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Production of high-titer infectious influenza pseudotyped particles with envelope glycoproteins from highly pathogenic H5N1 and avian H7N9 viruses
--Manuscript Draft--

1 **TITLE:**
2 **Production of High-Titer Infectious Influenza Pseudotyped Particles with Envelope**
3 **Glycoproteins from Highly Pathogenic H5N1 and Avian H7N9 Viruses**
4
5 **AUTHORS AND AFFILIATIONS:**
6 Fengwei Zhang[1], Yanan Wang[2], Xuechai Shang[1], Shanshan Wang[1], Rong Xiao[1], Hongjuan Zhou[1],
7 Long Cai[1]
8

9 [1]Central Laboratory, Hang Zhou Red Cross Hospital, Hangzhou, China
10 [2]Clinical Laboratory, Yuhang District Maternity and Child Health Care Hospital, Hangzhou, China
11
12 **Corresponding Authors:**
13 Fengwei Zhang   (Tome_Walker@zcmu.edu.cn)
14 Long Cai   (cailong@hz.cn)
15
16 **Email Addresses of Co-authors:**
17 Yanan Wang   (wynfy@163.com)
18 Xuechai Shang   (57435685@qq.com)
19 Shanshan Wang   (wangdshan@126.com)
20 Rong Xiao   (xiaorong7180@163.com)
21 Hongjuan Zhou   (zhouhjlan@qq.com)
22

23 **KEYWORDS:**
24 Influenza, HPAI, H5N1, hemagglutinin, neuraminidase, pseudotyped viral particle, infectivity,
25 envelope glycoproteins, reassortment, transfection
26
27 **SUMMARY:**
28 This protocol describes an experimental process to produce high-titer infectious viral
29 pseudotyped particles (pp) with envelope glycoproteins from two influenza A strains and how to
30 determine their infectivity. This protocol is highly adaptable to develop pps of any other type of
31 enveloped viruses with different envelope glycoproteins.
32
33 **ABSTRACT:**
34 The occasional direct transmission of the highly pathogenic avian influenza A virus H5N1 (HPAI
35 H5N1) and H7N9 to humans and their lethality are serious public health issues and suggest the
36 possibility of an epidemic. However, our molecular understanding of the virus is rudimentary,
37 and it is necessary to study the biological properties of its envelope proteins as therapeutic
38 targets and to develop strategies to control infection. We developed a solid viral pseudotyped
39 particle (pp) platform to study avian influenza virus, including the functional analysis of its
40 hemagglutinin (HA) and neuraminidase (NA) envelope glycoproteins, the reassortment
41 characteristics of the HAs and NAs, receptors, tropisms, neutralizing antibodies, diagnosis,
42 infectivity, for the purposes of drug development and vaccine design. Here, we describe an
43 experimental procedure to establish pps with the envelope glycoproteins (HA, NA) from two
44 influenza A strains (HAPI H5N1 and 2013 avian H7N9). Their generation is based on the capacity

45 of some viruses, such as murine leukemia virus (MLV), to incorporate envelope glycoproteins into
46 a pp. In addition, we also detail how these pps are quantified with RT-qPCR, and the infectivity
47 detection of native and mismatched virus pps depending on the origin of the HAs and NAs. This
48 system is highly flexible and adaptable and can be used to establish viral pps with envelope
49 glycoproteins that can be incorporated in any other type of enveloped virus. Thus, this viral
50 particle platform can be used to study wild viruses in many research investigations.
51
52 **INTRODUCTION:**
53 The mission of a viral particle is to transport its genome from an infected host cell to a non-
54 infected host cell and to deliver it into the cytoplasm or the nucleus in a replication-competent
55 form[1]. This process is initially triggered by binding to host cell receptors, followed by fusion of
56 virion and cellular membranes. For enveloped viruses, like influenza viruses, the spike
57 glycoproteins are responsible for receptor binding and fusion[1,2]. Viral envelope glycoproteins
58 (e.g., pyrogens, antigens), are involved in many important properties and events, such as virus
59 lifecycle initiation (binding and fusion), viral pathogenesis, immunogenicity, host cell apoptosis
60 and cellular tropism, the cellular endocytic pathway, as well as interspecies transmission and
61 reassortment[1,3–7]. Research on viral envelope glycoproteins will help us understand many aspects
62 of the viral infection process. Pseudotyped viral particles (pp), also termed pseudovirions or
63 pseudoparticles, can be generated through a pseudotyping technique[8–10]. This technology has
64 been used to develop pseudotyped particles of many viruses, including hepatitis C[11,12], hepatitis
65 B[13], vesicular stomatitis virus (VSV)[14,15], and influenza virus[16–19]. This technology is based on the
66 Gag-Pol protein of lentiviruses or other retroviruses.
67
68 Pseudotyped viral particles can be obtained using a three-plasmid system by cotransfecting a
69 viral envelope glycoprotein expression plasmid, a retroviral packaging plasmid missing the
70 envelope *env* gene, and a separate reporter plasmid into pp producer cells. The retrovirus is
71 assembled by its Gag protein, and it buds from an infected cell membrane that expresses the
72 virus envelope protein[1]. Therefore, it is possible to obtain high titer influenza pps using the
73 retrovirus Gag protein to produce buds on a cellular membrane expressing influenza HA and NA.
74 In our previous studies, HAs/NAs in all combinations were functional and able to perform their
75 corresponding functions in the viral life cycle[16–18,20–21]. These pps are used to investigate influenza
76 biological characteristics, including hemagglutination, neuraminidase activity, HA-receptor
77 binding tropism, and infectivity. Because HA and NA are both important surface functional
78 proteins in the viral life cycle, mismatched HAs and NAs derived from different strains of influenza
79 can partly demonstrate reassortment between them. Here, we generate eight types of influenza
80 pps by combining two HAs and two NAs (derived from the HPAI H5N1 strain and the H7N9 stain),
81 using a three-plasmid pseudotyping system. These eight types of pps include two native pps,
82 H5N1pp, H7N9pp; two mismatched pps, (H5+N9)pp, (H7+N1)pp; and four pps only harboring a
83 single glycoprotein (HA or NA), H5pp, N1pp, H7pp, N9pp. Studies on the influenza virus, such as
84 H5N1 and H7N9, are limited by biosafety requirements. All studies of the wild influenza virus
85 strains should be performed in a biosafety level 3 (BSL-3) laboratory. Pseudotyped viral particle
86 technology can be used to package an artificial virion in a biosafety level 2 (BSL-2) setting.
87 Therefore, pps represent a safer and useful tool to study the influenza virus processes depending
88 on its two major glycoproteins: hemagglutinin (HA) and neuraminidase (NA).

89

90   This protocol describes the generation of these pps with a three-plasmid cotransfection strategy
91   (overviewed in **Figure 1**), how to quantify pps, and infectivity detection. The pp production
92   involves three kinds of plasmids (**Figure 1**). The *gag-pol* gene, which encodes the retrovirus Gag-
93   Pol protein, was cloned from a retrovirus packaging kit and inserted into the pcDNA 3.1 plasmid
94   and named pcDNA-Gag-Pol. The enhanced green fluorescent protein (eGFP) gene, which encodes
95   Green Fluorescent Protein, was cloned from pTRE-EGFP vector, inserted into the pcDNA 3.1
96   plasmid, and called pcDNA-GFP. During cloning, a packaging signal (ψ) sequence was added via a
97   primer. The HA and NA genes were cloned into a pVRC plasmid, named pVRC-HA and pVRC-NA,
98   respectively. The last plasmid encodes the fusion protein and can be replaced with any other
99   fusion protein of interest. Our pseudotyping platform includes two glycoprotein expression
100 plasmids: pVRC-HA and pVRC-NA. This can simplify the research on reassortment between
101 different virus strains in a BSL-2 setting.

102

103 **PROTOCOL:**

104

105 **1. Day 1: Cell culture and seeding**

106

107 1.1. Cultivate human embryonic kidney (HEK) 293T/17 cells in 60 mm dishes with Dulbecco's
108 modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 100
109 U/mL penicillin-streptomycin (DMEM Complete Medium, DCM) in a 37 °C, 5% carbon dioxide
110 (CO_2) incubator until about 80% confluent.

111

112 NOTE: HEK 293T/17 low passage cells are recommended.

113

114 1.2. Carefully wash the cells with 5 mL of phosphate buffered saline (PBS) 1x.

115

116 NOTE: Manual handling of the HEK 293T/17 cells must be very gentle, because they easily detach.

117

118 1.3. Remove PBS and dissociate the cells with 1 mL of 0.25% trypsin-ethylene diamine tetraacetic
119 acid (EDTA) solution. Place the dish in a 37 °C, 5% CO_2 incubator for no more than 5 min until the
120 cells are dissociated.

121

122 1.4. Deactivate trypsin by adding 5 mL of DCM. Disperse the cells into a single-cell suspension by
123 pipetting up and down several times.

124

125 1.5. Transfer the cell suspensions to a prechilled 15 mL centrifuge tube. Collect the cells by
126 centrifugation at 250 x *g* for 5 min at 4 °C.

127

128 1.6. Decant as much of the supernatant as possible. Resuspend the cell pellet with 6 mL of DCM
129 medium and count the cells. Dilute the cells to 1×10^6 cells/mL with DCM medium.

130

131 1.7. Seed the cells into a 6 well plate with 1 mL of cell suspension per well. Gently pat the plate
132 to evenly distribute the cells. Incubate the plate overnight (14–16 h) in a 37 °C, 5% CO_2 incubator.

2. Day 2: Four-plasmid cotransfection mediated by lipofection

2.1. Check the cell morphology and density under an inverted light microscope. Ideally, cells should be approximately 85% confluent at transfection. Replace the medium with 1 mL of serum-free DMEM medium per well, and then put the plate back into the incubator.

NOTE: Manual handling of the HEK 293T/17 cells must be very gentle, because they easily detach.

2.2. For each well of cultured cells to be transfected, dilute 8 µL of the transfection reagent to a volume of 150 µL with Reduced Serum Medium (tube 1). Mix gently and incubate for 5 min at room temperature (RT, approximately 20 °C).

NOTE: For each transfection sample, prepare two 1.5 mL microcentrifuge tubes, numbered 1 and 2.

2.3. In tube 2, dilute 2.5 µg of plasmid DNA into 150 µL of Reduced Serum Medium.

NOTE: In tube 2, dilute each plasmid DNA as shown in **Table 1**. A plasmid encoding vesicular stomatitis virus (VSV) G glycoprotein (plasmid pLP-VSVG) was used as a positive control, because VSV is able to infect a wide range of cells. Negative control particles that lack influenza envelope glycoproteins (Δenv pps) were generated using pcDNA-Gag-Pol and pcDNA-GFP plasmids.

2.4. After a 5 min incubation, combine the diluted DNA with diluted transfection reagent. Mix gently and incubate for another 15 min at RT.

2.5. Add the DNA-lipid complex to the corresponding well containing the cells and serum-free medium. Mix gently by rocking the plate back and forth.

2.6. After incubation for 4–6 h in a 37 °C, 5% CO_2 incubator, remove the medium, and replace with 2 mL of DMEM. Incubate for another 36–48 h in a 37 °C, 5% CO_2 incubator.

NOTE: Replace with serum-free and antibody-free DMEM. In this protocol, two HAs and two NAs can be used to generate eight types of pps (shown in **Table 1**).

3. Day 3: Susceptible cells seeding

3.1. For the infectivity assay, seed each type of susceptible cells at 1 x 10^4 cells per well in a 96 well plate.

NOTE: Use two types of target cell to perform the infectivity assay in this article: an alveolar-derived human cell line (A549 cells) and the Madin-Darby Canine Kidney (MDCK) cells. MDCK cells are widely used in influenza research and can be a good control. This step is flexible. Any other target cell lines can be used according to the research requirements.

177

3.2. Incubate the plate overnight (14–16 h) in a 37 °C, 5% CO_2 incubator.

4. Day 4: Pseudotyped viral particle collection, quantification, and infectivity assay

4.1. Pseudotyped viral particle collection

4.1.1. Check the color of the medium. Ideally, it should be light pink or slightly orange. Examine the cells with an inverted fluorescent biological microscope under 440–460 nm.

4.1.2. At 36–48 h posttransfection, harvest the pps by passaging through a 0.45 μm polyvinylidene fluoride (PVDF) membrane filter to eliminate cell debris.

4.1.3. Divide the pps into small volume aliquots.

4.1.4. Store the pps at -80 °C.

NOTE: The protocol can be paused here. However, this is not encouraged, because the infectivity of the pps will sharply decline after freezing and thawing.

4.2. Pseudotyped viral particle quantification

4.2.1. Transfer 20 μL of purified pps to a 1.5 mL ribonuclease (RNase)-free microcentrifuge tube.

4.2.2. Add 1 μL of 0.24 U/mL benzonase nuclease. Incubate at 37 °C for 1 h to eliminate any DNA and RNA contamination.

NOTE: Target RNA, commonly downregulated cytomegalovirus (CMV)-GFP RNA, is packaged in the pps and can avoid being degraded by benzonase nuclease.

4.2.3. Freeze the sample at -70 °C to inactivate the benzonase nuclease.

4.2.4. Add 2 μL of proteinase K. Incubate at 50 °C for 30 min to digest the envelope proteins and release the CMV-GFP RNA. Inactivate the proteinase K at 100 °C for 3 min.

4.2.5. Quantify the pps by real-time quantitative reverse-transcription (qRT)-PCR with a Universal Probe One-Step RT-qPCR Kit, using the forward primer 5'-AACAAAAGCTGGAGCTCGTTTAA-3', the reverse primer 5'-GGGTCTCCTCAGAGTGATTGACTAC-3', and the probe 5'-FAM-CCCCCAAATGAAAGACCCCCGAG-TAM-3', on a Real-Time PCR thermocycler. Normalize the pps for RNA copy number before infectivity.

4.3. Infectivity assay

4.3.1. Dilute each type of pps in terms of the qRT-PCR data to 4 × 10^5 copies/mL (pp

221 normalization).

223 4.3.2. Add Tosyl-Phenylalanine Chloromethyl-Ketone (TPCK)-trypsin to a final concentration of
224 40 μg/mL into pps that harbor H7N9 HA. Incubate at 37 °C for 1 h to form its functional subunits
225 HA1 and HA2.

227 NOTE: There is no need to treat the pps that harbor H5 with TPCK-trypsin, because they have
228 multiple arginine and lysine residues at the HA1-HA2 cleavage site. This multi-basic cleavage site
229 can be cleaved by ubiquitous cellular proteases.

231 4.3.3. Mix normalized pps with DMEM medium (serum-free) at a 1:1 ratio (volume/volume).

233 4.3.4. Bring the plate containing susceptible cells to the biosafety cabinet.

235 4.3.5. Aspirate the supernatant and wash the cells once with 0.1 mL of prewarmed PBS.

237 4.3.6. Add 0.1 mL of pps-DMEM mixture to one well. Triplicate the infectivity tests of each type
238 of pps to one susceptible cell line (overviewed in **Figure 2**).

240 4.3.7. Incubate the 96 well plate for 4–6 h in a 37 °C, 5% CO_2 incubator.

242 4.3.8. Aspirate the supernatant and replace with 0.1 mL of DCM.

244 4.3.9. Incubate the 96 well plate for another 24–36 h in a 37 °C, 5% CO_2 incubator.

246 **5. Day 5 or 6: Infectivity detection**

248 5.1. Bring the 96 well plate to the biosafety cabinet.

250 5.2. Aspirate the supernatant and wash the cells 1x with 0.2 mL of prewarmed PBS.

252 5.3. Remove PBS and dissociate the cells with 0.1 mL of 0.25% trypsin-EDTA solution.

254 5.4. Place the dish in a 37 °C, 5% CO_2 incubator for 3 min until the cells are dissociated.

256 NOTE: Avoid incubating for more than 5 min, because this will lead to cell clumping.

258 5.5. Deactivate trypsin by adding 0.4 mL of DCM.

260 5.6. Disperse the cells into single-cell suspension by pipetting up and down several times.

262 5.7. Transfer the cell suspensions to a chilled 1.5 mL microcentrifuge tube.

264 5.8. Determine the GFP reporter-positive cells with Fluorescence Activated Cell Sorting (FACS).

265

266   NOTE: To determine the ratio of GFP reporter-positive target cells, set flow cytometer gates using
267   the control samples (pps-untreated A549 cells or MDCK cells), and then count the GFP reporter-
268   positive cells of 10,000 cells per sample.

269

270   **REPRESENTATIVE RESULTS:**
271   Depending on the general procedure described above, we have generated 10 types of pps
272   combining two group HAs/NAs or VSV-G glycoprotein or no-envelope glycoproteins (shown in
273   **Table 1**). Seven of them are infectious. The pps that harbor no-envelope glycoprotein or only
274   harbor NA did not show any infectivity here. The influenza pp production procedure is
275   overviewed in **Figure 1**. Transmission electron micrographs of pps (e.g., H5N1pp) are shown in
276   **Figure 3**. The results of infectivity assays of these pps are shown in **Figure 4**. The infectivity of the
277   seven types of pps was evaluated at a genome copies per cell (GCP) [similar to multiplicity of
278   infection (MOI)] value of 20. In the pps group harboring H5, the infectivity of H5N1, (H5+N9), and
279   H5 was 90.05 ± 4.05%, 17.78 ± 1.58%, 10.15 ± 2.85% for cell line A549 and 40.37 ± 4.92%, 5.24 ±
280   1.32%, 4.88 ± 0.27% for MDCK, respectively. In the pps group harboring H7, the infectivity of
281   H7N9, (H7+N1), and H7 was 10.45 ± 2.35%, 6.75 ± 1.37%, 1.23 ± 0.33% for cell line A549 and 7.61
282   ± 1.04%, 4.12 ± 1.29%, 1.08 ± 0.02% for MDCK, respectively. For the infectivity assays of the pps,
283   especially HApp (H5pp, H7pp), exogenous neuraminidase was not added. In this infectivity assay,
284   the MDCK cells were also used as the control cell line to test pps infectivity. The infectivity of
285   VSVGpp was 20.9 ± 2.00% for A549 and 16.02 ± 2.41% for MDCK cells. The delta-envelope
286   glycoproteins pp (Δenv pp) showed no infectivity in our study. These data also showed that
287   HAs/NAs from diverse virus strains are capable of successfully generate infectious viral particles.
288   Taken together, our pseudotyping platform can develop infectious pseudotyped viral particles
289   used in influenza research.

290

291   **FIGURE AND TABLE LEGENDS:**
292   **Figure 1: Overview of influenza pp production procedure.** (**A**) The packaging plasmid pcDNA-
293   Gag-pol, the reporter plasmid pcDNA-GFP, and the envelope glycoprotein expression plasmids
294   pVRC-HA and pVRC-NA, are cotransfected into pp-producing HEK 293/17 cells. (**B**) In HEK 293/17
295   cells, the Gag-Pol polyproteins are synthesized and transported by an unknown mechanism to
296   the cell membrane. Glycoproteins HA and NA are transported and anchored onto the cell
297   membrane via the secretory pathway. Reporter plasmid pcDNA-GFP is transcribed into the single-
298   stranded ψ-GFP genomic RNA. (**C**) During or after transport, the Gag-Pol protein recruits the
299   single-stranded ψ-GFP genomic RNA via the psi-RNA packaging signals, thereby forming the pre-
300   budding complexes. (**D**) The assembled Gag-pol-ψ-RNA complex induces membrane curvature,
301   leading to the formation of a bud. During budding, the viral envelope glycoproteins HAs/NAs are
302   incorporated into the nascent particles. Budding is completed as the particle pinches off from the
303   cell membrane.

304

305   **Figure 2: Overall arrangement in infectivity assay.** The infectivity tests of each type of pps to one
306   susceptible cell line were measured in triplicate. Alveolar-derived human cells A549 and MDCK
307   are used as susceptible cells.

308

309 **Figure 3:** Transmission electron micrographs of pps. Unconcentrated (left) and concentrated
310 (right) supernatant.
311
312 **Figure 4: Infectivity of normalized pps.** Infectivity is presented as the mean ± standard deviation
313 (SD) percentage of infected cells (n = 3). Infectivity of pps was assessed in two cell lines, A549 and
314 MDCK cells. Seven types of pps displayed various infectivity profiles in two target cells. Pps that
315 only harbor NA are not shown here as they did not manifest any infectivity. Pps that harbor no-
316 envelope glycoproteins (Δenv) also showed no infectivity in our study. The percent of infectivity
317 denoted the ratio of GFP reporter-positive cells in 10,000 cells per sample.
318
319 **Table 1: Combinations of HA and NA proteins derived from H5N1 and H7N9 viruses and the**
320 **required quantities (volume) of plasmid DNA for one well transfection of a 6 well plate.**
321 According to the concentration of plasmid preparations, calculate the required volume of each
322 plasmid DNA. The total amount of plasmid DNA for tranfection of one well is 2.5 µg. To obtain
323 the highest transfection efficiency and lowest non-specific effects, we optimized the transfection
324 conditions by varying plasmid DNA and tranfection reagent concentrations. After optimizing, the
325 ratio of the four plasmids quantities was set as 16:16:1:1. The volume of transfection reagent
326 used in one well transfection of a 6 well plate is 8 µL (not shown in the table). Two HAs and two
327 NAs from H5N1 and H7N9 viruses can be used to generate eight types of pps: H5N1pp, (H5 +
328 N9)pp, H5pp, N1pp, H7N9pp, (H7 + N1)pp, H7pp and N9pp. The pps that harbor no-envelope
329 glycoproteins (Δenv) or only harbor NA glycoproteins showed no infectivity in our study.
330
331 **DISCUSSION:**
332 In this protocol, we describe a method to produce influenza virus pseudotyped particles (pp) in
333 a BSL-2 setting. The reporter plasmid pcDNA-GFP is incorporated into the pps and can be used to
334 quantify pps by FACS in an infectivity assay. We chose two types of susceptible cell lines because
335 they are widely used in influenza research. MDCK cells would provide a good control to the
336 variable immortalized human cells used in these studies.
337
338 This protocol is based on the retrovirus MLV, which can incorporate a GFP reporter and produce
339 buds on the cellular membrane. This technique has been widely developed for packaging
340 influenza virus particles[4,22–24]. Some other systems are based on the lentiviral HIV-1 pseudotyping
341 system[19,25–26] or the baculovirus-insect cell expression system[27–29]. Many other reporter genes
342 have also been used for pseudoparticle production, such as luciferase (luminescence)[30–33], β-
343 gal/LacZ (by colorimetry)[34–35], and secreted alkaline phosphatase[36–37]. Millet et al. used the
344 luciferase assay to quantify the infectivity of coronavirus pseudotyped particles[33]. Compared with
345 luciferase, GFP's green fluorescence is easily observed with an inverted fluorescent biological
346 microscope. This is a convenient way to check transfection and infection efficiencies. For this
347 study, the infectivity can be determined directly by detecting the GFP-positive cells with FACS.
348
349 In this method, several steps critically affect the results. Optimized cell density is a critical factor
350 for successful transfection. In this protocol, a cell density in the range of 80–90% confluent was
351 found to be optimal for influenza virus pp production. Higher or lower cell density will result in
352 lower transfection efficiency. Good physiological status of producer cells is also very important

for high particle production. This is why lower cell passage HEK 293T/17 cells are recommended to produce pps in this protocol. Also, the volume of transfection reagent and the ratio of the four plasmids quantities can also affect the transfection efficiency. To obtain the highest transfection efficiency, it is recommended to optimize these factors by varying and testing their amount. We set the ratio of four plasmids quantities as 16:16:1:1 and the volume of transfection reagent as 8 µL for one well tranfection in a 6 well plate. Another issue is the handling of the cells. The HEK 293T/17producer cells are low-adhesion, so manual handling of the cells must be very gentle. Lipofection can lead to a cell permeability increase, and serum and antibodies in the medium may increase cytotoxicity. It is best to use serum-free and antibody-free DMEM to culture post-transfected HEK 293T/17 cells. Furthermore, collection time is important. At 36–48 h post-transfection, the color of the cell supernatant must be checked to make sure that it is pink or orange-pink before pp collection. Yellow supernatant indicates that the medium is too acidic to support cell growth and usually leads to poor pp yields.

We performed the transfection assay in a 6 well plate. To obtain more pp volume the number of transfection wells can be increased and supernatants that contain the same pps can be pooled together. Alternatively, some other kind of vessels can be used to increase pp yield. For instance, 18 µL of transfection reagent and 5.5 µg of plasmid DNA can be used to perform transfection with 2.2×10^6 cells in one 60 mm dish. Similarly, an infectivity assay can be carried out in a 24 well plate.

The success of this technique can be influenced by many factors. Therefore, the procedure should be optimized for particle packaging of different kinds of viruses.

In this protocol, pseudotyped viral particles of HPAI H5N1 and H7N9, as well as their reassortants, were generated. In this method, we use qRT-PCR of the GFP-RNA in the infectivity assay at the single-cell level (as genome copies per cell) instead of Tissue Culture Infectious Dose 50 (TCID50) or traditional MOI[23,38–39]. Virus infectivity titration methods are based on plaque assays or TCID50 assays, which are both time-consuming and labor-intensive. Both assays rely on the measurement of visible cytopathic effects (CPE) caused by virus infection in cell lines, so it may be hard to titrate viruses of low infectivity (i.e., H7pp in this study). We think it is a convenient, effective, and rapid method to normalize pps with qRT-PCR of the GFP RNA contained in influenza pps.

Taken together, our technique is safe and adaptable, and can be used to study enveloped viruses, including their receptors, tropisms, envelope glycoprotein function, neutralizing antibodies, antivirus drug development, diagnosis, and vaccine design and evaluation.
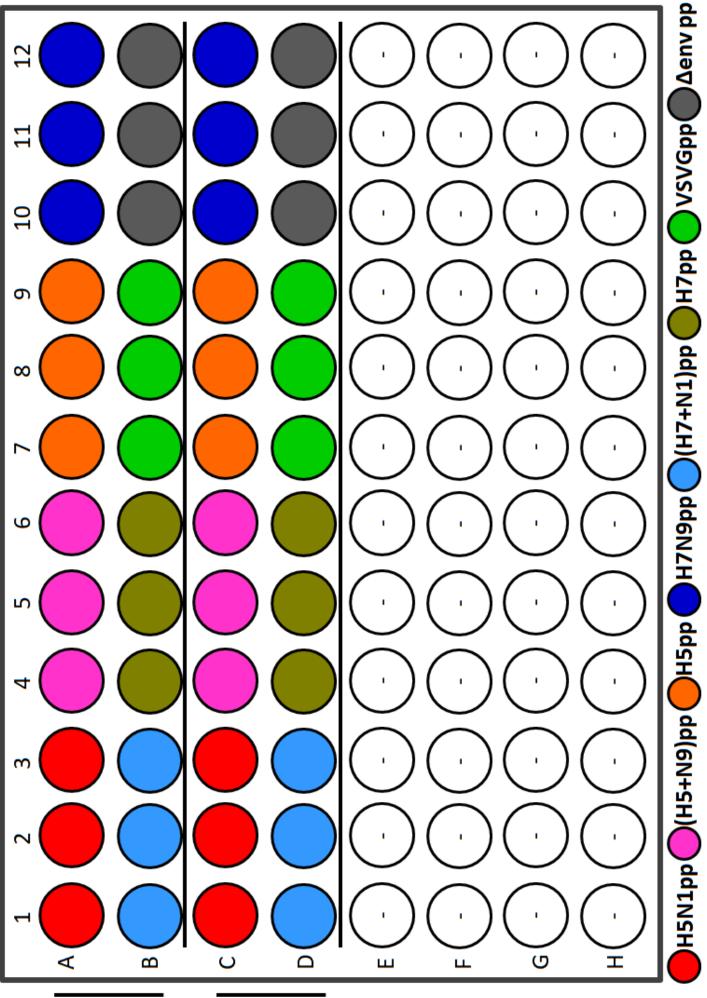
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DISCLOSURES:
The authors have nothing to disclose.

REFERENCES:
1. Knipe, D. M., Howley, P. M. *Fields Virology (6th)*. Lippincott Williams & Wilkins Immunology. Philadelphia, PA (2013).

2. White, J. M., Delos, S. E., Brecher, M., Schornberg, K. Structures and mechanisms of viral membrane fusion proteins: multiple variations on a common theme. *Critical Reviews in Biochemistry and Molecular Biology*. **43** (3), 189–219 (2008).

3. Bright, R. A. et al. Cross-clade protective immune responses to influenza viruses with H5N1 HA and NA elicited by an influenza virus-like particle. *PLoS One*. **3** (1), e1501 (2008).

4. Yang, J. et al. Reliability of pseudotyped influenza viral particles in neutralizing antibody detection. *PLoS One*. **9** (12), e113629 (2014).

5. Wyatt, R., Sodroski, J. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. *Science*. **280** (5371), 1884–1888 (1998).

6. Joe, A. K., Foo, H. H., Kleeman, L., Levine, B. The transmembrane domains of Sindbis virus envelope glycoproteins induce cell death. *Journal of Virology*. **72** (5), 3935–3943 (1998).

7. Albecka, A., Laine, R. F., Janssen, A. F., Kaminski, C. F., Crump, C. M. HSV-1 Glycoproteins Are Delivered to Virus Assembly Sites Through Dynamin-Dependent Endocytosis. *Traffic*. **17** (1), 21–39 (2016).

8. Huang, A. S., Palma, E. L., Hewlett, N., Roizman, B. Pseudotype formation between enveloped RNA and DNA viruses. *Nature*. **252** (5485), 743–745 (1974).

9. Rubin, H. Genetic Control of Cellular Susceptibility to Pseudotypes of Rous Sarcoma Virus. *Virology*. **26**, 270–276 (1965).

10. Steffen, I., Simmons, G. Pseudotyping Viral Vectors With Emerging Virus Envelope Proteins. *Current Gene Therapy*. **16** (1), 47–55 (2016).

11. Bartosch, B., Dubuisson, J., Cosset, F. L. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *Journal of Experimental Medicine*. **197** (5), 633–642 (2003).

12. Bian, T., Zhou, Y., Bi, S., Tan, W., Wang, Y. HCV envelope protein function is dependent on the peptides preceding the glycoproteins. *Biochemical and Biophysical Research Communications*. **378** (1), 118–122 (2009).

13. Gudima, S., Meier, A., Dunbrack, R., Taylor, J., Bruss, V. Two potentially important elements of the hepatitis B virus large envelope protein are dispensable for the infectivity of hepatitis delta virus. *Journal of Virology*. **81** (8), 4343–4347 (2007).

14. Yoshida, Y., Emi, N., Hamada, H. VSV-G-pseudotyped retroviral packaging through adenovirus-mediated inducible gene expression. *Biochemical and Biophysical Research Communications*. **232** (2), 379–382 (1997).

15. Burns, J. C., Friedmann, T., Driever, W., Burrascano, M., Yee, J. K. Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*. **90** (17), 8033–8037 (1993).

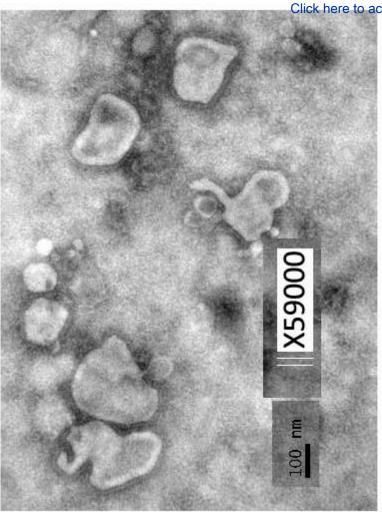16. Zhang, F. et al. Characterization of pseudoparticles paired with hemagglutinin and

neuraminidase from highly pathogenic H5N1 influenza and avian influenza A (H7N9) viruses. *Virus Research*. **253**, 20–27 (2018).

17. Zhang, F. et al. Infectivity of Pseudotyped Particles Pairing Hemagglutinin of Highly Pathogenic Avian Influenza a H5N1 Virus with Neuraminidases of The 2009 Pandemic H1N1 and a Seasonal H3N2. *Journal of Bioterrorism & Biodefense*. **2**,104 (2011).

18. Wu, J. et al. Characterization of neuraminidases from the highly pathogenic avian H5N1 and 2009 pandemic H1N1 influenza A viruses. *PLoS One*. **5** (12), e15825 (2010).

19. Nefkens, I. et al. Hemagglutinin pseudotyped lentiviral particles: characterization of a new method for avian H5N1 influenza sero-diagnosis. *Journal of Clinical Virology*. **39** (1), 27–33 (2007).

20. Zhang, Y. et al. Hemagglutinin and neuraminidase matching patterns of two influenza A virus strains related to the 1918 and 2009 global pandemics. *Biochemical and Biophysical Research Communications*. **387** (2), 405–408 (2009).

21. Lin, X. et al. Oseltamivir boosts 2009 H1N1 virus infectivity in vitro. *Biochemical and Biophysical Research Communications*. **390** (4), 1305–1308 (2009).

22. McKay, T., Patel, M., Pickles, R. J., Johnson, L. G., Olsen, J. C. Influenza M2 envelope protein augments avian influenza hemagglutinin pseudotyping of lentiviral vectors. *Gene Therapy*. **13** (8), 715–724 (2006).

23. Pan, H. et al. Autophagy mediates avian influenza H5N1 pseudotyped particle-induced lung inflammation through NF-kappaB and p38 MAPK signaling pathways. *American Journal of Physiology-Lung Cellular and Molecular Physiology*. **306** (2), L183–195 (2014).

24. Szecsi, J. et al. Induction of neutralising antibodies by virus-like particles harbouring surface proteins from highly pathogenic H5N1 and H7N1 influenza viruses. *Virology Journal*. **3** 70 (2006).

25. Garcia, J. M., Lagarde, N., Ma, E. S., de Jong, M. D., Peiris, J. S. Optimization and evaluation of an influenza A (H5) pseudotyped lentiviral particle-based serological assay. *Journal of Clinical Virology*. **47** (1), 29–33 (2010).

26. Garcia, J. M., Lai, J. C. Production of influenza pseudotyped lentiviral particles and their use in influenza research and diagnosis: an update. *Expert Review of Anti-infective Therapy*. **9** (4), 443–455 (2011).

27. Haynes, J. R. et al. Influenza-pseudotyped Gag virus-like particle vaccines provide broad protection against highly pathogenic avian influenza challenge. *Vaccine*. **27** (4), 530–541 (2009).

28. Schmeisser, F. et al. Production and characterization of mammalian virus-like particles from modified vaccinia virus Ankara vectors expressing influenza H5N1 hemagglutinin and neuraminidase. *Vaccine*. **30** (23), 3413–3422 (2012).

29. Liu, Y. V. et al. Chimeric severe acute respiratory syndrome coronavirus (SARS-CoV) S glycoprotein and influenza matrix 1 efficiently form virus-like particles (VLPs) that protect mice against challenge with SARS-CoV. *Vaccine*. **29** (38), 6606–6613 (2011).

30. Moeschler, S., Locher, S., Conzelmann, K. K., Kramer, B., Zimmer, G. Quantification of Lyssavirus-Neutralizing Antibodies Using Vesicular Stomatitis Virus Pseudotype Particles. *Viruses*. **8** (9), pii: E254 (2016).

31. Lai, A. L., Millet, J. K., Daniel, S., Freed, J. H., Whittaker, G. R. The SARS-CoV Fusion Peptide Forms an Extended Bipartite Fusion Platform that Perturbs Membrane Order in a Calcium-Dependent Manner. *Journal of Molecular Biology*. **429** (24), 3875–3892 (2017).

32. Millet, J. K. et al. Middle East respiratory syndrome coronavirus infection is inhibited by griffithsin. *Antiviral Research*. **133**, 1–8 (2016).

485 33. Millet, J. K. et al. Production of Pseudotyped Particles to Study Highly Pathogenic
486 Coronaviruses in a Biosafety Level 2 Setting. *Journal of Visualized Experiments*. (145), e59010
487 (2019).
488 34. Ma, M. et al. Murine leukemia virus pseudotypes of La Crosse and Hantaan Bunyaviruses: a
489 system for analysis of cell tropism. *Virus Research*. **64** (1), 23–32 (1999).
490 35. Wool-Lewis, R. J., Bates, P. Characterization of Ebola virus entry by using pseudotyped viruses:
491 identification of receptor-deficient cell lines. *Journal of Virology*. **72** (4), 3155–3160 (1998).
492 36. Chen, C. M. et al. Production and design of more effective avian replication-incompetent
493 retroviral vectors. *Developmental Biology*. **214** (2), 370–384 (1999).
494 37. Kaku, Y. et al. Second generation of pseudotype-based serum neutralization assay for Nipah
495 virus antibodies: sensitive and high-throughput analysis utilizing secreted alkaline phosphatase.
496 *Journal of Virological Methods*. **179** (1), 226–232 (2012).
497 38. Rudiger, D., Kupke, S. Y., Laske, T., Zmora, P., Reichl, U. Multiscale modeling of influenza A
498 virus replication in cell cultures predicts infection dynamics for highly different infection
499 conditions. *PLOS Computational Biology*. **15** (2), e1006819 (2019).
500 39. Petiot, E. et al. Influenza viruses production: Evaluation of a novel avian cell line DuckCelt(R)-
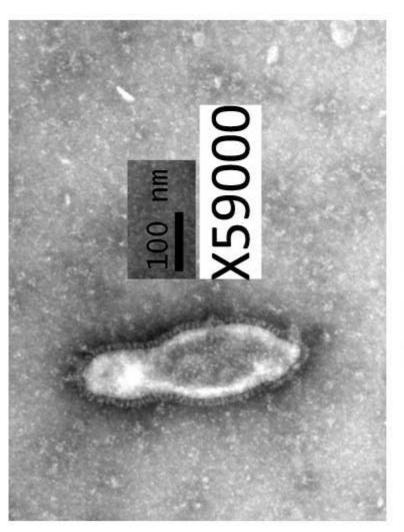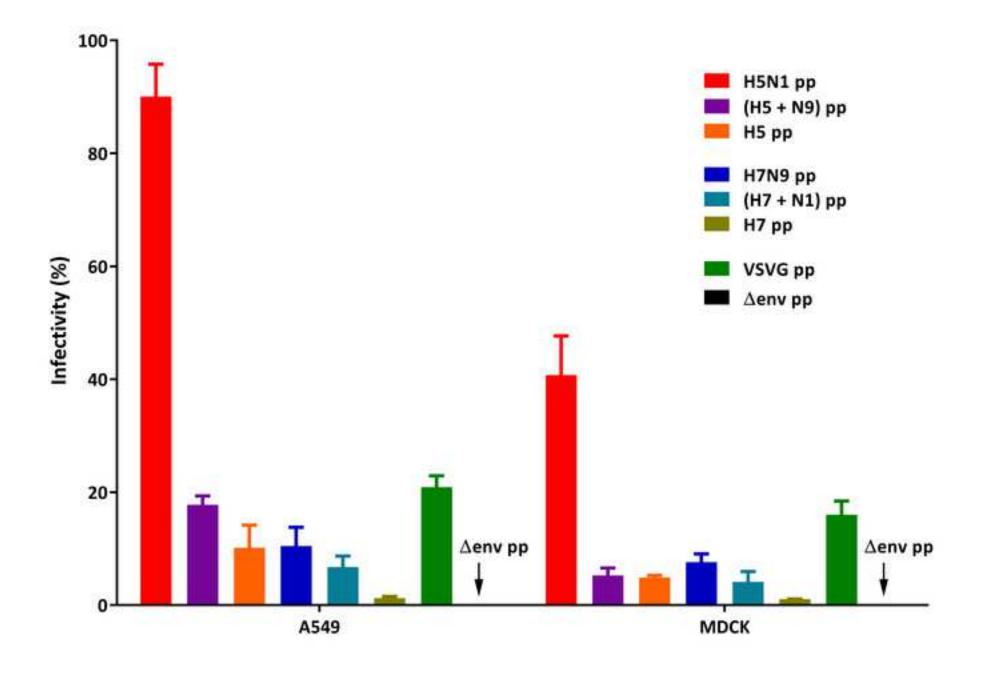501 T17. *Vaccine*. **36** (22), 3101–3111 (2018).

Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

X59000

100 nm

Concentrated

X59000

100 nm

Supernatant

Figure 4

Table 1　　　　Click here to access/download;Table;Table 1.xlsx ⬇

plasmid (µL, 0.1 µg/µL)	H5N1	(H5+N9)	H7N9	(H7+N1)	pp			
					H5	N1	H7	N9
pcDNA-Gag-pol	10.53	10.53	10.53	10.53	11.43	11.43	11.43	11.43
pcDNA-GFP	10.53	10.53	10.53	10.53	11.43	11.43	11.43	11.43
pVRC-HA	1.98	1.98	1.98	1.98	2.14	-	2.14	-
pVRC-NA	1.98	1.98	1.98	1.98	-	2.14	-	2.14
pcDNA-VSVG	-	-	-	-	-	-	-	-

VSVG	Δenv
11.43	12.5
11.43	12.5
-	-
-	-
2.14	-

Name of Material/ Equipment	Company	Catalog Number
Benzonase Nuclease	Millipore	70664
Clear Flat Bottom Polystyrene TC-treated Microplates (96-well)	Corning	3599
Clear TC-treated Multiple Well Plates (6-wells)	Costar	3516
Dulbecco's modified essential medium (DMEM)	Gibco	11965092
Fetal bovine serum	Excell	FND500
Fluorescence Activated Cell Sorting (FACS)	Beckman coulter	cytoflex
Human alveolar adenocarcinoma A549 cells	ATCC	CRM-CCL-185
Human embryonic kidney (HEK) HEK-293T/17 cells	ATCC	CRL-11268
Inverted fluorescent biological microscope	Olympus	BX51-32P01-FLB3
Inverted light microscope	Olympus	CKX31-12PHP
Lipofectamine 2000 Transfection Reagent	Invitrogen	11668019
Luna Universal Probe One-Step RT-qPCR Kit	NEB	E3006L
Madin-Darby Canine Kidney (MDCK) cells	ATCC	CCL-34
MaxyClear Snaplock Microcentrifuge Tube (1.5 mL)	Axygen	MCT-150-C

Millex-HV Syringe Filter Unit, 0.45 µm, PVDF	Millipore	SLHV033RS
Opti-MEM I Reduced Serum Medium	Gibco	11058021
penicillin-streptomycin	Gibco	15140122
PP Centrifuge Tubes (15 mL)	Corning	430791
Proteinase K	Beyotime	ST532
TC-treated Culture Dish (60mm)	Corning	430166
TPCK-trypsin	Sigma	T1426
Trypsin-EDTA (0.25%), phenol red	Gibco	25200056

Comments/Description

Effective viscosity reduction and removal of nucleic acids from protein solutions

Treated for optimal cell attachment
Sterilized by gamma radiation and certified nonpyrogenic
Individual alphanumeric codes for well identification

Individual alphanumerical codes for well identification
Treated for optimal cell attachment
Sterilized by gamma irradiation

A widely used basal medium for supporting the growth of many different mammalian cells
fetal bovine sera that can offer excellent value for basic cell culture, specialty research, and specific assays

A versatile transfection reagent that has been shown to effectively transfect the widest variety of adherent and suspension cell lines

Rapid, sensitive and precise probe-based qPCR detection and quantitation of target RNA targets.
Will withstand up to 14,000 RCF
RNase-/DNase-free Nonpyrogenic

33 mm, gamma sterilized

an improved Minimal Essential Medium (MEM) that allows for a reduction of Fetal Bovine Serum supplementation by at least 50% with no change in cell growth rate or morphology. Opti-MEM I medium is also recommended for use with cationic lipid transfection reagents, such as Lipofectamine reagent.

The antibiotics penicillin and streptomycin are used to prevent bacterial contamination of cell cultures due to their effective combined action against gram-positive and gram-negative bacteria.

Maximum RCF is 12,500 xg

Temperature range from -80°C to 120°C

RNase-/DNase-free

Sterile

 a stable and highly reactive serine protease

Treated for optimal cell attachment

Sterilized by gamma radiation and certified nonpyrogenic

Trypsin from bovine pancreas

TPCK Treated, essentially salt-free, lyophilized powder, ≥10,000 BAEE units/mg protein

This liquid formulation of trypsin contains EDTA and phenol red. Gibco Trypsin-EDTA is made from trypsin powder, an irradiated mixture of proteases derived from porcine pancreas. Due to its digestive strength, trypsin is widely used for cell dissociation, routine cell culture passaging, and primary tissue dissociation. The trypsin concentration required for dissociation varies with cell type and experimental requirements.

October 11, 2019

Editor

Journal of Visualized Experiments

Dear Dr. Wu,

Thank you for your letter. We all appreciate the editor and reviewers very much for their positive and constructive comments and suggestions on our manuscript entitled "Production of high-titer infectious influenza pseudotyped particles with envelope glycoproteins from highly pathogenic H5N1 and avian H7N9 viruses". According to the editor's suggestions, we have revised the manuscript intensively and made corresponding corrections point-by-point following comments. All authors agree to list "Rong Xiao, M.D." (He comes from another research group in our Lab) in the Article Authors on account of his contributions in editing and revising this manuscript for this time. With these revisions, we hope our revised manuscript could satisfy the quality standard for publication in JoVE.

POINT-BY-POINT RESPONSE TO THE EDITORIAL COMMENTS

Editorial comments

QUESTION 1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

RESPONSE1. Thank you for your comment. According to the Editorial suggestions, we have thoroughly proofread the manuscript. Please see details in Revised Manuscript.

QUESTION 2. *Please number all figures in the order of their appearance in the manuscript. Figure 3 should be numbered as Figure 2 as it was cited before Figure 2.*

RESPONSE 2. Thanks for your suggestions. We have re-number this two Figures. Please see details in Revised Manuscript.

QUESTION 3. *Please ensure that the references appea r as the following:* [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage (YEAR).] *For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:*

Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2 -[(hydroxyimino)methyl]imidazole. *Journal of Medicinal Chemistry*. **32** (2), 493-503 (1998).

RESPONSE3. Thanks for the comment. We have revised the references throughout the Reference Part, and please see details in Revised Manuscript.

Looking forward to hearing from you soon.

With kindest regards,

Sincerely,

Fengwei Zhang, M.D.

Central Laboratory

Hang Zhou Red Cross Hospital

East road, Hangzhou Ring, Zhejiang Province 310003

People's Republic of China

Tel: 86-571-56108777, Fax: 86-571-56108778

E-mail addresses: Tome_Walker@zcmu.edu.cn