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Establishment and characterization of a Madin-Darby canine kidney reporter cell line for influenza A virus assays

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ABSTRACT

Influenza virus diagnosis has traditionally relied on virus isolation in chicken embryo or cell cultures. Many laboratories have adopted rapid molecular methods for detection of influenza viruses and discontinued routine utilization of the relatively slow viral culture methods. We describe an influenza A reporter cell line that contributes to more efficient viral detection in cell culture. Madin-Darby canine kidney (MDCK) cells were engineered to constitutively produce an influenza genome-like luciferase reporter RNA driven by the canine RNA polymerase I promoter. Induction of high level of luciferase activity was detected in the Luc9.1 cells upon infection with various strains of influenza A virus including 2009 H1N1-pandemic and highly pathogenic H5N1 virus. In contrast, infection with influenza B or human Adenovirus type 5 did not induce significant levels of reporter expression. The reporter Luc9.1 cells were evaluated in neutralizing antibody assays with convalescent H3N2 ferret serum yielding a neutralization titer comparable to that obtained by the conventional microneutralization assay suggesting that the use of the reporter cell line might simplify neutralization assays by facilitating the establishment of infectious virus endpoints. Luc9.1 cells were also used to determine the susceptibility of influenza A viruses to a model antiviral drug. The equivalence with conventional antiviral assay results indicated that the Luc9.1 cells could provide an alternative cell-based platform for high throughput drug discovery screens. In summary, the MDCK-derived Luc9.1 reporter cell line is highly permissive for influenza A virus replication and provides a very specific and sensitive approach for simultaneous detection and isolation of influenza A viruses as well as functional evaluation of antibodies and antiviral molecules.

INTRODUCTION

Influenza viruses cause respiratory tract infections associated with substantial morbidity and mortality. Seasonal influenza epidemics affect between 5 and 15% of the world population, causing 3-5 million cases of severe disease and approximately 0.5 million deaths per year (54). Influenza pandemics have also caused sporadic large-scale morbidity and mortality in the past century (15). Type A influenza viruses are responsible for most of the influenza disease burden in human populations (46). A novel H1N1 virus emerged in 2009 caused a still ongoing pandemic with excess morbidity and mortality(52). The genome of influenza A viruses consists of eight negative-sense RNA molecules (35) with highly conserved termini comprising the core promoter for transcription and replication (9, 22, 30, 56). Each viral RNA segment associated with nucleoprotein (NP) and RNA polymerase subunits (PB2, PB1 and PA) forming ribonucleoprotein (RNP) complexes.

Clinical and public health reference laboratories generally rely on embryonated chicken eggs or cell cultures of mammalian origin for isolating and propagating influenza viruses (41, 43, 47). However, culture of subtype H3N2 viruses from clinical specimens by inoculation into eggs is becoming increasingly problematic; currently very few specimens yield an isolate (34). Viral isolation in cell cultures is handicapped by the relatively longer times required to obtain test results (5-7 days) and substantial requirements for specialized materials, equipment and labor (42), although culture systems such as R-Mix cells provide results faster (1-2 days) (2, 10) . Cell lines expressing reporter genes inducible upon viral infection could mitigate this problem (23, 24, 32, 33). These reporter cells exploit the specificity of viral transcription factors for their target promoters in combination with the extreme sensitivity of reporter enzymes such as luciferase (31). This approach expedites detection of specific viruses and amplifies the virus

present in the clinical specimen, providing a live virus stock to be stored for further analyses. However, reporter cell lines have not been widely used for influenza, perhaps because the available HEK-293T reporter cells are not a favored substrate for virus isolation due to their susceptibility to the toxic effects of trypsin, which is required for the production of infectious influenza viruses in cultured cell lines (16, 20). MDCK cells have become a most widely used substrate for isolation of influenza viruses since they are known to be highly permissive for propagation of influenza viruses (47) and resistant to the toxic effects of trypsin supplementation .

Some clinical virology laboratories continue to isolate influenza viruses in cultured cells but the faster immunochromatographic or EIA (enzyme immunoassay)-based devices or PCR are most widely used for laboratory diagnosis of influenza (8, 17, 19, 28, 36, 44, 48, 55). This trend has become problematic for influenza surveillance programs because virus isolates are indispensable for monitoring antigenic drift, vaccine seed development and drug sensitivity testing (12, 13, 34). In addition, phenotypic analyses of viral isolates are critical to fully interpret results from vaccine and antiviral effectiveness clinical trials (3). We report here on the development of a cell-based reporter system for influenza A virus using highly permissive MDCK cells expressing a luciferase-encoding amplicon controlled by canine polymerase I promoter elements. This cell-based reporter system provides a sensitive method for the detection and isolation of influenza A viruses and it is also useful for the screening of antiviral drugs or neutralizing antibody assays.

MATERIALS AND METHODS

Cells and viruses

Madin-Darby canine kidney-London (MDCK-London) cells were obtained from CDC Scientific Resources Program and human lung adenocarcinoma (A549; CCL-185) cells from the American Type Culture collection (Manassas, VA), cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA), containing 10% fetal bovine serum, 1% penicillin/streptomycin and incubated at 37ºC in a humidified CO_2 incubator. Influenza type A viruses, subtype H1N1 (A/WS/33, A/WSN/33, A/swine/Missouri/001187/06, A/Ohio/83, A/Solomon Islands/6/06, A/New Caledonia/20/99, A/Brisbane/59/07), 2009 pandemic H1N1 (A/New York/18/09, A/New York/4/09, A/Ohio/7/09, A/Michigan/10/09), H2N2 (cold adapted A/Ann Arbor/6/60)(26), H3N2 (A/swine/Minnesota/001170/06, A/Memphis/102/72, A/Wisconsin/67/05), H3N8 (A/equine/Montana/07, A/canine/Florida/43/04), highly pathogenic H5N1 (A/Vietnam/1203/04, A/Vietnam/JP 12-2/05, A/Hong Kong/213/03), H9N2 (A/turkey/Wisconsin/66), and type B viruses (B/Jiangsu/10/03, B/Brisbane/33/2008) were used in this study. Virus stocks were prepared in 10-day-old embryonated eggs or in MDCK cell culture as indicated. Influenza titers were determined by plaque assay or endpoint dilution on MDCK cells whereas Adenovirus titers were determined by plaque assay on A549 cells.

Plasmids and construction of reporter vectors.

pSV-Luc was obtained from Promega (Madison, WI). Plasmids expressing PB2, PB1, PA, and NP proteins of influenza A/WSN/33 were described previously (37). The canine RNA polymerase I (POL-I) promoter reporter plasmid was constructed by replacing the DNA region encoding chloramphenicol acetyltransferase (CAT) gene in pCAT3-basic vector (Promega, Madison, WI) with the region encoding Renilla luciferase (Rluc) gene flanked by non-coding

regions (NCR) from the NP segment of influenza A/WSN/33 with minor modification, as described previously (25, 30). To generate a viral RNA-like RLuc amplicon under the control of POL-I, the canine POL-I promoter and terminator sequences flanking RLuc were fused upstream to the 5' NCR or downstream to the 3' NCR, respectively (Figure 1A). For selection of stable transfectant cells expressing the reporter amplicon, constitutively active transcriptional cassettes expressing neomycin phosphotransferase gene and enhanced green fluorescent protein (EGFP; derived from pEGFP-N1, Clontech, Mountain View, CA) were inserted into the pk9POLI-RLuc vector, resulting in pk9POLI-RLuc-NeoGFP.

Selection of stable transfectant MDCK cell lines

For transient expression of the reporter amplicon and/or viral ribonucleoprotein (PB2, PB1, PA, and NP of WSN/33 virus), 10^6 MDCK cells seeded in each well of 6-well cluster plate were transfected with 2.0 µg of each plasmid using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions (37). Reporter plasmid pSV-Luc (Promega, Madison) that constitutively expresses firefly luciferase was co-transfected (0.5 µg/ well) as needed to normalize transfection efficiency. Twenty four hours after the transfection, cells were rinsed with phosphate buffered saline (PBS) and maintained in culture with fresh medium. Renilla luciferase activities were measured in lysates from cells harvested at 48 hours after transfection.

Transiently transfected cells were inoculated with influenza viruses (multiplicity of infection, MOI = 0.001) at 24 hours after transfection. After virus adsorption for 1 hour, cells were washed with PBS and then incubated for additional 24 hours in Opti-MEM media (Invitrogen) containing 0.5µg/ml TPCK (tosylsulfonyl phenylalanyl chloromethyl ketone)-

trypsin (Sigma, St. Louis, MO), followed by dual-luciferase reporter assays (Promega) or fluorescence microscopy analysis.

For development of stable MDCK transfectant cells that constitutively produce the viral RNA-like RLuc amplicon transcripts, 10 µg of linearized pk9POLI-RLuc-NeoGFP reporter plasmids DNA was transfected into MDCK cells as described above. The stably transfected cells were selected by adding 500 µg/ml of G418 (Invitrogen, Carlsbad, CA) to the culture medium with medium replacement every 3 days for a total of two weeks. G418-resistant cell colonies were detached from the dishes and cloned twice by use of cloning cylinders. Candidate cell clones were infected with A/WS/33 at an MOI of 0.01 to examine reporter gene inducibility by infection. Luciferase levels were measured at 24-72 hours post infection as appropriate.

Induction of luciferase expression by virus infection in Luc9.1 cell line.

A stable MDCK transfectant cell clone expressing the k9POLI-RLuc amplicon, termed Luc9.1, was cultured in 100uL DMEM (10^4 cells/well, 96 well cluster plate) supplemented with 10% FBS and 500µg/mL G418. Infections were performed 24 hours later, by rinsing the monolayers with PBS followed by inoculation with 100µL Opti-MEM containing 0.5µg/mL TPCK trypsin and the appropriate amounts of virus (e.g. A/Ohio/83; within desired multiplicity of infection range). Twenty four hours after infection, the cell culture medium containing amplified virus was collected and stored for subsequent studies whereas cell monolayers were rinsed once with PBS, and harvested by lysis with Renilla luciferase detection reagent (Promega, Madison, WI) according to the manufacturer's instruction. For the time-course study, Luc9.1 cells were infected with 10 PFU A/Ohio/83 (H1N1), A/Wisconsin/67/05(H3N2) or B/Jiangsu/10/03 viruses (MOI 0.001) and cell monolayers were harvested at 24, 48 and 72 hours post infection for

Renilla luciferase activity measurements. Luminescence intensity was measured with a Centro LB960 luminometer (Berthold, Germany) or a Victor multilabel reader (Perkin-Elmer, MA). All experiments with live highly pathogenic H5N1 viruses were performed in biosafety level 3 containment, including enhancements required by the U.S. Department of Agriculture and the Select Agents program http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm .

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Antibody neutralization assay

Normal or convalescent (A/Sydney/5/97 (H3N2)) ferret sera were prepared as described previously and treated with receptor-destroying enzyme (RDE) (4, 53). Serial two-fold dilutions of RDE-treated antiserum were carried out in Opti-MEM. A viral inoculum of 1,000 PFU diluted in Opti-MEM was added to each antibody dilution and incubated at 37ºC for 1 hour with brief gentle mixing at 15 min intervals. Luc 9.1 cells in 96 well plates were rinsed with PBS and virus-antibody mixtures supplemented with 0.5µg/mL TPCK-treated trypsin were then added to the wells followed by incubation at 37ºC. Intracellular Renilla luciferase activity was measured at 24 hours after inoculation using a Renilla luciferase assay system according to the manufacturer's instructions. Conventional ELISA-based microneutralization assay was performed as described by Rowe (40).

Antiviral activity assay

Luc 9.1 cells cultured in 96 well plates as described above were rinsed twice with PBS and cultured in 50 µL Opti-MEM containing the appropriate concentration of amantadine hydrochloride (Sigma, St. Louis, MO) for 30 min at 37ºC. Next, a volume of 50µL Opti-MEM

containing 1000 PFU of the appropriate virus and 0.5µg/mL TPCK trypsin was added to the well and the plate was incubated at 37ºC. Luciferase activity was measured at 24 hours after inoculation. Alternatively, parental MDCK cells were infected with viruses as described above. The cell culture supernatant was collected at 48 hours after infection, and virus titer was determined by hemagglutination assay using 0.5% turkey red blood cells. IC_{50} values , calculated from GraphPad Prism software, represent the concentration of drug that was required to inhibit virus-induced reporter activity to 50% of untreated cultures.

RESULTS

Functional characterization of an influenza A reporter amplicon in MDCK cells.

A human embryonic kidney cell line (HEK-293) was previously used as a platform to develop a reporter cell system based on a negative-sense RNA amplicon comprising a luciferase coding region flanked by the NCR (non-coding regions) of influenza genomic RNA segments (24). In this study, we aimed to develop a reporter cell system based on the Madin-Darby canine kidney (MDCK) cell line because it is a preferred substrate for influenza virus isolation in many clinical and research laboratories. Previous studies demonstrated that the widely used human RNA polymerase I (POL-I) promoter is virtually inactive in canine cells and most non-primate cells (29, 49). To circumvent this restriction, we engineered an influenza NCR-luciferase reporter amplicon driven by the canine POL-I promoter (Fig. 1A). The reporter function of the resulting pk9POL-I plasmid was evaluated by transient transfection along with four other plasmids expressing the influenza A proteins required for viral transcription (PB2, PB1, PA and NP) (25). Transfected MDCK cells yielded 8,000-fold higher Renilla luciferase activity than control cells (without influenza RNP expression; Fig. 1B). To determine whether luciferase

reporter activity was also induced by viral infection, MDCK cells transfected 24 hours earlier with pk9POLI-RLuc plasmid were infected with a panel of influenza A viruses (MOI= 0.001) of different subtypes. Viral infection induced increased luciferase expression between 100-fold and 20,000 fold in the transfected MDCK cells ($p<0.05$) (Fig. 1C) indicating that the reporter amplicon is highly specific and responsive to influenza A infection (Fig. 1C and data not shown).

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Development of influenza reporter MDCK cell line Luc 9.1

In order to establish a reporter cell line for rapid isolation and detection of replicating influenza A virus, MDCK cells were transfected with an influenza luciferase reporter plasmid (pk9POLI-RLuc-NeoGFP) containing neomycin phosphotransferase and EGFP expression cassettes. Twenty four cell colonies were individually recovered using cloning cylinders from cultures in G418 selective medium, which kills all cells that do not carry the transfected plasmid. The resulting cell clones were examined by fluorescence microscopy to determine expression of GFP indicative of plasmid retention in the cells and also tested for induction of luciferase activity by influenza A virus infection. Clone Luc9.1 was selected for further characterization because it showed the highest luciferase inducibility upon virus infection (data not shown).

Constitutive expression of an influenza amplicon RNA in the Luc 9.1 cells might trigger host cell antiviral responses that could inhibit viral replication (45). In addition, the many population doublings (~ 40) required for cloning from the original transfectant could reduce cellular permissiveness to influenza virus infection (14). To assess the susceptibility of the Luc9.1 reporter cell line to influenza viruses, we analyzed the plaquing efficiency and morphology of A/Ohio/83 (H1N1), A/Wisconsin/67/05 (H3N2) and B/Jiangsu/03 viruses on Luc 9.1 cells. No significant differences in plaque number, size and morphology were noted between

Luc 9.1 and parental MDCK cells, suggesting that amplicon expression and cloning procedures did not compromise their permissiveness for influenza virus replication (data not shown).

To determine the influenza type specificity of luciferase induction in the Luc9.1 reporter cell line, we infected 10^4 cells with 1000 PFU of A/Ohio/83 (H1N1), A/Wisconsin/67/05(H3N2) or B/Jiangsu/10/03 viruses at MOI of ~0.1. Luciferase reporter induction by A/Wisconsin/67/05 infection (121-fold increase; $p<0.005$) was similar to that of A/Ohio/83 virus (124-fold; $p<0.005$) at 24 hours after infection (Fig. 2A), while influenza B virus infection failed to induce significant reporter levels (2-fold). Luciferase induction by adenovirus, which is often detected in clinical specimens from patient with respiratory illness, was only 1 to 2-fold when infected with an infection dosage of 10^2 to 10^5 PFU (data not shown).

To determine the lower limit of virus detection by the Luc9.1 cells at 24 hours after infection, we conducted an infection dose-response study with H1N1 influenza A virus. The maximum induction (110 fold) of luciferase activity was detected in cells infected with 400 PFU (MOI of ~0.04, Fig. 2B) of A/Ohio/83. Significant luciferase expression was still detectable with an infectious dose of 16 PFU (MOI of ~0.0016) ($p<0.005$). These results suggest that the stable reporter cell line Luc9.1 is highly sensitive for rapid detection, within 24 hours, of low doses of infectious influenza A virus.

Luciferase levels from Luc 9.1 cultures infected with ~16 PFU A/Ohio/83 were moderately increased (~ 8 fold) (Fig. 2B), suggesting that longer incubation times may allow further viral replication and improve the reporter activity. Luc 9.1 cells infected with 10 PFU of A/Ohio/83 (MOI of 0.001), revealed a 60-fold induction at 48 hours post infection (Fig. 3). Similarly, A/Wisconsin/67/05 (H3N2) infection yielded a 48-fold increase. A slight increase of luciferase activity was measured at 72 hours post infection. However, the increased background

activity at 72 hours results in similar or lower levels of induction; 46-fold for A/Ohio/83 and 33 fold for A/Wisconsin/67/05 (Fig. 3). These results indicated that with longer incubation, theLuc9.1 reporter cell line can detect as few as 10 PFU (MOI: 0.001) of influenza A virus from the two circulating influenza subtypes.

Next, we tested a set of 2009 pandemic H1N1 viruses and highly pathogenic avian H5N1 isolates in the Luc9.1 cells (Fig.4). Cells were inoculated at MOI of 0.01 and luciferase activity was measured after 24 hours of incubation. We detected a 20 to 1200-fold increase of luciferase activity depending on the virus isolates (Fig.4A). Relatively high induction of luciferase activity is evident upon pandemic H1N1 virus infection. Infection to Luc9.1 cells with highly pathogenic H5N1 virus with a MOI of 0.01 induced 287 to 1041-fold luciferase activity within 24 hours of infection (Fig.4B). These results corroborate the permissiveness of the Luc9.1 cell line to various subtypes of influenza A virus and its potential as an alternative method to detect influenza A virus infection.

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Antibody neutralization and drug sensitivity assays

The most widely used methods to measure the specificity and potency of antiviral antibodies are neutralization assays that establish endpoints for reduction of plaque numbers or proportion of infected cultures in microtiter plates. However, plaque assays are extremely laborious and cannot be automated. Similarly, the 96-well microtiter plate assay is complicated by the need to perform a secondary assay such as ELISA to establish virus neutralization endpoints (40). Both methods pose a significant challenge especially for studies involving large number of sera and multiple virus strains. We reasoned that the Luc9.1 MDCK cells could greatly simplify neutralizing antibody assays in clinical or research samples by facilitating the

establishment of infectious virus endpoints. In this study, we performed a neutralization assay by the constant virus-variable serum approach. Convalescent H3N2 ferret serum dilutions were incubated with 1000 PFU of A/Sydney/5/97(H3N2) virus and the presence of infectious virus was tested by inoculating these mixtures onto multiple cultures of Luc9.1 cells seeded in 96 well plates. Luc9.1 cell lysates revealed luciferase activity at high serum dilutions due to absence of virus neutralization by antibody. In contrast, no induction of reporter activity was recorded at low serum dilutions, indicative of absence of virus infectivity due to neutralization (Fig. 5). Complete suppression of reporter activity was detected at dilutions of the H3N2 ferret serum of 1 in 5,120 or lower. This neutralization titer was comparable to that obtained by the conventional microneutralization assay (data not shown) (40) The H3N2 specificity of the ferret antisera was supported by the presence of high luciferase activity in Luc9.1 cells inoculated with A/Ohio/83 (H1N1) pre-incubated with ferret anti-H3N2 antiserum at dilutions of 320 or lower. These results indicated that the Luc 9.1 MDCK cells provide a promising alternative to quantify neutralizing antibodies in biological samples.

To investigate whether Luc 9.1 MDCK cells are suitable to determine the susceptibility of influenza viruses to antiviral drugs, we used amantadine hydrochloride, a blocker of influenza virus M2 protein ion-channel as a model. Luc9.1 MDCK cells pre-incubated with 5µM amantadine were infected with 1,000 PFU of A/Ohio/83, A/Sydney/5/97 (amantadine sensitive), or A/Wisconsin/67/05 virus, which carries an amantadine-resistance mutation in M2. Luciferase activity was suppressed more than 40-fold for the amantadine-sensitive viruses whereas A/Wisconsin/67/06 infection induced high levels of luciferase activity in treated Luc9.1 cells (Fig 6A). To determine the median inhibitory concentration (IC_{50}) of amantadine for A/Ohio/83

virus, different drug concentrations were pre-incubated with Luc9.1 MDCK cells. Cells treated with 0.625 µM amantadine showed 80% reduction in luciferase activity at 24 hours after A/Ohio/83 infection (Fig. 6B). However, further increases in amantadine concentration resulted in modest additional suppression of luciferase activity. The IC_{50} results from Luc9.1 cells (~0.4 µM) were similar to those derived by the conventional hemagglutination titer reduction assay (Fig.6C), indicating that the Luc9.1 cell line is a suitable reporter for antiviral sensitivity assays (6, 18).

DISCUSSION

Reporter cell lines expressing viral RNA-like amplicons provide an alternative approach to detect and isolate a variety of RNA viruses from diverse specimens (21). The sensitivity of reporter assays such as luciferase reduce testing turnaround times. In addition, the simplicity of these assays relative to ELISA or realtime PCR reduces labor, supplies and space requirements. Despite these advantages, reporter cell lines have not been widely used for detection and isolation of influenza viruses (24). The high susceptibility of HEK-293T to trypsin toxicity may have discouraged the use of these reporter cells for influenza diagnosis. Multi-cycle replication of influenza A viruses requires trypsin to cleave the HA of newly produced virions and enable a new round of infection (20). In the absence of proteases to cleave the HA, influenza reporter cell systems can only support a single round of infection, reducing their sensitivity. Thus, such systems are expected to require larger amounts of infectious virus to induce reporter gene expression.

In this study, we developed an alternative cell-based influenza reporter system based on the MDCK cells since it is compatible with trypsin supplementation and reportedly a most

328 permissive substrate for isolation and propagation of human influenza viruses (39). To this end,
329 we first developed a plasmid that produces a transcript resembling an influenza genome segment
330 directed by the strong constitutive canine RNA polymerase I promoter (25, 29, 38). This reporter
331 plasmid was then modified to include a mammalian cell drug-selection marker and used to
332 identify a stable and sensitive influenza reporter cell line. Further characterization of the
333 resulting Luc9.1 reporter cell line showed excellent sensitivity for isolation and detection of
334 influenza A virus in samples with as few as 10 PFU with extended test time. These data
335 represents a 1000-fold gain in sensitivity relative to the previously reported 293T cell-based
336 reporter system, providing improved signal-to-noise ratios for diagnosis (24). Diagnosis of
337 influenza A virus using Luc9.1 cells can be achieved as early as 24 hours post infection with a
338 virus inoculum of approximately 16 PFU.

339 Luc9.1 cells also provide an approach to simplify the detection of neutralizing antibodies
340 in clinical specimens or research samples. The most widely used neutralization protocol with
341 authentic influenza viruses relies on a laborious ELISA test (40). Replacement of conventional
342 MDCK cells with the Luc9.1 cells eliminates the lengthy ELISA procedure for virus detection.

343 The MDCK reporter cell line generated in this study provides an alternative platform for
344 high throughput virus detection assays and a highly attractive anti-viral assay for influenza virus
345 drug discovery. The recent emergence of drug resistant viruses, either spontaneously or after
346 therapy underscores for the need to identify novel antiviral drugs effective against influenza (7).
347 In vivo cell-based screening approaches may yield broad anti-viral lead compounds that target
348 virus entry, endocytosis, and replication for further development.

349 In summary, the MDCK reporter cell line established in this study is highly permissive
350 for influenza virus replication and provides a highly specific and sensitive approach for

simultaneous detection and isolation of influenza viruses. Simplified neutralization assays and high throughput anti-viral drug screening can also be implemented using this reporter cell system.

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FIGURE LEGENDS

Figure 1. Functional analysis of the influenza reporter amplicon with a canine polymerase I promoter in MDCK cells. (A) The negative-sense Renilla luciferase [R-LUC(-)] coding region is flanked by modified non-coding regions (NCR, hatched box) from the nucleoprotein gene of A/WSN/33 virus (24, 30). The sequences of the canine POL-I promoter and the canine Pol-I terminator (k9POL-I and k9TI, gray box) were fused upstream to the 5' NCR or downstream to the 3' NCR, respectively. (B) MDCK cells were co-transfected with reporter plasmid pk9POLI-RLuc and influenza RNP expressing plasmids, or control plasmid. (C) MDCK cells transfected with pk9POLI-RLuc plasmid for 24 hours were infected with influenza viruses at MOI of 0.001. Luciferase activities in whole cell lysates collected at 24 hours post infection are shown as average of luciferase activity of cells from three independent wells. Values shown are the Renilla firefly activities from 10^4 cells after normalization using firefly luciferase expression from a co-transfected plasmid to account for variation in transfection efficiency. Error bars depict standard error, brackets denote p values from Student t test: * $p>0.9$, ** $p<0.05$, *** $p<0.005$.

Figure 2. Induction of luciferase activity in the reporter Luc 9.1 cells upon influenza virus infection. (A) Specificity of luciferase activation in Luc9.1 reporter cells. Luc 9.1 cells were infected with A/Ohio/83 (H1N1), A/Wisconsin/67/05 (H3N2) or B/Jiangsu/10/03 viruses at MOI of 0.1. (B) Detection limits of Luc9.1 cells upon influenza virus infection. Luc9.1 cells were inoculated with the indicated amounts of infectious A/Ohio/83 virus. Luciferase activity in each culture was measured in lysates harvested at 24 hours post infection. Data represent average luciferase activity of 10^4 cells from three independent wells. Error bars depict standard error, brackets denote p values from Student t test; * denotes $p <0.005$.

Figure 3. Time course of luciferase activity induction in the reporter Luc9.1 cells upon influenza virus infection. Luc 9.1 cells were inoculated with A/Ohio/83(H1N1), A/Wisconsin/67/05 (H3N2) or B/Jiangsu/10/03 with an MOI 0.001. Renilla luciferase activity was measured at different times after infection. Data represent normalized luciferase activity of 10^4 cells from three independent wells.

Fig 4. Luciferase activity in Luc9.1 cells upon infection with seasonal, avian and 2009 pandemic H1N1 influenza A viruses.

(A) Luc9.1 cells were infected with the seasonal H1N1, H3N2, pandemic H1N1 or virus isolated from animal species with the MOI of 0.01. Renilla luciferase activity was measured at 24 hours post infection. Error bars depict standard error, brackets denote p values from Student t test: * $p<0.001$. (B) Luc9.1 cells were inoculated with highly pathogenic H5N1 (A/Vietnam/1203/04, A/Vietnam/JP 12-2/05, A/Hong Kong/213/03) with an MOI of 0.01. Renilla luciferase activity was measured at 24 hours post infection. Error bars depict standard error, brackets denote significance per Student t test: * $p<0.05$.

Figure 5. Performance characteristics of the Luc 9.1 cells in a virus neutralization assay. Two-fold dilutions of convalescent A/Sydney/5/97 ferret antiserum were incubated with 1000 PFU of H1N1 (A/Ohio/83), or H3N2 (A/Sydney/5/97) viruses. Intracellular Renilla luciferase was measured 24 hours after infection. Data represent normalized luciferase activity of 10^4cells from three independent wells. Error bars depict standard error, brackets denote p values from Student t test: * $p<0.05$.

Figure 6. Performance characteristics of the Luc9.1 cells in antiviral drug assays. (A) Amantadine resistant virus (A/Wisconsin/67/05) and amantadine sensitive viruses (A/Sydney/5/97 and A/Ohio/83) were incubated with 5 µM amantadine and added to Luc9.1 cells. Luciferase activity was measured at 24 hours post infection. (B,C) A/Ohio/83 virus was incubated with different concentrations of amantadine and added to (B) Luc9.1 cells, and (C) parental MDCK cells. Luciferase activity was measured 24 hours post-infection from Luc 9.1 cells. MDCK cell supernatants were collected 48 hours later for HA titration. Data represent normalized luciferase activity of 10^4 cells from three independent wells. Error bars depict standard error, brackets denote p values from Student t test; * $p<0.001$.

Fig.1

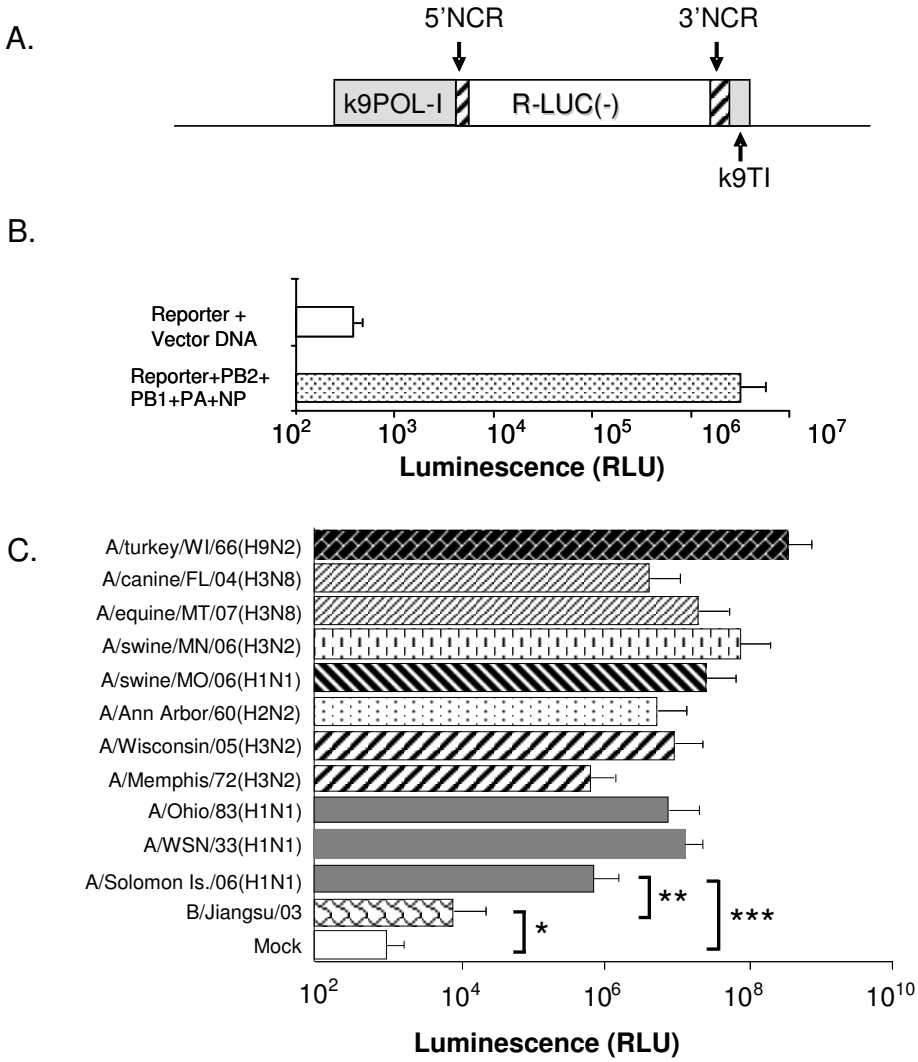
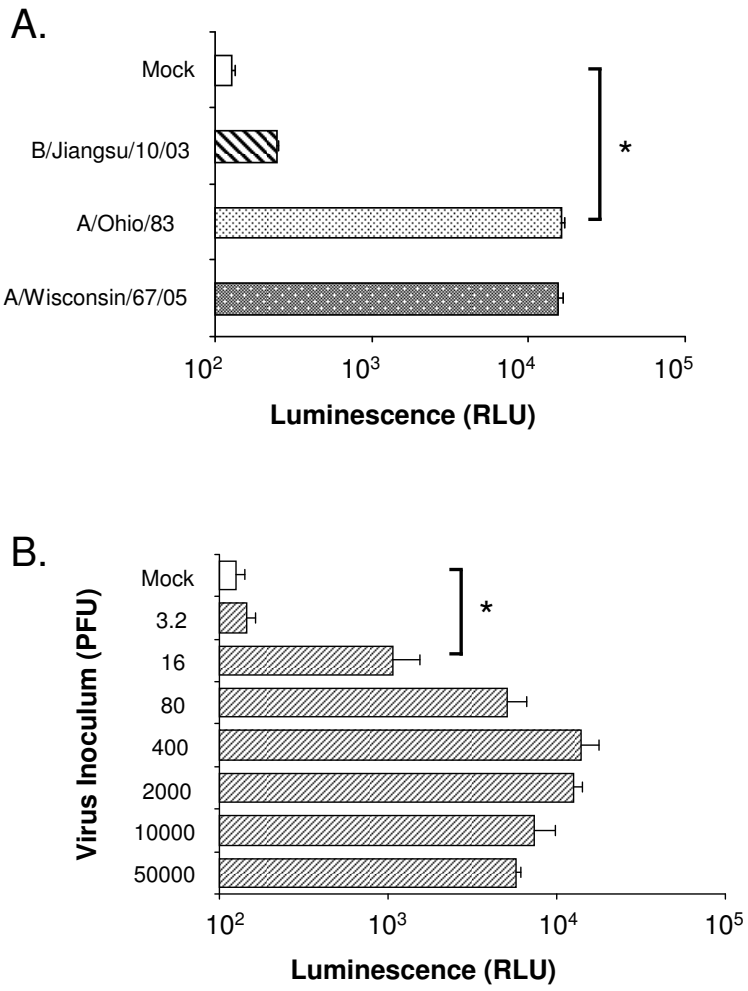


Fig 2.



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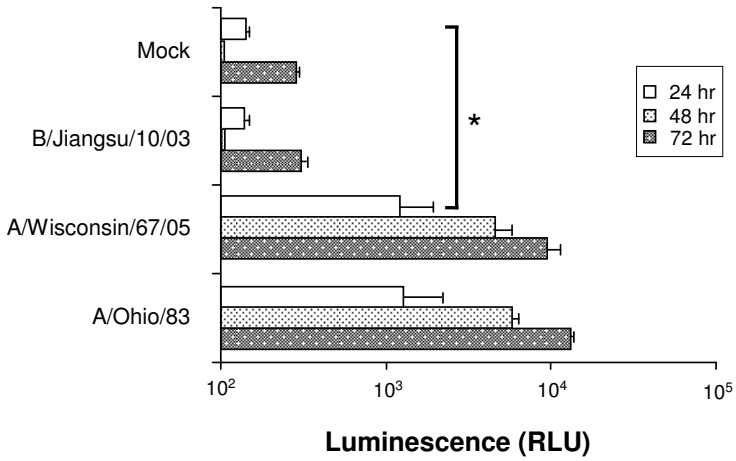

Fig.4

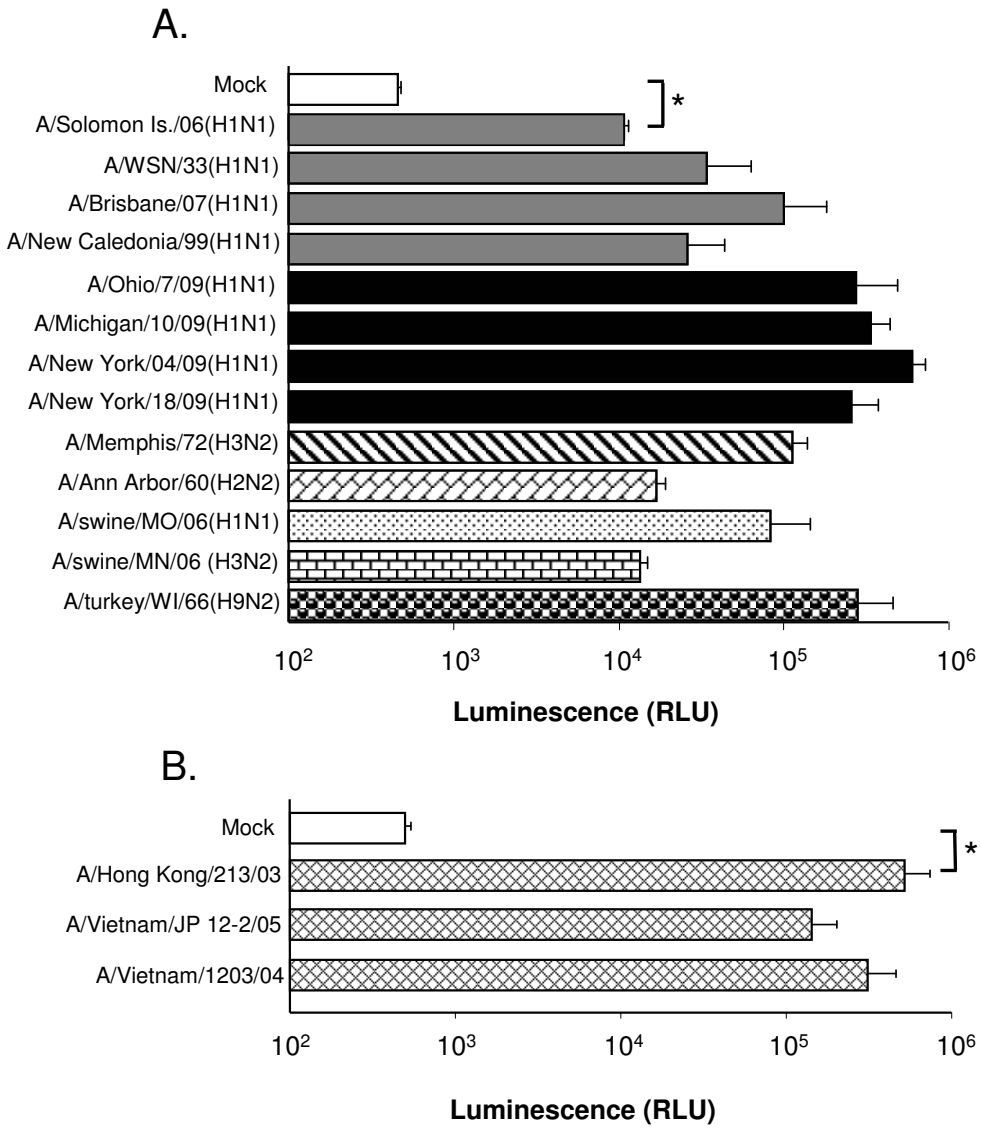

Fig. 5

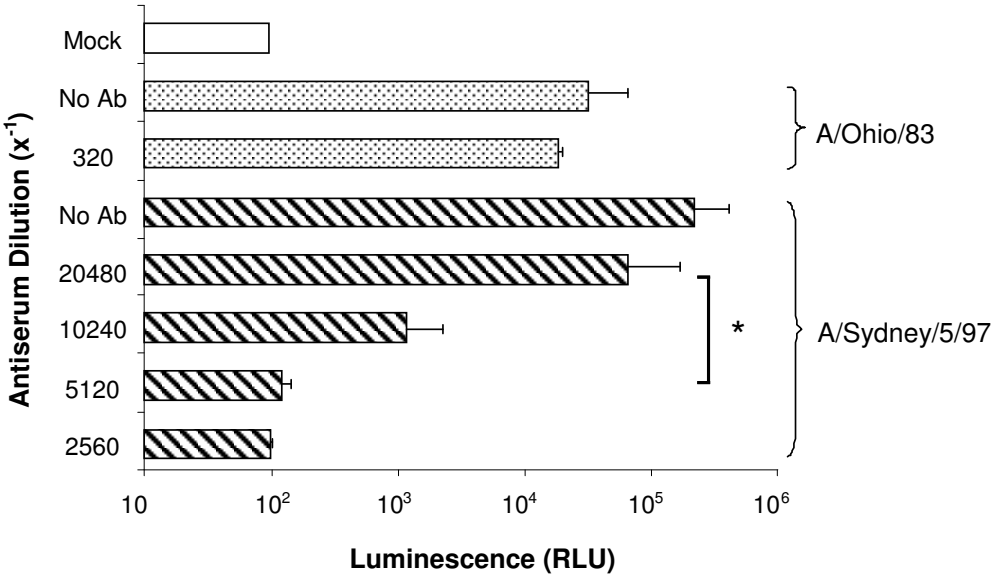

Fig.6