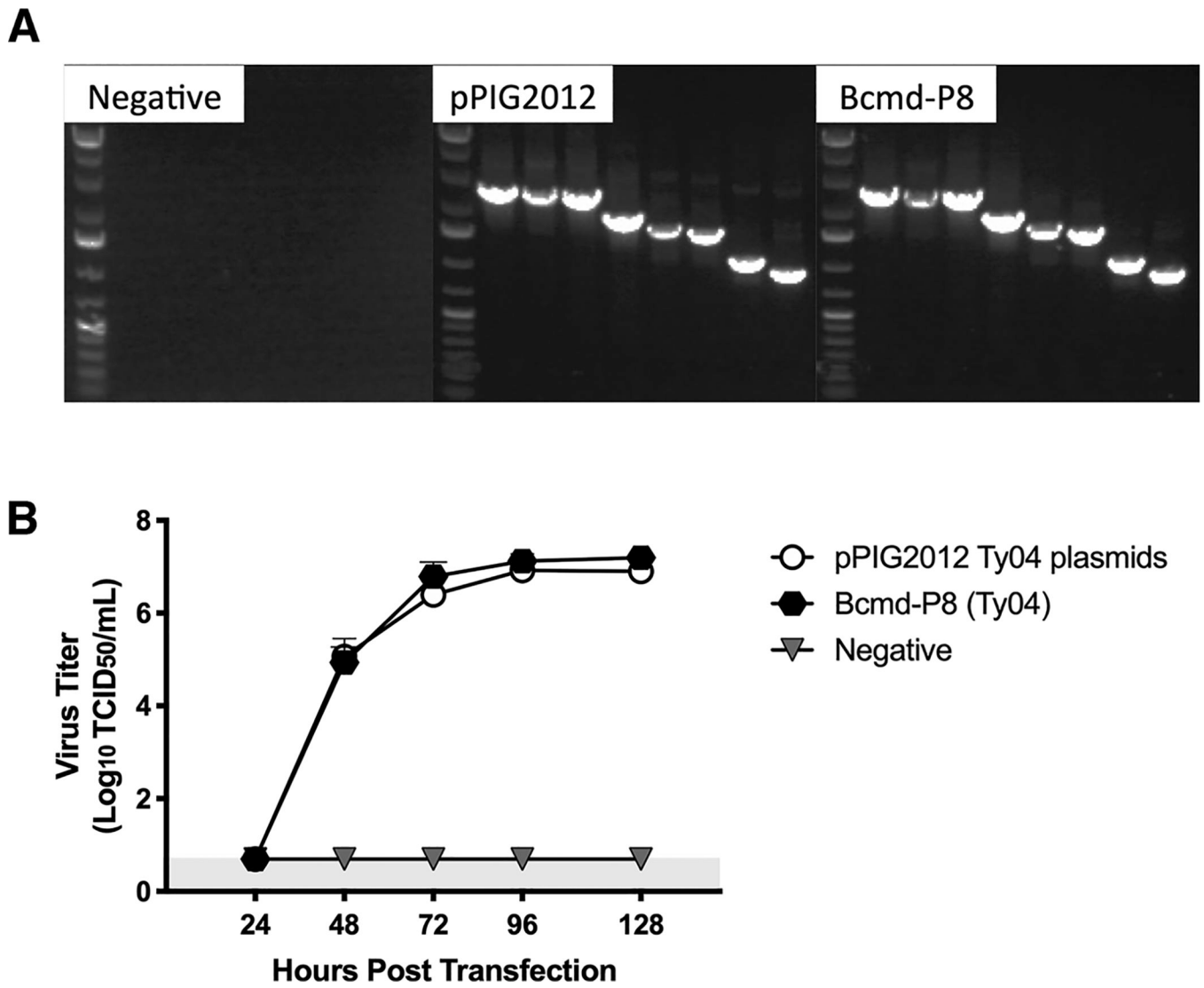

Fig. 5. Bacmid stability and rescue of Ty04 from pΔFast-P8 (Ty04).

A) PCR amplification of gene segments using segment specific primers shows the presence of full-length segments for all 8 genes in the pΔFast-P8 (Ty04) bacmid clone. B) Rescue kinetics of pΔFast-P8 and the 8-plasmid system for Ty04 in PK(15)/MDCK co-culture and a 1:4 ratio of cells. Negative control was Ty04 8 plasmid system lacking the PB1 segment.

Table 1

Primers used to clone IAV Ty04, SGML, and CA04 into pPIG2012.

Gene	Virus Strain	RE	Forward Primer		Reverse Primer	
PB2	Ty04, SGML, CA04	BsaI	Ba-PB2 1F	TATT*GGTCTC*AGGGAGCGAAAGCAGGTC	BaPIG-PB2 2341R	ATAT*GGTCTC*GAGATAGTAGAAACAAGGTCGTTT
PB1	Ty04, SGML, CA04	BsmBI	Bm-PB1 1F	TATT*CGTCTC*AGGGAGCGAAAGCAGGCA	BmPIG-PB1 2341R	ATAT*CGTCTC*GAGATAGTAGAAACAAGGCATTT
PA	Ty04	BsaI	Ba-PA-1F	TATT*GGTCTC*AGGGAGCGAAAGCAGGTAC	B2PIG-PA 2233R	ATAT*GGTCTC*GAGATAGTAGAAACAAGGTACTT
	SGML	AarI	Aar_PA_1	TATT*CACCTGC*CTCAGGGAGCGAAAGCAGGTAC	AarPIG_PA_1R	ATAT*CACCTGC*CTCAAGATAGTAGAAACAAGGTACTT
	CA04	BtgZI	BtgZI_PA_1F	TATT*GCGATG*ATATCGTGGTGGGAGCGAAAGCAGGTAC	BtgZI_PA_1R	ATAT*GCGATG*ACTAGATCTAAGATAGTAGAAACAAGGTACTT
HA	Ty04	AarI	Aar-HA 1F	TATT*CACCTGC*CTCAGGGAGCAAAAGCAGGGG	AarPIG-NS 890R	
	SGML, CA04	BsmBI	Bm-HA-1F	TATT*CGTCTC*AGGGAGCAAAAGCAGGGG	BmPIG-NS 890R	
NP	Ty04, SGML, CA04	BsaI	Ba-NP-1F	TATT*GGTCTC*AGGGAGCAAAAGCAGGGTA	BaPIG-NP 1565R	ATAT*GGTCTC*GAGATAGTAGAAACAAGGGTATTTTT
NA	Ty04, CA04	BsmBI	Bm-NA 1F	TATT*CGTCTC*AGGGAGCAAAAGCAGGAGT	BmPIG-NA 1413R	ATAT*CGTCTC*GAGATAGTAGAAACAAGGAGTTTTTT
	SGML	BsmBI	BmN3–1	TATT*CGTCTC*AGGGAGCAAAAGCAGGTGC	BmPIG_N3_1420R	ATAT*CGTCTC*GAGATAGTAGAAACAAGGTGCTTTTT
M	Ty04, SGML, CA04	BsmBI	Bm-M-1F	TATT*CGTCTC*AGGGAGCAAAAGCAGGTAG	BmPIG-M 1027R	ATAT*CGTCTC*GAGATAGTAGAAACAAGGTAGTTTTT
NS	Ty04	AarI	Aar-NS 1F	TATT*CACCTGC*ATCGGGGAGCAAAAGCAGGGTG	AarPIG-NS 890R	ATAT*CACCTGC*TATGAGATAGTAGAAACAAGGGTGTTTT
	SGML, CA04	BsmBI	Bm-NS-1F	TATT*CGTCTC*AGGGAGCAAAAGCAGGGTG	BmPIG-NS 890R	ATAT*CGTCTC*GAGATAGTAGAAACAAGGGTGTTTT

Primer sequences used to amplify eight gene segments from IAV Ty04, SGML, and CA04 for cloning into pPIG2012. Shown in italics is the restriction enzyme recognition sequence and in bold is the primer sequence that encodes the 12 conserved and 13 conserved nucleotides on the 5′ and 3′ end of the cDNA. Sequences specific for individual genes are underlined. 5′ of all conserved sequences is a BsmBI (Bm), BsaI (Ba), AarI (Aar) or BtgZI restriction site (RE) for cloning.

Table 2

Primers used to clone IBV B/Bris into pPIG2012.

Gene	RE	Forward Primer		Reverse Primer	
PB2	BsmBI	Bm-PB2b-1F_AK	TATT*CGTCTC*AGGG**AGCAGAAGCGGAGCGTTTTC**	pPig_B_Bris_PB2_R_AK	ATAT*CGTCTC*GAGAT**AGTAGAAACACGAGCATTTTTCACTC**
PB1	BsmBI	Bm-PB1b-1	TATT*CGTCTC*AGGG**AGCAGAAGCGGAGCCTTTAAGATG**	pPig_B_Bris_PB1_R_AK	ATAT*CGTCTC*GAGAT**AGTAGAAACACGAGCCTTTTTTCAT**
PA	BsmBI	Bm-Pab-1	TATT*CGTCTC*AGGG**AGCAGAAGCGGTGCGTTTGA**	pPig_B_Bris_PA_R_AK	ATAT*CGTCTC*GAGAT**AGTAGAAACACGTGCATTTTTGATTC**
HA	BsmBI	MDV-B 5′ BsmBI-HA	TATT*CGTCTC*AGGG**AGCAGAAGCAGAGCATTTTCTAATATC**	pPig_B_Bris_HA_R_AK	ATAT*CGTCTC*GAGAT**AGTAGTAACAAGAGCATTTTTCAAT**
NP	BsaI	Ba-NPb-1F_AK	TATT*GGTCTC*AGGG**AGCAGAAGCACAGCATTTTCTTGTG**	pPig_B_Bris_NP_R_AK	ATAT*GGTCTC*GAGAT**AGTAGAAACAACAGCATTTTTTACAT**
NA	BsmBI	Bm-Nab-1	TATT*CGTCTC*AGGG**AGCAGAAGCAGAGCA**	pPig_B_Bris_NA_R_AK	ATAT*CGTCTC*GAGAT**AGTAGTAACAAGAGCATTTTTCAG**
M	BsaI	Ba-Mb-1F_AK	TATT*GGTCTC*AGGG**AAGCAGAAGCACGCAC**	pPig_B_Bris_M_R_AK	ATAT*GGTCTC*GAGAT**AGTAGAAACAACGCACTTTTTCC**
NS	BsmBI	MDV-B 5′ BsmbI-NS F	TATT*CGTCTC*AGGG**AGCAGAAGCAGAGGATTTGTTTAGTC**	pPig_B_Bris_NS_R_AK	ATAT*CGTCTC*GAGAT**AGTAGTAACAAGAGGATTTTTATTTT**

Primer sequences used to amplify eight gene segments from IBV B/Bris for cloning into pPIG2012. Shown in italics is the restriction enzyme recognition sequence and in bold is the primer sequence that encodes the 12 conserved and 13 conserved nucleotides on the 5′ and 3′ end of the cDNA. Sequences specific for individual genes are underlined. 5′ of all conserved sequences is a BsmBI (Bm) or BsaI (Ba) restriction site for cloning.

Table 3

Rescue of IBV using pPIG2012 and pDP2002 in PK-15/MDCK and HEK293 T/MDCK co-culture cells.

Plasmid Vector	Cell Type	hpt	$TCID_{50}$/mL (Log10)
pPIG2012	PK(15)/MDCK	120	6.39 ± 0.16^{a}
pDP2002	PK(15)/MDCK	120	7.23 ± 0.28^{b}
pPIG2012	HEK293 T/MDCK	120	$2.93 \pm 0.63^{\dagger}$
pDP2002	HEK293 T/MDCK	120	$6.42 \pm 0.72^{\ddagger}$

Co-cultures of PK-15/MDCK (5:2) or HEK293 T/MDCK (3:1) were transfected with plasmids encoding B/Bris PB2, PB1, PA, HA, NP, NA, M and NS in pPig2012 or pDP2002 vector overnight (14 h) using TransIT transfection reagent. The media was replaced and then supplemented with TPCK-treated trypsin at 30 hpt and 72 hpt. Cell culture supernatants were collected at 120 hpt for the quantification of virus titers by $TCID_{50}$ assays using the Reed-Muench method. Data represents means ± standard errors. Multiple t tests with correction for multiple comparisons using Holm-Sidak method was performed to calculate significance between the two groups in each cell line co-culture. Superscript of letters or symbols indicate that the mean comparison between those two groups is significantly different.