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TITLE:

Preparation of Pseudo-typed H5 Avian Influenza Viruses with Calcium Phosphate Transfection Method and Measurement of Antibody Neutralizing Activity

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KEYWORDS:

H5 influenza pseudovirus, pseudovirus packaging, pseudovirus titration, pseudovirus-based neutralization assays

SUMMARY:

Here we describe a protocol for pseudovirus packaging and the measurement of antibody neutralizing activity.

ABSTRACT:

Since 1996, A/goose/Guangdong/1/96-lineage highly pathogenic avian influenza (HPAI) H5 viruses have been causing flu outbreaks in poultry and wild birds. Occasionally, humans also fall victim to it, which results in high mortality. Nonetheless, HPAI virus research is often hindered, considering that it must be handled within biosafety level 3 laboratories. To address this issue, pseudoviruses are adopted as an alternative to wild-type viruses in some experiments of H5 HPAI studies. Pseudoviruses prove to be the ideal tools to study neutralizing antibodies against H5 HPAI viruses. This protocol describes the procedures and critical steps of H5 HPAI pseudovirus preparations and pseudovirus neutralization assays. Also, it discusses the troubleshooting, limitation, and modifications of these assays.

INTRODUCTION:

Since 1996, A/goose/Guangdong/1/96-lineage highly pathogenic avian influenza (HPAI) H5 viruses have been causing continual flu outbreaks in poultry and wild birds, accounting for enormous socio-economical losses in the global poultry industry. Sometimes, humans also get infected with it, faced with a high fatality rate[1,2]. However, HPAI virus research is often hindered, given that it cannot be handled outside of biosafety level 3 laboratories. To address this issue, pseudoviruses are adopted as an alternative to wild-type viruses in some experiments of H5 HPAI studies. Pseudoviruses are safe enough to practice in biosafety level 2 laboratories.

H5 HPAI pseudoviruses belong to chimeric viruses consisting of surrogate virus cores, lipid envelopes with the surface glycoproteins from influenza viruses, and reporter genes. Pseudovirus cores are usually derived from lentiviral human immunodeficiency virus (HIV), retroviruses such as murine leukemia virus (MLV), and vesicular stomatitis virus (VSV)[3]. Specifically, the HIV-1 packaging system is widely used to produce influenza pseudovirus, where the primary genes provided are *gag* and *pol*. The HIV gag gene expresses core proteins. The pol gene expresses the integrase and reverse transcriptase, both of which are necessary for the expression of the reporter gene in transduced cells. Mimicking the genome of the surrogate virus, the reporter gene is embraced into the pseudovirus core in RNA form. The reporter gene will express the protein in host cells. The gene expression levels of reporter genes can be used to measure pseudovirus infection efficiency[3,4]. The primary reporter is firefly luciferase to measure the relative luminescence units (RLU) or relative luciferase activity (RLA) in transduced cells. Other reporters such as lacZ, Gaussia, and Renilla luciferase are also used, only to a lesser extent[5].

Pseudoviruses are ideal tools to study neutralizing antibodies against H5 HAPI viruses. To measure the neutralizing antibody potency, pseudovirus neutralization (PN) assays[6] are used. Hemagglutinin (HA) and neuraminidase (NA) are glycoproteins on the surface of the influenza A virus[7,8]. The HA is composed of a globular head domain for receptor binding and a stem domain for membrane fusion. The NA protein has the sialidase activity to facilitate virus release[7,8]. A PN assay can measure neutralizing antibodies directed to HA proteins. Neutralizing antibodies directed to the head and stem region of HA can also be detected by viral attachment and entry assays. Compared with wild-type viruses, pseudovirus neutralization experiments have more sensitive detection values, can be safely handled in a Level 2 biosafety laboratory, and are generally easier to operate in practice.

This protocol presents in detail the procedures and critical steps of H5 HPAI pseudovirus preparations and PN assays. Also, it discusses the troubleshooting, limitation, and modifications of these assays. In this study, the A/Thailand/1(KAN)-1/2004(TH) strain from H5N1 HPAI viruses was used as an example. To obtain the immune sera used in assays, this protocol selected the HA protein originating from the TH strain as the immunogen to immunize mice.

PROTOCOL:

All pseudovirus-related experimental operations were performed under ABSL2 condition in the Institut Pasteur of Shanghai Chinese Academy of Sciences (IPS, CAS). Animal experiments were performed based on Institutional Animal Care and Use Committee-approved animal protocols of IPS, CAS.

1. Pseudovirus packaging with Calcium-phosphate transfection

1.1. Make single-cell suspensions of HEK293FT cells (9×10^5 per mL) in complete DMEM medium (**Table 1**). Add 10 mL of the single-cell suspensions to a T75 flask, incubate the cells in a 37 °C, 5% carbon dioxide (CO_2) incubator for 20 h before the transfection.

NOTE: HEK293FT low passage (<20 passages) is recommended. Ensure that the cell monolayer is 80–90% confluent.

1.2. Replace the medium with 10 mL of fresh complete medium including chloroquine (100 μM) 2 h before transfection.

1.3. Mix the reagents and plasmid DNA as shown in **Table 2** and **Figure 1**: ddH_2O, pCMV/R-HA, pCMV/R-NA, pHR-Luc, pCMV Δ 8.9, 2.5 M $CaCl_2$, and 2x HEPES buffer. Pipette up and down 5 times gently, incubate the mixture at room temperature (RT) for 20 min.

1.4. Transfer the mixture into the medium above the cells, and rock the flask gently. Incubate the cells in the cell incubator for 15 h.

1.5. Replace the medium with 15 mL of fresh complete DMEM medium. Incubate the cells in a 37 °C, 5% CO_2 incubator for 65 h.

NOTE: The color of the medium will be light orange or slightly yellow, and at least 80% of the cell should show the cytopathic effect (CPE) under the inverted light microscope.

1.6. Harvest the supernatant, and centrifuge it at 2095 x *g* for 20 min at 4 °C. Collect the supernatant, aliquot it, and store it at -80 °C.

2. Detection of the HA, NA and HIV-1 p24 protein expression of influenza pseudovirus.

2.1 Detect the HA protein expression by Hemagglutinin (HA) assay.

2.1.1. Add 50 µL of PBS into columns 2–12, rows A, B, and C of a 96-well round-bottom plate.

NOTE: Row A, B, and C belong to 3 independent replication tests, and column 12 is used as the negative control.

2.1.2. Add 100 µL of pseudovirus into the wells of column 1. Transfer 50 µL from column 1 into Column 2, mix well by pipetting up and down 5 times, perform the two-fold dilutions until the last column 11, and discard the extra 50 µL from column 11.

2.1.3. Add 50 µL of 0.5% erythrocyte into each well, pipette it up and down gently twice. Incubate the plate for 30–60 min at RT or until the erythrocytes of the negative control form the regular spots at the bottom of the well.

2.1.4. Observe and record HA titers of the pseudovirus.

NOTE: HA units of the pseudoviruses is the highest dilution factor of the virus that can cause 100% hemagglutination of the red blood cells.

2.2 Detect HA and NA protein expression by the western-blot assay.

NOTE: All the western-blot steps are performed according to the manual of Molecular cloning (4^{th} edition).

2.3 Detect the HIV-1 p24 protein expression by the HIV-1 p24 ELISA kit (**Table of Materials**).

2.3.1. Add 50 µL of the lysis buffer into 450 µL of pseudovirus sample. Mix it thoroughly.

2.3.2. Add 300 µL of the wash buffer to each well of the microplate. Strike the plate inverted to remove the wash buffer completely. Repeat the wash 5 times.

2.3.3. Add 200 µL of the standard and samples to each well separately. Incubate them at 37 °C overnight or for 2 h.

2.3.4. Aspirate the contents of the plate and wash the plate as described in step 2.3.2.

NOTE: Leave one well of the assay plate empty to use as a substrate blank.

2.3.5. Add 200 µL of the p24 detector antibodies to each well. Incubate them at 37 °C for 1 h. Aspirate and wash the plate as described in step 2.3.2.

2.3.6. Add 100 µL of the streptavidin-Peroxidase working solution to each well, and incubate them at 37 °C for 30 min. Aspirate and wash the plate as described in step 2.3.2.

2.3.7. Add 100 µL of the substate working solution to each well, including the substrate blank well, and incubate them at 37 °C for 30 min.

NOTE: Blue color will develop in the wells.

2.3.8. Add 100 µL of the stop buffer to each well.

NOTE: The blue color will change to yellow immediately.

2.3.9. Read the optical density at 450 nm within 15 min. Record the p24 titers of the pseudovirus

3. Pseudovirus titration

3.1. Make single-cell suspensions of MDCK cells (5 x 10^4 per mL) in complete DMEM medium, add 1250, 2500, 5000, 10000, 20000, and 40000 cells to different wells of a 96-well flat-bottom plate. Incubate the cells in a 37 °C, 5% CO_2 incubator for 20 h.

3.2. Take out the pseudovirus from the -80 °C refrigerator, thaw it on the ice, and vortex the pseudovirus.

3.3. Add 6 HAU (6x HA units) pseudoviruses to each well of 96-well round-bottom plate and replenish each well with complete DMEM up to 120 µL.

3.4. Pipette up and down the mixture 5 times to mix thoroughly. Incubate the mixture plate in a 37 °C, 5 % CO_2 incubator for 1 hour.

3.5. Transfer 100 µL of the mixture to the cells in the 96-well plate. Put the cell culture plate back into the incubator for 48 h, 60 h, and 72 h after virus infection.

3.6. Take the plate out of the incubator and perform the luciferase assay (**Table of Materials**).

3.7. Remove the medium from the wells of the plate carefully. Rinse the cells with 200 µL of PBS and remove the PBS as much as possible.

NOTE: Flip the plate and discard the supernatant by reverse slapping it on absorbent paper. If the testing cell line could not firmly attach to the bottom, remove the medium aspiration carefully.

3.8. Add 50 µL of the lysis buffer to each well, and rock the culture plate several times. Store the plate at -80 °C overnight.

NOTE: Mix 1 volume of 5x lysis buffer with 4 volumes of water to make the 1x lysis buffer. Equilibrate the 1x lysis buffer to RT before use. Rock the plate to ensure that the cells are covered with the lysis buffer completely. The single freeze-thaw process can help the cell be lysed

completely.

3.9. Take the plate out, equilibrate at RT for 2 h.

NOTE: The cell should be lysed completely.

3.10. Rock the plate several times gently, and transfer all the cell lysates to opaque 96-well plates.

3.11. Add 50 µL of the substrate to each well, rock the plate several times gently, and mix substrate and lysis buffer thoroughly.

3.12. Measure the light produced within 5 min using a luminometer. Record the luciferase titers of the pseudovirus.

NOTE: When a proper substrate is added, light is emitted as a by-product via a chemical reaction in which luciferin is converted to oxyluciferin by the luciferase enzyme. The amount of light produced is proportional to the amount of the luciferase enzyme.

4. Immune sera preparation

NOTE: The immune serum will be used for cell-related experiments, and the experimental operation should be carried out under aseptic conditions.

4.1. Prepare 12 eight-week-old female BALB/c mice. Divide equally the mice into two groups.

NOTE: One group was DDV group and the other was the negative control group.

4.2. DDV group immunization: Prime twice intramuscularly with 100 µg of a codon-optimized DNA plasmid encoding A/Thailand/(KAN-1)/2004 (TH) HA protein at week 0 and week 3 and boost once intraperitoneally with 512 HAU TH virus-like particles (VLP) at week 6.

4.3. Control group immunization: Prime twice intramuscularly with 100 µg empty vector plasmid DNA at week 0 and week 3 and boost once intraperitoneally with HIV-1 gag VLP at week 6.

4.4. Collect the mouse blood 2 weeks after the last immunization.

NOTE: Here, the mice were anesthetized with pentobarbital sodium (65 mg/kg) at the time of blood collection. After blood collection from the submandibular vein, the mice were euthanized immediately with CO_2.

4.5. Keep the blood at RT for 2 h, then 4 °C overnight. Centrifuge at 900 x *g* for 10 min at 4 °C and collect the supernatant. Inactivate the sera by placing it at 56 °C for 30 min.

4.6. Store the immune sera in a -80 °C refrigerator for use.

NOTE: Refer to **Supplementary Figure 1** for the flowchart that describes the major procedures of mouse immunization.

5. Pseudovirus neutralization (PN) assay.

5.1. Make single-cell suspensions of MDCK cells (5 x 10^4 per mL) in complete DMEM medium, and add 100 µL of the cell suspension to each well of a 96-well flat-bottom plate. Incubate the cells in a 37 °C, 5% CO_2 incubator for 20 h.

5.2. Take out the pseudovirus and sera from the -80 °C refrigerator, thaw it on the ice, and vortex thoroughly.

5.3. Dilute the sera in the complete medium 20 times, and add 100 µL of complete DMEM medium to the wells of a 96-well round-bottom plate from columns 2–10.

5.4. Add 200 µL of the diluted sera into the wells of column 2, transfer 100 µL from column 2 to column 3, dilute the sera as 1:20, 1:40, 1:80, etc., to 1:10240 successively from column 2 to column 10, and discard the 100 µL from column 10.

5.5. Add 100 µL of DMEM complete medium to the wells of column 11 as the negative control.

5.6. Add 100 µL of pseudoviruses to each well, pipette up and down the mixture 5 times thoroughly, and incubate the mixture plate in a 37 °C, 5% CO_2 for 1 h.

5.7. Transfer 100 µL of the mixture from the 96-well round-bottom plate to the corresponding well of the 96-well cell culture plate. Put the plate back in the incubator (37 °C, 5% CO_2) for 60 h.

5.8. Take the plate out of the incubator. Perform the luciferase assay as described in steps 3.7–3.11.

6. Pseudovirus attachment assay

6.1. Make single-cell suspensions of MDCK cells (4 x 10^4 per mL) in complete DMEM medium, and add 100 µL cell suspensions to each well in a 96-well flat-bottom plate. Incubate the cells at 37 °C, 5% CO_2 for 20 h.

6.2. Incubate the pseudovirus with sera in DMEM containing 1% BSA at 4 °C overnight.

NOTE: The volume of the mixture is 100 µL.

6.3. Block the MDCK cells with 100 µL per well of DMEM containing 1% BSA at 4 °C for 1 h.

6.4. Inoculate the pseudovirus and serum mixture (100 µL) onto the MDCK monolayer and incubate it at 4 °C for 2 h.

6.5. Wash the cell plate 4 times with PBS containing 1% BSA to remove any unbound virus.

6.6. Lyse the cells with 100 µL of luciferase lysis buffer at RT for 30 min.

6.7. Quantify the amount of bound virus on the cells with an HIV-1 p24 ELISA kit as described above (section 2.3).

7. Assessment of viral entry

7.1. Make single-cell suspensions of MDCK cells (5 x 10^4 per mL) in complete DMEM medium, and add 100 µL of the cell suspension to each well of a 96-well flat-bottom plate. Incubate the cells at 37 °C, 5% CO_2 for 20 h before the assay.

7.2. Inoculate the pseudovirus (100 µL) onto an MDCK monolayer in a 96-well plate at 4 °C for 6 h.

7.3. Wash the cell plate 4 times with PBS containing 1% BSA to remove unbound pseudoviruses.

7.4. Add 100 µL of the serially diluted immune sera or sera from sham vaccination mice in DMEM containing 1% BSA to the monolayer, and incubate cell at 4 °C for 2 h.

7.5. Remove the cell supernatant, and wash the cell culture plate 4 times with PBS containing 1% BSA.

7.6. Add fresh DMEM to the cells and incubate for 60 h in a 37 °C, 5% CO_2 incubator.

7.7. Measure the relative luciferase activity (RLA) of the cells as described in steps 3.7–3.11.

NOTE: Viral entry, as indicated by RLA, was expressed as a percentage of the reading obtained in the absence of sera, which was set as 100%.

REPRESENTATIVE RESULTS:

HA, NA and HIV-1 p24 protein expression of influenza pseudovirus

To identify the viral packaging efficiency, influenza pseudovirus stocks were first detected by HA assay (**Figure 2A**). HA units per milliliter of influenza pseudoviruses are 643 (**Table 3**). Western-blot assay and sandwich ELISA assays were used to test HA, NA, and HIV-1 p24 protein expression. Then the ratios of HA unit and the amount of gag p24 for pseudoviruses were calculated. The results of the western-blot assay showed that there were 4 protein types: HA0, HA1, HA2, and NA proteins (**Supplementary Figure 2**). This indicates that the envelop proteins of influenza pseudoviruses are similar to that of wild-type viruses. The ratios of HA and Gag p24 were within a normal range as reported (**Table 3**)[9].

[Place **Table 3** here]

Pseudovirus titration

To measure the concentration of functional viral particles, relative luciferase activity (RLA) of pseudovirus was detected. RLA readings are dependent on many variables. To identify the effect of transduced cell amount on RLA readings, we seeded the cells of 1250, 2500, 5000, 10000, 20000, and 40000 per well of 96-well plate (**Figure 2**). Results showed that RLA readings of pseudovirus are the highest, and the standard error of mean is the lowest when 5000 cells are seeded in a well of 96-well plate (**Figure 4A**). To identify the effect of incubation time, RLA readings are detected at 48 h, 60 h, and 72 h after virus infection. Results showed that when incubated for 60 h, the RLA readings of pseudovirus are higher in values and better in repeatability with low standard error of the mean (**Figure 4B**). Results indicate it is suitable to seed 5000 cells in a well of a 96-well plate and detect RLA readings 60 h after virus infection.

PN assays

Neutralization titers of immune sera from the control group and the DDV group were measured by PN assays (**Figure 5**). As shown in **Figure 5A**, the immune sera from the DDV group exhibited high neutralization titers against pseudovirus A/Thailand/1(KAN)-1/04 strain. In contrast, sera from the control group did not exhibit any neutralization activity (**Figure 5B**). Compared with traditional serological assays (HI and MN assays), PN assays are more sensitive (Data are not showed).

Attachment neutralization assay

Some neutralization antibodies can hinder viruses' attachment to the sialic acid receptors. These antibodies are directed to the HA head and are predominant after immunization by commercial vaccine or infection of influenza virus. To identify the neutralizing activity of these antibodies, attachment neutralization assays are performed (**Figure 3**). Compared with the control sera, the neutralizing activity of immune sera is potent which indicates there are a lot of antibodies directed to the HA head in the immune sera (**Figure 6A**).

Assessment of viral entry

This assay is used to identify antibodies that hinder the fusion of virus envelop and endosomal membranes during influenza virus infection. These antibodies are directed to the HA stalk domain and are commonly less potent. To measure the neutralization titers of these antibodies, post-attachment assays are performed (**Figure 3**). There are significant differences between the immune sera and the control sera, which indicates there are antibodies directed to the HA stem region in the immune sera (**Figure 6B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Flowchart of Calcium mediated transfection. This flowchart is used to describe major procedures of Calcium mediated transfection.

Figure 2: Quantification and titration of pseudovirus. (**A**) Flowchart of pseudovirus HA assay describing the major steps of pseudovirus HA assay. (**B**) Flowchart of Pseudovirus P24 assay describing the major steps of pseudovirus P24 assay. (**C**) Flowchart of Pseudovirus titration assay describing the major steps of pseudovirus titration assay.

Figure 3: Pseudovirus-based neutralization assays. (**A**) Flowchart of PN assay describing the major steps of PN assay. (**B**) Flowchart of pseudovirus attachment assay describing the major steps of attachment assay. (**C**) Flowchart of Assessment of viral entry assay describing the major steps of viral entry assay.

Figure 4: Pseudovirus titration. (**A**) Different cell amounts per well in a 96-well plate influence RLA readings. 1250, 2500, 5000, 10000, 20000, and 40000 cells were seeded in a 96-well plate. (**B**) Different harvest time influences RLA readings. RLA readings are detected at 48 h, 60 h, and 72 h after virus infection. Data collected from three independent experiments are presented as mean ± SEM; error bars represent the standard error of the mean (SEM). One-way ANOVA with Tukey test was performed. *** $P < 0.0001$; ns, not significant.

Figure 5: H5 neutralizing antibody responses detected with pseudovirus. (**A**) Titration of neutralizing antibody responses of the immune sera from the DDV group. (**B**) Titration of neutralizing antibody responses of the immune sera from the control group. The IC_{50} is defined as the reciprocal dilutions of the neutralizing antibody that can inhibit 50% of the pseudovirus. Data collected from three independent experiments are presented as mean ± SEM; error bars represent the standard error of the mean (SEM). VSVG pseudovirus was used as a negative control.

Figure 6: Detection of neutralization antibody potency by binding and viral entry assays. (**A**) Immune sera from the DDV group, not that from the control group, inhibit viral attachment to cells. (**B**) Immune sera from the DDV group, not that from the control group, partially inhibit viral post-attachment infection. Data collected from three independent experiments are presented as mean ± SEM; error bars represent the standard error of the mean (SEM).

Table 1: Complete DMEM medium composition.

Table 2: Pseudovirus packaging system.

Table 3: Quantification of H5 pseudovirus.

Supplementary Figure 1: Flowchart of mouse immunization. This flowchart describes the major procedures of mouse immunization. The immune sera were used as samples.

Supplementary Figure 2: HA, NA and HIV-1 p24 protein expression of influenza pseudovirus. Western-blot of pseudovirus proteins.

DISCUSSION:

HEK293FT cells are usually used as packaging cells to produce pseudoviruses. Regular mycoplasma detection is essential during the cell culture. Mycoplasma contamination can drastically decrease the pseudovirus yields and sometimes close to zero. Compared with other contaminations, mycoplasma contaminations do not lead to pH value changes or turbidity of the cell culture medium. Even a high concentration of mycoplasma is not visible with naked eyes or under a microscope. Three popular methods of mycoplasma detection are mycoplasma culture, DNA staining with fluorescent DAPI or Hoechst 33258, and polymerase chain reaction (PCR). PCR amplifying the mycoplasma DNA in culture samples is widely used for fast, easy, and reliable mycoplasma testing[10].

Calcium phosphate-mediated transfection has been used for influenza pseudoviruses packaging for many years due to its high efficiency, low cost, and easy operation[11]. However, this method is very sensitive to the pH value of a HEPES-buffered solution, the HEK293FT cell culture medium, and the double-distilled H_2O. pH value affected the formation of precipitate and the duration time of the precipitate's stay on HEK293FT cells. Thus, it can determine the DNA amount that is taken up by HEK293FT cells. The pH for a HEPES-buffered solution is extremely narrow restricted, ranging from 7.05–7.12[12–14], in which condition, a precipitate containing calcium phosphate and DNA is slowly formed in HEPES-buffered solution. If the pH is too high, the size of the precipitate will become so big that it can even kill the HEK293T cells. On the contrary, if the pH is too low, the precipitate cannot be formed, or the precipitate will be too small to settle down to the cell surface. Moreover, the pH of the HEK293FT cell culture medium also plays a role in transfection efficiency. During the transfection, the cell medium should be maintained between a pH of 7.2–7.4. Both hyper acidic and alkaline media will change the precipitate's size and the adsorption time of the DNA mixture on the cell surface. Additionally, double-distilled H_2O with different origins used in the DNA mixture can alter the pH of a HEPES-buffered solution. Thus, keeping a stable pH value of transfection solution and culture medium is crucial to guarantee high transfection efficiency. In addition, the transfection efficiency is also affected by 2.5 M $CaCl_2$ solution and the concentration of carbon dioxide (CO_2) in the cell incubator. During the transfection process, it is crucial to store the $CaCl_2$ solution properly and stabilize the CO_2 concentration in the incubator.

The most popular packaging system of influenza pseudovirus involves a multiple plasmids co-transfection approach. HA, NA, reporter, and lentiviral *gag* and *pol* genes are cloned into plasmid expression vectors separately, and then they are transfected into the cells simultaneously. To increase the protein expression, a Kozak consensus sequence is often added before the transcription start site.

It is necessary to prepare pure, supercoiling, and endotoxin-free plasmid DNA[15]. Since plasmid ratio directly affects the pseudovirus yields. This protocol optimized the plasmid ratio of the "core: reporter: HA: NA" to 28:28:4:1 and compared it to other transfection methods. It is required that the amount of the plasmid DNA in calcium phosphate transfection should reach up to 30.5 μg per 100 mm dish.

Pseudovirus titers are detected by quantitative real-time PCR (qRT-PCR), enzyme-linked

immunosorbent assay (ELISA), hemagglutination assay, and RLA detection assays. qRT-PCR assay is used to estimate copy numbers of RNA genes of pseudovirus interior[5]. ELISA assay is used to detect the content of p24 protein, which is the principal component of the HIV core. Hemagglutination assay measures HA spike quantity on the surface of the pseudovirus particles. These three assays are employed to quantify unfunctional viral particles. RLA detection assay is the major method for measuring the concentration of functional viral particles which can infect transduced cells and release reporter genes in pseudovirus stock. MDCK cells are commonly used as transduced cells. Transduced cells are seeded in 96-well culture plates, infected by pseudovirus, lysed by lysis buffer, and then transferred to 96-well luminometer plates to read values. Different cell amounts and incubation times influence RLA values. Based on the results, the reporter gene expression is high when 5000 cells are seeded per well on a 96-well plate, and RLA is detected at 60 h after virus infection.

H5 HPAI pseudovirus neutralization (PN) assays have been widely applied for antibody detection because of their safety, higher sensitivity, accuracy, and suitability for high-throughput screening. A general protocol of PN assay includes the following four steps: pseudovirus quantities, serum dilution, pseudovirus-antibody incubation and luciferase activity detection. The quantities of pseudovirus are normalized based on RLA readings. RLA values of 10^6 per well in a 96-well plate are used to obtain reproducible experiment results. The start point of serum sample dilution must be over 40. The serum sample component will obviously increase luciferase values of pseudovirus when the dilution factor of the serum sample is less than 40. Pseudovirus-antibody mixture is incubated for 1 h at 37 °C and then transferred to MDCK cells, followed by RLA readings detection in 60 h. Luciferase levels are generally taken as a tool to determine pseudovirus infection efficiency.

In PN assay, there are four important elements: the luciferase reporter protein, the assay chemistry, a luminometer, and microplates[16]. Firstly, the luciferase gene is codon-optimized to improve protein expression levels in transduced cells. Engineering the reporter and vector backbone genes reduces the number of consensus transcription factor binding sites to cut down the anomalous transcription risk. Secondly, proper detection reagents are selected according to the experiment's aim. PN assays are generally used as high-throughput methods to detect neutralizing responses of serum or antibody samples. This protocol used the bright-Glo luciferase assay system, which offers the brightest signal, wide dynamic range, and high sensitivity. More importantly, the half-life of the signal is approximately 10 min, and the cells are immediately tested after the substrate is added. A luminometer is used to detect luciferase concentration. Many different types of luminometers are manufactured. The basic principle is that the machine used can precisely identify differences in luciferase activity between different samples. Black or white polystyrene microplates should be used for opacity and low luminescent backgrounds. White microplates can enhance luminescent signals and have low background luminescence, while black microplates can minimize light scattering to reduce well-to-well cross-talk[17].

PN assays are in line with traditional methods for detecting neutralizing antibody responses. However, including only HA and NA proteins from influenza wild-type viruses, pseudoviruses are far from the wild-typed virus. HA can bind to the sialic acid receptor of the recipient cell to initiate

infection. Then the HA conformational changes in the endosome trigger the fusion of the viral and endosomal membranes, releasing the viral ribonucleoproteins (vRNPs) into the cytoplasm. Influenza pseudoviruses can only imitate these processes and are applied in related researches.

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DISCLOSURES:

The authors have nothing to disclose.

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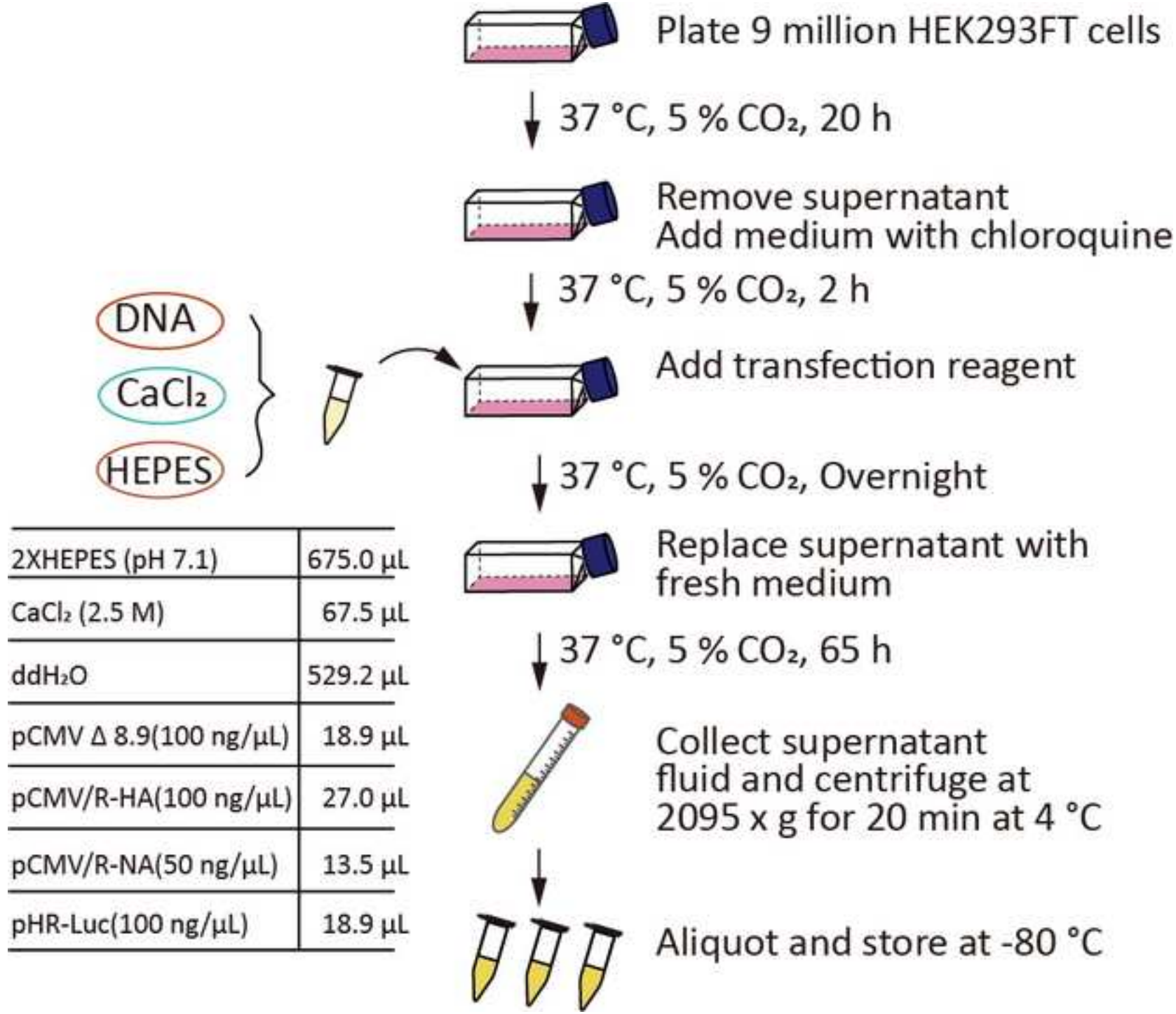
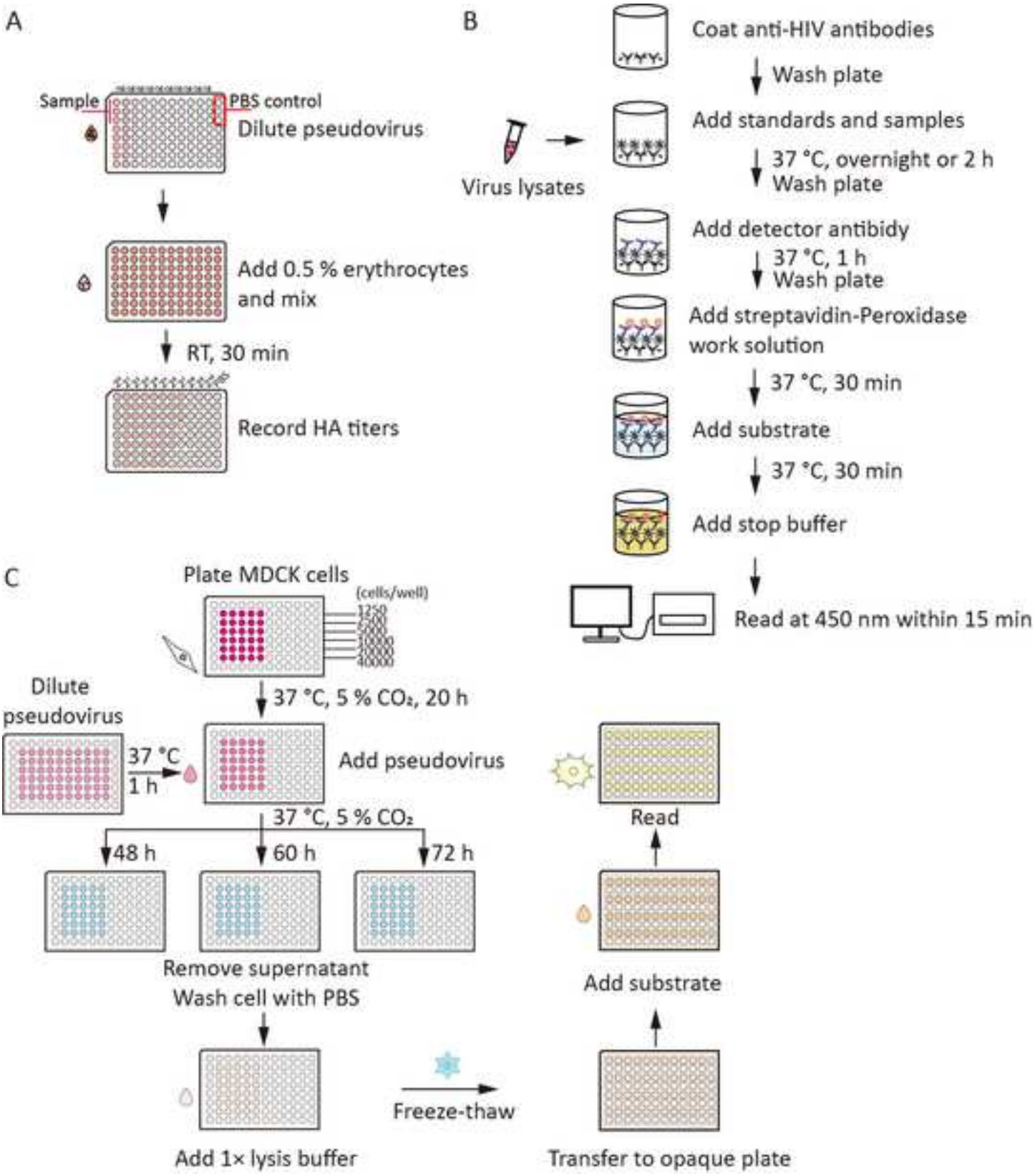
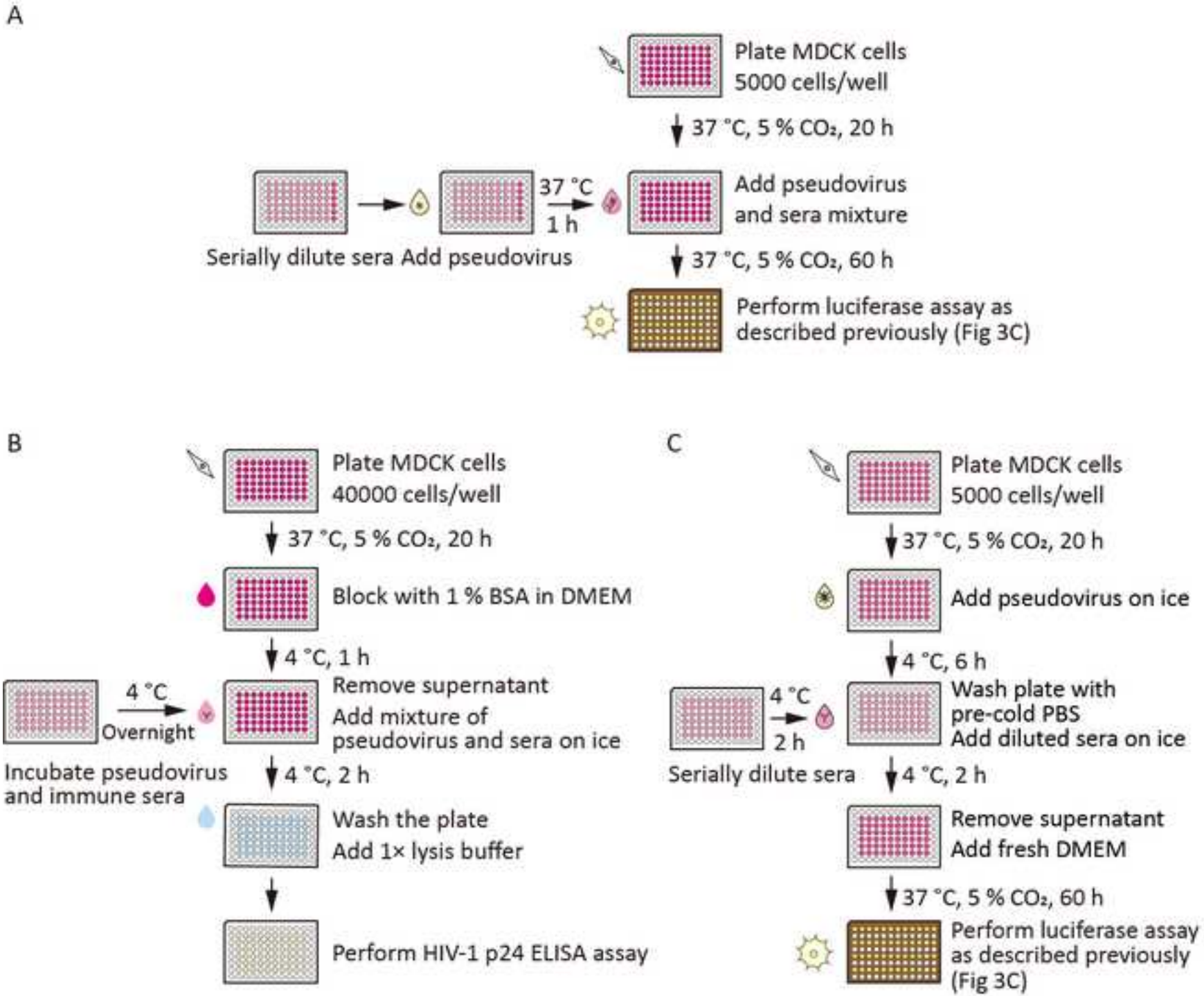


2XHEPES (pH 7.1)	675.0 µL
$CaCl_2$ (2.5 M)	67.5 µL
ddH_2O	529.2 µL
pCMV Δ 8.9(100 ng/µL)	18.9 µL
pCMV/R-HA(100 ng/µL)	27.0 µL
pCMV/R-NA(50 ng/µL)	13.5 µL
pHR-Luc(100 ng/µL)	18.9 µL

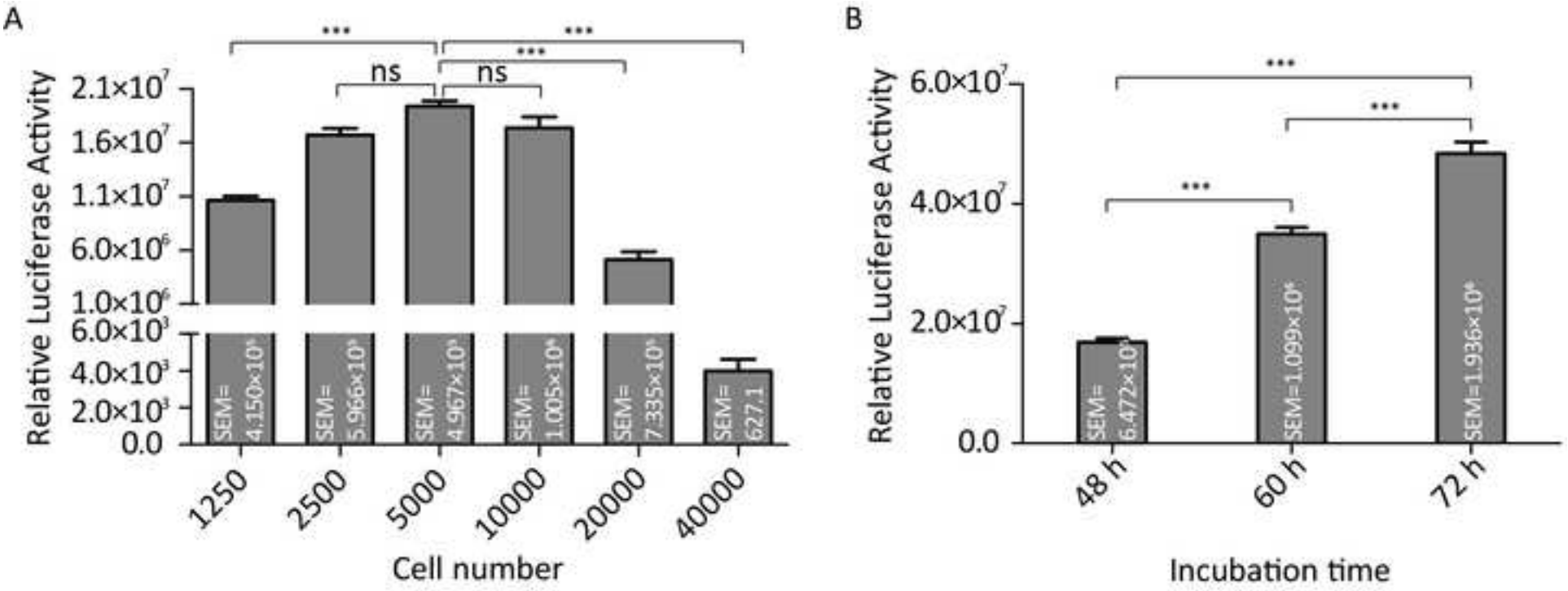


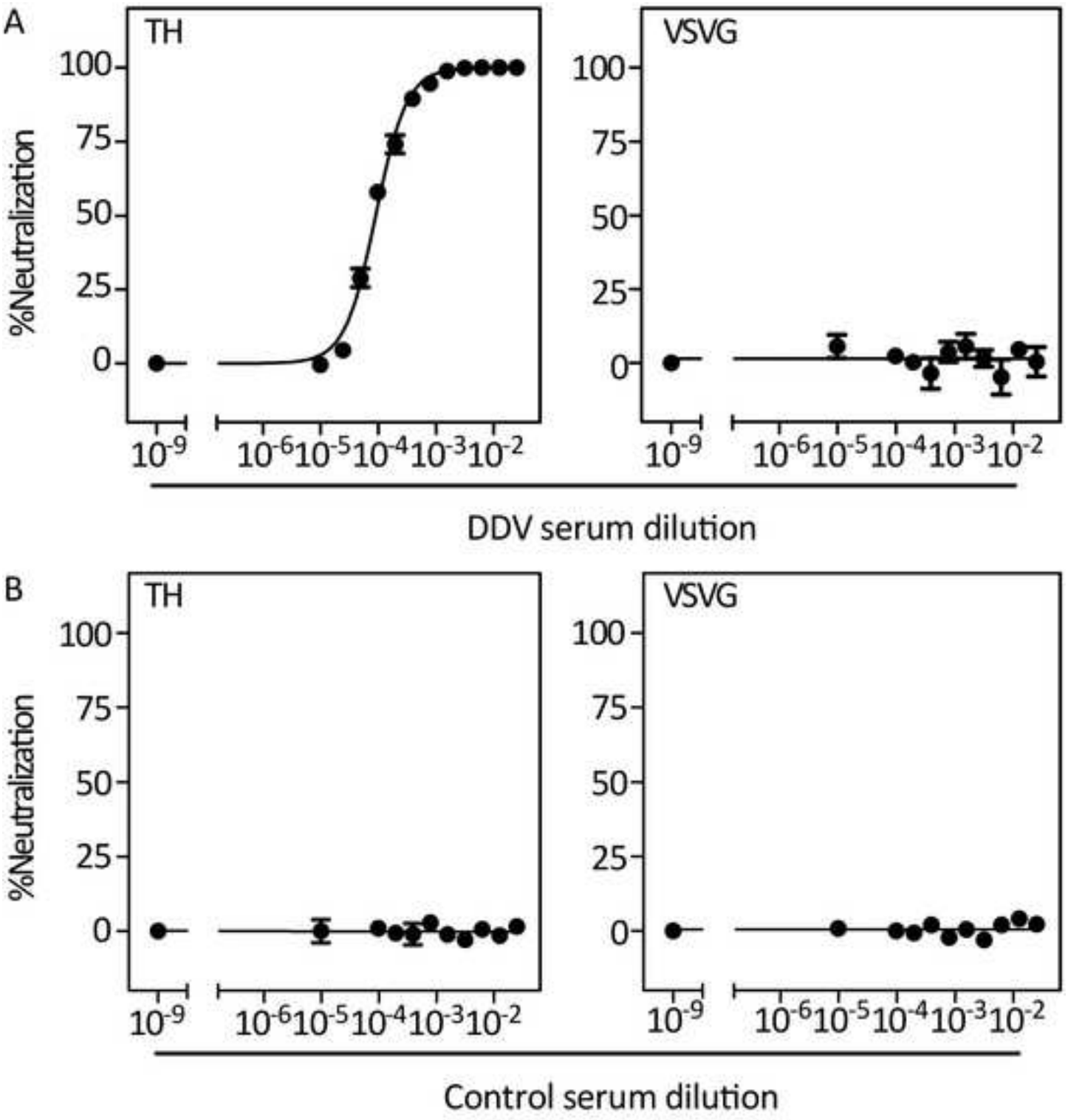
Flowchart

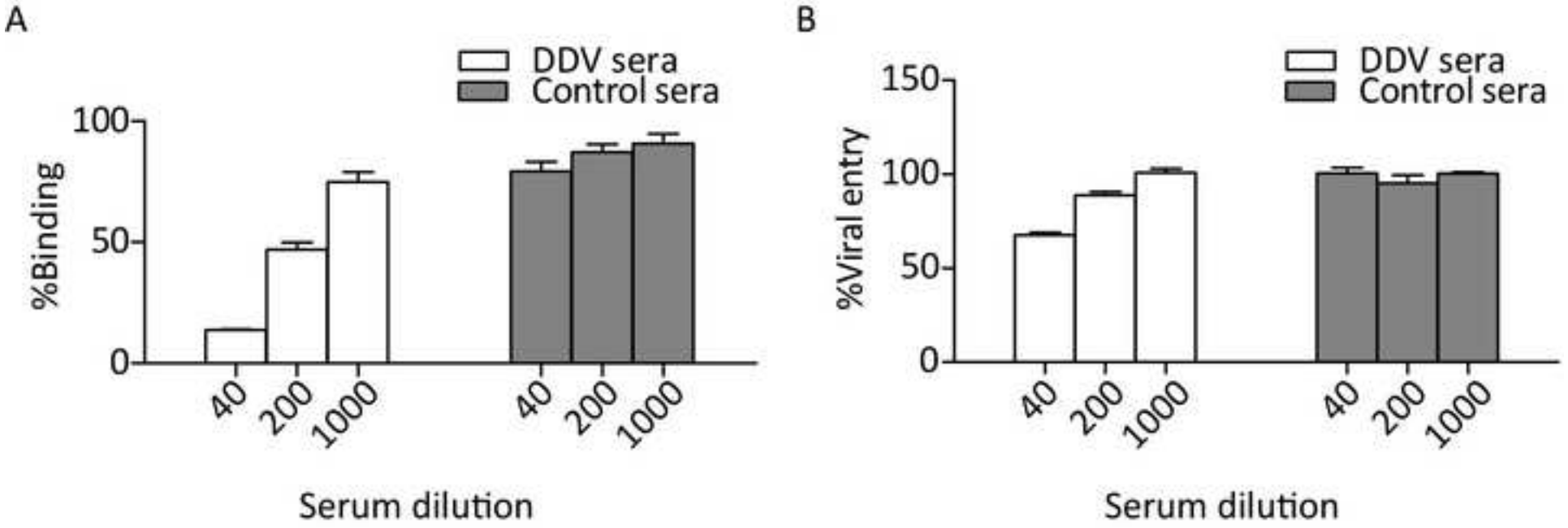


Result

Click here to access/download;Figure;Figure 4.jpg







Complete DMEM Medium Composition	Concentration
Dulbecco's Modified Eagle Medium (DMEM)	1x
Fetal Bovine Serum	10%
Penicillin(1 U/mL)/Streptomycin	1 µg/ mL

Materials	Concentration	Volume
2x HEPES (pH 7.1)	--	675.0 µL
$CaCl_2$ (2.5 M)	--	67.5 µL
ddH_2O	--	529.2 µL
pCMV Δ 8.9	1000 ng/µL	18.9 µL
pCMV/R-HA	100 ng/µL	27.0 µL
pCMV/R-NA	50 ng/µL	13.5 µL
pHR-Luc	1000 ng/µL	18.9 µL

Table 3

Pseudovirus Strains	HAU/mL	1.00E + 05 pg/mL
P A/Thailand/(KAN-1)/2004	642.52 ± 30.26	4.20 ± 0.17

*The ratios of the HA units and the amount of HIV-1 Gag p24 in pseudoviruses w
amount of Gag p24.

Ratio of HAU/1.00E+05 pg*
152.98

were calculated as follows: HA units/the

Dear editor:

Thanks for your reply.

Our responses are as follows:

For the Editorial comments:

1. Please note that the manuscript has been formatted to fit the journal standard.

2. Please revise the lines to avoid the issue of plagiarism: 151-153, 389-392.

Response:

We reword the contents as follows:

Line 151-153:

Original:

NOTE: One HA unit in the haemagglutinin titration is the minimum amount of virus that will cause complete agglutination of the red blood cells. The last well that shows complete agglutination is the well that contains one HA unit.

Editorial:

NOTE: HA units of the pseudoviruses is the highest dilution factor of the virus that can cause 100% hemagglutination of the red blood cells.

Line 389-392:

Original:

The IC50 (dashed line) is defined as the reciprocal dilutions that result in 50% inhibition. Data collected from three independent experiments are presented as mean ± SEM; error bars represent the standard error of the mean (SEM).

Editorial:

The IC_{50} is defined as the reciprocal dilutions of the neutralizing antibody that can 50% inhibition of the pseudovirus. Based on three independent experiments, the data are presented as mean ± SEM and the error bars mean the standard error of the mean (SEM).

Additionally, we reworded the contents in line 397

Line 397-398:

Original:

Data collected from three independent experiments are presented as mean ± SEM; error bars represent the standard error of the mean (SEM).

Editorial:

Based on three independent experiments, the data are presented as mean ± SEM and the error bars mean the standard error of the mean (SEM).

3. Since the protocol focuses on using immune sera for the assays, please consider including a brief section/step in the protocol describing how the animals were prepared for the injections, the volume of injections, route of delivery, the requirement of anesthesia, etc. based on the Supplementary figure 2.
Regarding animal treatment in the protocol, please add the following information to the text:
a) Please specify the euthanasia method if any. Please mention how animals are anesthetized and how proper anesthetization is confirmed
b) Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.
c) For survival strategies, discuss post-surgical treatment of animals, including recovery conditions and

treatment for post-surgical pain.
d) Discuss maintenance of sterile conditions during survival surgery/injections.
e) Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.
f) Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

Response：
We added the protocol "4 immune sera preparation" under the protocol "3 pseudovirus titration"
And renumbered the following protocols.

The contents of protocol "4 immune sera preparation" as follows:

4. Immune sera preparation

NOTE: The immune serum will be used for cell-related experiments, and the experimental operation should be carried out under aseptic conditions.

4.1 Prepare 12 eight-week-old female BALB/c mice. Divide equally the mice into two groups.

NOTE: one group was DDV group and the other was negative control group.

4.2 DDV group immunization: prime twice intramuscularly with 100 μg of codon-optimized DNA plasmid encoding A/Thailand/(KAN-1)/2004 (TH) HA protein at week 0 and week 3 and boost once intraperitoneally with 512 HAU TH virus-like particles (VLP) at week 6.

4.3. Control group immunization: prime twice intramuscularly with 100 μg empty vector plasmid DNA at week 0 and week 3 and boost once intraperitoneally with HIV-1 gag VLP at week 6.

4.4. Collect mouse blood 2 weeks after the last immunization.

NOTE: Blood was collected through submandibular vein and the mice will be immediately euthanized with CO_2. The mice were anesthetized with pentobarbital sodium (65 mg/kg) at the time of blood collection.

4.5 Keep the blood at room temperature for 2 h, then 4°C overnight. Centrifuge at 900 x *g* for 10 min at 4°C and collect the supernatant. Inactivate 56°C for 30 min.

4.6 Store immune sera in -80°C refrigerator for use.

In addition, we deleted the repeated contents in line 92, the changes as follows:
Line 91-95:
Original:
To obtain the immune sera used in assays, this protocol selected the HA protein originating from the TH strain as the immunogen to immunize mice. In the immune group, BALB/c mice were primed twice with DNA plasmid encoding H5 TH HA and boosted once with virus-like particles

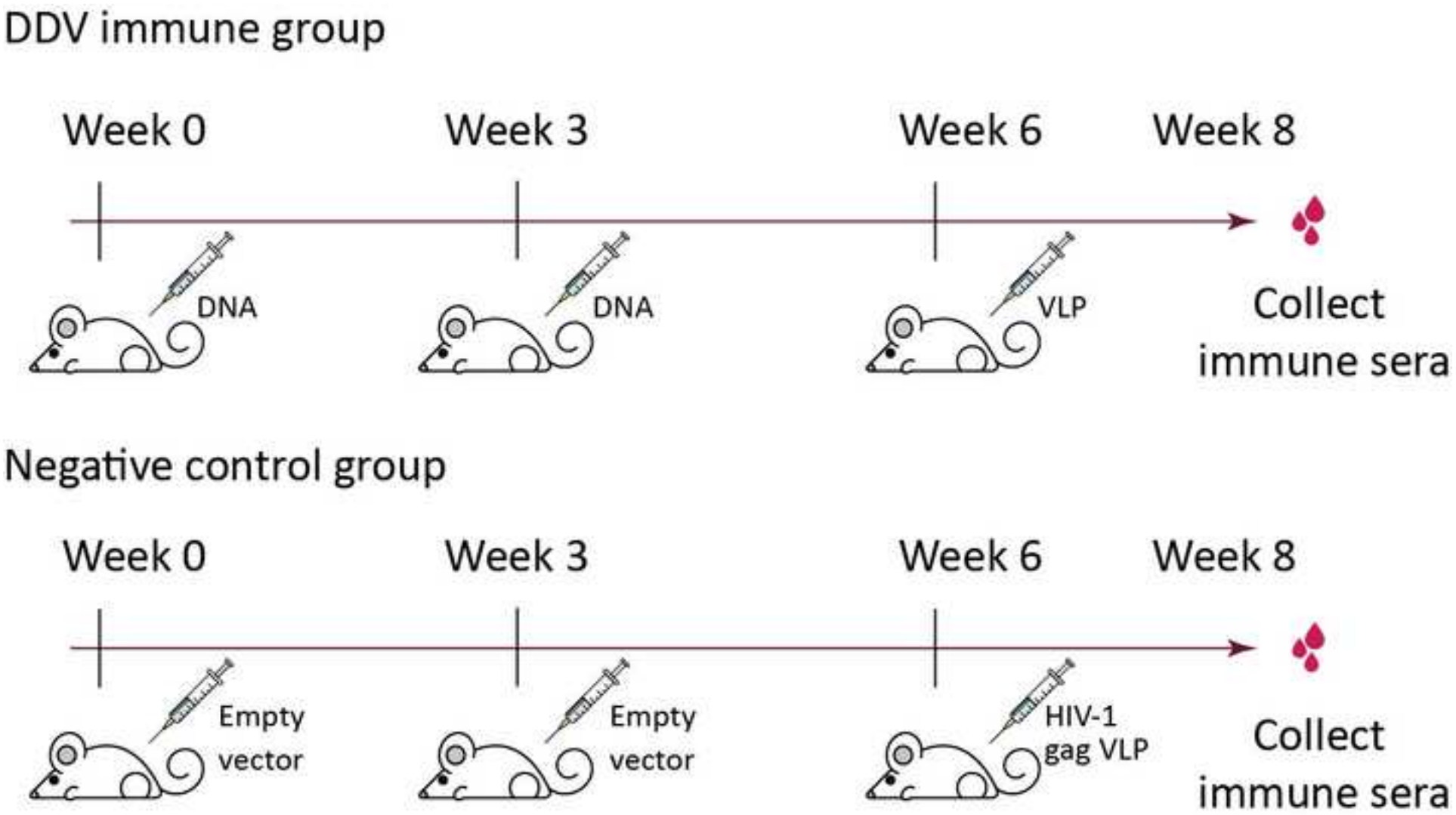
(VLPs) from the same strain. While for the control group, BALB/c mice were primed twice with the DNA plasmid of the empty vector and boosted with HIV-1 gag alone.

Editorial:
To obtain the immune sera used in assays, this protocol selected the HA protein originating from the TH strain as the immunogen to immunize mice.

Besides, the product information of pentobarbital sodium is added to the material table.

The protocols' number changes as follows
Original:
1. Pseudovirus packaging with Calcium-phosphate transfection
2. Detection of the HA, NA and HIV-1 p24 protein expression of influenza pseudovirus
3. Pseudovirus titration
4. Pseudovirus neutralization (PN) assay.
5. Pseudovirus attachment assay
6. Assessment of viral entry

Editorial:
1. Pseudovirus packaging with Calcium-phosphate transfection
2. Detection of the HA, NA and HIV-1 p24 protein expression of influenza pseudovirus
3. Pseudovirus titration
4. immune sera preparation
5. Pseudovirus neutralization (PN) assay.
6. Pseudovirus attachment assay
7.Assessment of viral entry



Supplementary Figure 2

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