

# Simple and rapid plaque assay for recombinant baculoviruses expressing influenza hemagglutinin

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## Abstract

Recombinant baculoviruses (rBVs) have been extensively used to generate virus-like particles, and baculoviruses expressing antigenic proteins have become efficient tools for inducing protective immunity. However, current methods for generating baculoviruses are costly and inefficient. Thus, the development of a simple, rapid, and accurate method of baculovirus titration is critically important. We established a method of plaque assay using an immunostaining method by which plaques can be easily visualized in Sf9 cells under a light microscope. Sf9 cells were infected with recombinant baculoviruses expressing influenza hemagglutinin surface proteins from H1N1 (A/California/04/09) or rH5N1 (A/Vietnam/1203/04). The infected cells were incubated with anti-HA antibody and the plaques were visualized using the chromogen 3′3-diaminobenzidine (DAB). Plaques were observed from days 1 to 6 post-infection, and differences in Sf9 cell seeding densities resulted in variations in the final plaque quantification. Sf9 cells seeded at a concentration of  $5.5 \times 10^4$  cells/well or  $7.5 \times 10^4$  cells/well showed the higher plaque titers at days 3, 4, and 5 post-infection than those found at days 1, 2, and 6 post-infection. With  $5.5 \times 10^4$  cells/well or  $7.5 \times 10^4$  cells/well of cell concentrations, recombinant baculovirus for rBV-HA (H1N1) showed  $6 \times 10^7$  pfu/ml of titer and rBVs for rBV-HA (rH5N1) showed  $5.4 \times 10^7$  pfu/ml of titer. Three days of baculovirus incubation with a certain concentration of Sf9 cells seeded are required for a rapid,

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simple, and accurate plaque assay, which could significantly contribute to all baculovirus-related studies.

### Keywords

Baculovirus, immunostaining, plaque assay, influenza virus, hemagglutinin

### Highlights

- Baculovirus plaques can be observed on day 1 post-infection in Sf9 cells.
- The highest plaque tiers were detected on days 3, 4, and 5 post-infection.
- Cell numbers at  $5.5 \times 10^4$  cells/well or  $7.5 \times 10^4$  cells/well showed the highest plaque titers.

### Introduction

Baculovirus expression vector system (BEVS) is a useful and convenient platform for the generation of recombinant proteins in laboratories and industries. BEVS has been used extensively in vaccine production<sup>1–3</sup> and pharmaceutical production,<sup>4</sup> and it is capable of producing high-yields of eukaryotic proteins that are similar in biological and immunological activity when compared to naturally occurring proteins.<sup>5</sup>

Baculovirus titration is necessary to determine the optimal dose for infecting host cells.<sup>5</sup> Researchers have reported various assays for the quantifying viruses, including plaque assay,<sup>5</sup> end-point dilution assay,<sup>6,7,8</sup> and 50% tissue culture infectivity dose assay.<sup>9</sup> However, current baculovirus titration methods are major time-consuming processes, requiring 6–10 days for plaque formation,<sup>10</sup> which increases the risk of cell damage.<sup>11</sup> It also requires the use of expensive equipment, such as polymerase chain reaction (PCR) or quantitative PCR (qPCR),<sup>12</sup> flow cytometry,<sup>13</sup> and enzyme-linked immune absorbent spot (ELISpot).<sup>14</sup> Additionally, the plaque assay is difficult to perform for an inexperienced researcher. For these reasons, it is critically important to develop simple and rapid baculovirus titration methods.

The hemagglutinin assay (HA) is one of the most important parameters in characterizing influenza virus activity. The hemagglutinin glycoprotein on the surface of the influenza virus binds to the sialic acid receptors on the surface of red blood cells (RBC), resulting in an agglutinated lattice structure which is shown as HA titer within a few hours by using chicken red blood cells (cRBCs).<sup>15</sup> We used recombinant baculoviruses expressing hemagglutinin proteins from influenza virus H1N1 or H5N1 since influenza hemagglutinin assay (HA activity) can be easily detected using chicken RBC. Thus, in the current study, recombinant baculoviruses expressing H1N1 HA or H5N1 HA were confirmed by HA assay and then plaque titers of rBVs were determined.

In this study, immunostaining method was applied in the plaque assay of rBVs expressing hemagglutinin (HA) from H1N1 (A/California/04/09) or rH5N1

(A/Vietnam/1203/04). We found that the concentration of Sf9 cells seeded in 96-well plates and the incubation period for rBVs were critical in plaque formation.

## Materials and methods

### *Viruses, cells, and antibodies*

A/California/04/09 (H1N1) and A/Vietnam/1203/04 (rH5N1) were propagated from 11-day old embryonated chicken eggs as described previously.<sup>16,17</sup> Allantoic fluid containing influenza viruses was collected and purified using ultracentrifugation and sucrose gradient (15%, 30%, and 60%) centrifugation. *Spodoptera frugiperda* (Sf9) cells were cultured in a serum-free SF900II medium (Invitrogen, Carlsbad, California, USA) at 27°C at a speed of 130–135 rpm.<sup>18</sup> Polyclonal antibodies against influenza virus were obtained by infecting mice with the influenza virus. Horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (IgG) (Southern Biotech, Birmingham, AL, USA) and diaminobenzidine (DAB) (Invitrogen) were prepared for the immunostaining process.

### *Cloning and generation of recombinant baculovirus*

Plasmid constructs containing HA genes from A/California/04/09 (H1N1) or A/Vietnam/1203/04 (rH5N1) were cloned, and baculoviruses were generated as described.<sup>18,19–21</sup> Briefly, total RNA from A/California/04/09 (H1N1) or A/Vietnam/1203/04 (rH5N1) were extracted and cDNA was synthesized. Then HA genes from the two viruses were PCR-amplified and cloned into pFastBac plasmid and transformed into DH10Bac competent cell. Transformant DNA was transfected into Sf9 cells and rBVs were produced.

### *Hemagglutination (HA) assay*

Influenza viruses A/Vietnam/1203/04 (rH5N1) and A/California/04/09 (H1N1) were two-fold serially diluted and added into a round-bottom 96-well plate. Then, 0.5% cRBCs were added and incubated at room temperature for 1 h. HA titer, which is defined as the reciprocal value of the highest viral dilution point at which cRBCs become completely agglutinated by viruses, was measured after incubation.

### *Seeding of Sf9 cells and baculovirus virus infection*

Sf9 cells were seeded into the wells of 96-well plate at various concentrations including  $1 \times 10^4$ ,  $4 \times 10^4$ ,  $5.5 \times 10^4$ ,  $7.5 \times 10^4$ ,  $8.5 \times 10^4$ , and  $9 \times 10^4$  cells/well. After 1 hour, baculoviruses serially diluted in SF900II media ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  dilution) were added to the wells. After 1 h of viral inoculation at 27°C, baculoviruses were removed, and 0.8% agar was added as the overlay. Overlaid agar was removed from each well of the 96-well plates on days 1, 2, 3, 4, 5, and 6 post-

infection. Before proceeding to the immunostaining process, cytopathological changes were monitored to confirm viral infectivity, and images were acquired under 200× magnification (Leica DMI8; Leica, Wetzlar, Germany) daily.

### *Immunostaining for plaque visualization*

After the overlay was removed, cells in 96-well plates were fixed with 37% formaldehyde for approximately 15 min and subsequently washed with PBST after formaldehyde removal. After incubating the cells with a blocking solution (3% skim milk) for 10 min at room temperature, they were washed with PBST and incubated with 100 µl of polyclonal anti-influenza antibodies at 37°C, 1 h. After 1 h, the wells were washed with PBST, and the plates were incubated at 37°C with HRP-conjugated goat anti-mouse secondary antibodies for 1 h. Next, the wells were washed with PBST and incubated with DAB substrate at room temperature (37°C) for 20 min. DAB substrate was removed by distilled water, and the plate was dried. Finally, plaques were counted and photographed under a 100× magnification on days 1, 2, 3, 4, 5, and 6 post-infection.

### *Statistical analysis*

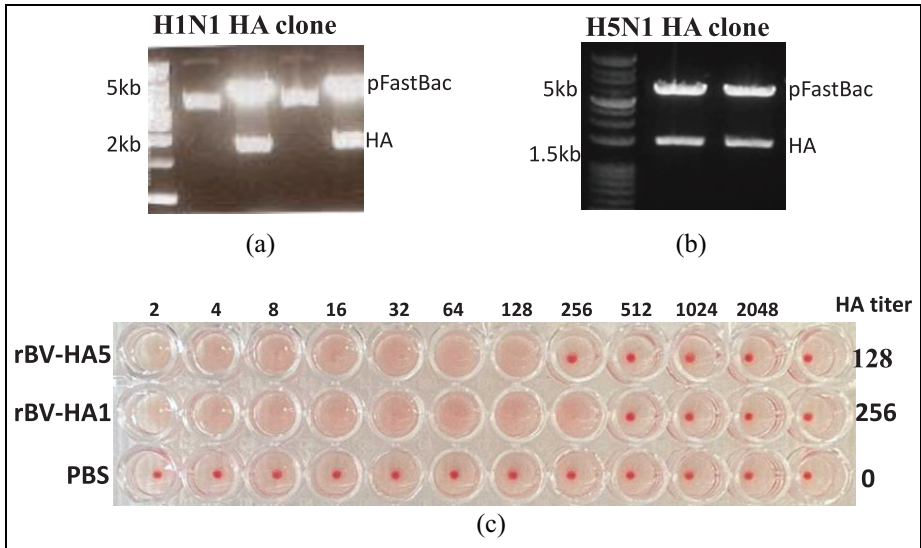
Statistical analysis was performed using GraphPad prism version 5 (San Diego, CA). Data was analyzed using one-way ANOVA with Tukey's post hoc test or 2-way ANOVA with Bonferroni's test. The data was considered statistically significant if  $p$  value \* < 0.05, \*\* < 0.01, \*\*\* < 0.001.

## **Results**

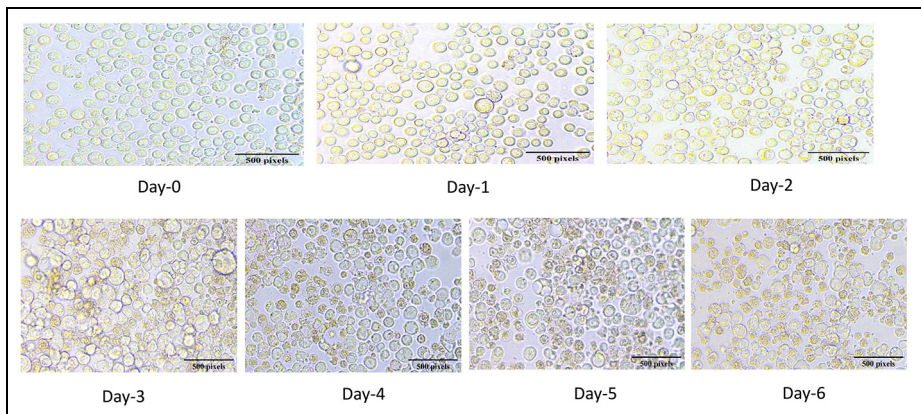
### *Recombinant baculoviruses were generated*

Constructs containing influenza HA from A/California/04/09 and A/Vietnam/1203/04 are shown in Figure 1(a) and (b). Incorporation of both HA genes into the pFastBac vectors was confirmed by using restriction enzyme digestion (EcoRI and XhoI) followed by gel electrophoresis and DNA sequencing (Figure 1(a) and (b)). Recombinant baculoviruses expressing influenza HA from A/California/04/09 showed 1:256 titer, and recombinant baculoviruses expressing influenza HA from A/Vietnam/1203/04 showed 1:128 titer (Figure 1(c)).

After restriction enzyme digestion, A/California/04/09 (H1N1) HA gene incorporation into the pFastBac vectors was confirmed. (A) Influenza HA gene from A/California/04/09 was incorporated into the pFastBac vectors. (B) Influenza HA gene from A/Vietnam/1203/04 (VN/04) gene was incorporated into the pFastBac vectors. (C) HA titers of baculovirus expressing HA from A/California/04/09 (H1N1) and from A/Vietnam/1203/04 (rH5N1) are shown. HA titers were found to be 1:256 for HA from A/California/04/09 and 1:128 for HA from A/Vietnam/1203/04.



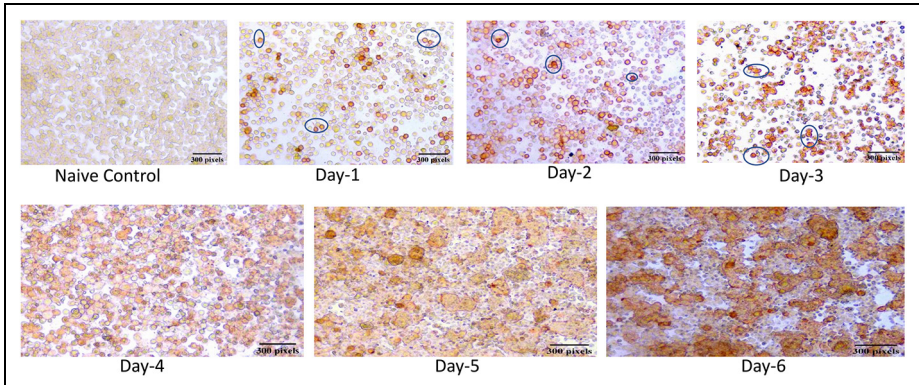
**Figure 1.** Gene cloning and HA titer for baculovirus.



**Figure 2.** Sf9 cells infected with recombinant baculovirus.

### *Cytopathological changes were observed*

Sf9 cells seeded in 96-well plates at various concentrations ( $1 \times 10^4$ ,  $4 \times 10^4$ ,  $5.5 \times 10^4$ ,  $7.5 \times 10^4$ , and  $9 \times 10^4$  cells/well) were infected with serially diluted baculoviruses ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$ ). Cytopathological changes were monitored under  $100\times$  or  $200\times$  magnification from days 1 to 6 post-infection to see changes in the size and shape of Sf9 cells. As seen in Figure 2, Sf9 cells showed



**Figure 3.** Visualization of plaques obtained from baculovirus expressing H1N1-HA.

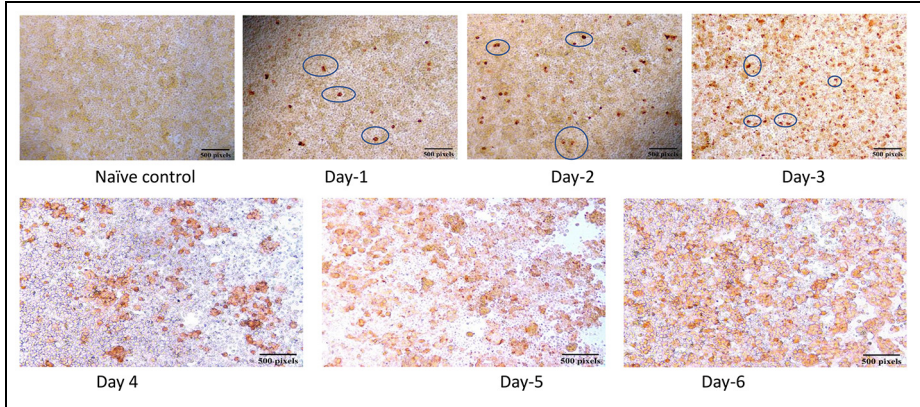
irregular cell shape and became larger at days 3 and 4 post-infection than they did at days 0 and 1 post-infection, indicating that Sf9 cells were infected with recombinant baculoviruses. When compared to naïve control cells, Sf9 cells infected with baculovirus showed gradual changes in terms of cell size and shape from days 1 to 6.

Sf9 cells were infected with baculovirus expressing HA from A/California/04/09 at serial dilutions ( $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ). Sf9 cells infected with the baculoviruses gradually increased in size with remarkable morphological changes. At days 2–3, cellular enlargement was observed from approximately 30% of the Sf9 cells. At days 4–5, most of the cells were dead. Cells were observed under  $200\times$  magnification with  $10^3$  viral dilution.

### Plaque determination

Sf9 cells were infected with recombinant baculoviruses. At days 1–6 post-infection, plaques were incubated with mouse polyclonal antibodies and goat anti-mouse HRP-conjugated IgG antibodies, and plaques were stained with DAB. As seen in Figures 3 and 4, plaques were clearly visible from days 1 to 6 post-infection for both baculoviruses expressing influenza H1N1 and H5N1, but more plaques were found at days 3, 4, and 5 when compared to days 1 and 6 (Figure 6(b)).

All images were acquired under  $100\times$  magnification and then cropped in the same ratio. Images show the plaque titration of wells in which  $7.5 \times 10^4$  cells/well concentration of seeded Sf9 cells were infected with  $10^{-3}$  viral dilution of H1N1-HA expressing baculovirus. This was done to show gradual changes in the size and shape of the plaques from days 1 to 6. Immunostaining was performed on days 1 to 6 for visualization of plaques, and naïve cells were included as control. No plaques were visible in the naïve control well. Plaques were clearly visible on day 1. From days 2 to 6, more plaques were observed. The plaques were circled in blue.



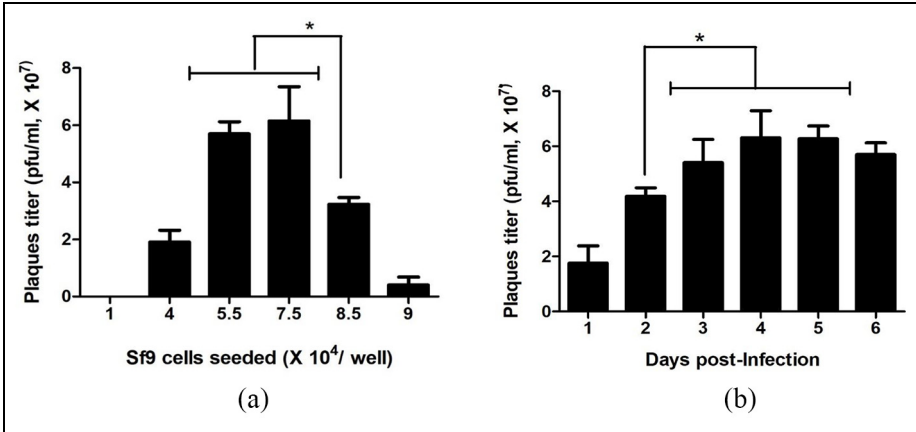
**Figure 4.** Visualization of plaques obtained from baculovirus expressing rH5N1-HA.

All images were acquired under  $100\times$  magnification and then cropped in the same ratio. Images show the plaque titration of wells in which  $7.5 \times 10^4$  cells/well concentration of seeded Sf9 cells were infected with  $10^{-3}$  viral dilution of VN/04-HA expressing baculovirus. This was done to show gradual changes in size and shape of the plaques from days 1 to 6. Immunostaining was performed on days 1 to 6 for visualization of the plaques, and naïve cells were included as control. No plaques were visible in the naïve control well. Plaques were clearly visible on day 1. From days 2 to 6, more plaques were observed. The plaques were circled in blue.

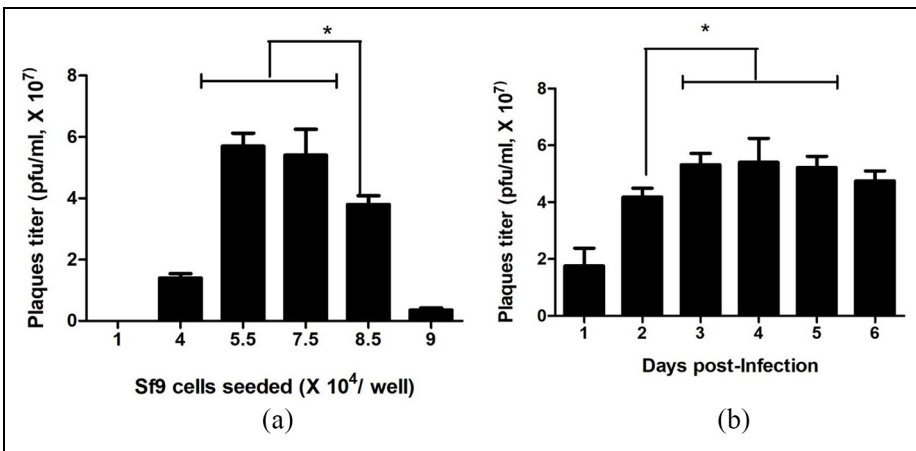
#### *Plaque visualization was affected by seeded Sf9 cell concentration*

Plaque titration was determined at various Sf9 cell concentrations ( $1 \times 10^4$ ,  $4 \times 10^4$ ,  $5.5 \times 10^4$ ,  $7.5 \times 10^4$ ,  $8.5 \times 10^4$ , and  $9 \times 10^4$  cells/well) to determine the optimum concentration at which plaques could be counted. As seen in Figures 5(a) and 6(a), plates seeded with  $5.5 \times 10^4$  cells/well and  $7.5 \times 10^4$  cells/well of Sf9 cells exhibited the highest number of plaques when compared to other concentrations of seeded cells. Negligible numbers of plaques were counted when cells were seeded at a concentration of  $1 \times 10^4$  cells/well. Multiple plaque titers were measured for each concentration of Sf9 cells seeded, and the optimum concentration was found to be  $6 \times 10^7$  pfu/ml for rBVs expressing HA from H1N1 and  $5.6 \times 10^7$  pfu/ml for rBVs expressing HA from H5N1.

Sf9 cells were infected with H1N1-HA expressing baculovirus. Figure 5(a) indicated that the highest plaque titer was obtained when  $7.5 \times 10^4$  cells/well of Sf9 cells were seeded, followed by  $5.5 \times 10^4$  cells/well, then  $8.5 \times 10^4$  cells/well. However, there was no significant difference in plaque titer between cell seeding densities of  $5.5 \times 10^4$  cells/well and  $7.5 \times 10^4$  cells/well. Figure 5(b) shows plaque titers determined on days 1–6 post-infection with Sf9 cells seeded at a concentration of  $7.5 \times 10^4$  cells/well. Plaque numbers gradually increased from days 1 to 5. There



**Figure 5.** Plaque titration of baculovirus expressing H1N1-HA.



**Figure 6.** Plaque titration of baculovirus expressing rH5N1-HA.

were no significant differences in plaque titer between days 3 and 4 and between days 4 and 5.

Sf9 cells were infected with baculovirus expressing HA from A/Vietnam/1203/04 (rH5N1). Figure 6(a) indicated that the highest plaque titer was obtained when  $7.5 \times 10^4$  cells/well of Sf9 cells were seeded. Figure 6(b) shows plaque titers determined on days 1 to 6 post-infection with Sf9 cells seeded at a concentration of  $7.5 \times 10^4$  cells/well. Plaque numbers gradually increased from days 1 to 5. There were no significant differences in plaque titer between days 3, 4, and 5.

## Discussion

In order to produce large amounts of recombinant protein by BEVS and generate virus-like particles, it is necessary to correctly quantify the existing infectious agents in viral stock. Although many laboratories have developed protocols for baculovirus titration, baculovirus plaque assay has always been a challenge and remains a problem. The plaques are unclear or difficult to obtain even after long periods of incubation with neutral red and crystal violet staining. Current protocols for plaque assay are time-consuming and inefficient, with plaque titration taking either 6–10 days or 4–7 days.<sup>22,23</sup> In this study, we developed a protocol for baculovirus plaque assay, in which clear plaques can be observed as early as day 1 post-infection by immunostaining.

In this study, baculovirus plaques were obtained on day 1 post-infection by an immunostaining method, and a reliable value of PFU/ml was obtained on days 3, 4, and 5 post-infection. We used immunostaining methods to visualize the plaques using a primary antibody against baculovirus expressing the protein of interest, which was followed by the addition of an enzyme-conjugated secondary antibody. Plaques were subsequently generated within 20 min by adding a chromogenic substrate. Anti-influenza polyclonal mouse antibodies were used as primary antibody, goat anti-mouse HRP-conjugated IgG antibodies were used as secondary antibody, and DAB was used as a substrate. This immunostaining method was more accurate than any dye staining method due to antigen-antibody specificity. Using this method, we concluded that 3 days of incubation is the optimum incubation time to obtain the highest number of plaques.

The rBV plaque assay has been designed using influenza HA expressing baculovirus because HA assay is a very important parameter for characterizing HA expressing baculovirus. Thus, rBV was confirmed first by HA assay then rBV plaque titers were determined. In our study, the plaque assays were performed at days 1, 2, 3, 4, 5, and 6 post-infection, providing detailed information on plaque assay from 6 different time points. Importantly, plaques can be observed as early as 1 day post-infection which has not been reported. Various Sf9 cell densities including  $1 \times 10^4$ ,  $4 \times 10^4$ ,  $5.5 \times 10^4$ ,  $7.5 \times 10^4$ ,  $8.5 \times 10^4$ , and  $9 \times 10^4$  cells/well were seeded to determine the cell numbers which showed the highest number of plaques. We found that cell densities  $5.5 \times 10^4$  cells/well or  $7.5 \times 10^4$  cells/well are optimal for showing the highest number of plaques. These results were not found in publications currently reported to date.

In this study, 96-well plates were used so that many samples could be tested using low amounts of primary and secondary antibodies, and low amounts of substrate were needed compared to the amounts needed for 6 or 12-well plates. There was no need for FBS in the Sf9 cell culture media, and 0.8% agar was used for the overlay instead of using methyl cellulose, which is expensive. Most importantly, no expensive equipment was needed. Therefore, this assay was extremely cost-effective.

It has been reported that baculovirus-infected insect cells increase in size, and some cells become larger than others due to the occurrence of nuclear

hypertrophy.<sup>24</sup> Also, some cells exhibited several cytopathological changes such as irregular cell morphology. Cytopathological changes were monitored to confirm that the infection of Sf9 cells with baculovirus was successful.

In this study, we compared plaques titers when different concentrations of Sf9 cells were seeded. We found that  $7.5 \times 10^4$  or  $5.5 \times 10^4$  cells/well were the optimum concentration to generate the highest plaque titer, and plaque numbers were reduced when  $9 \times 10^4$  cells/well were seeded. We found that different plaque titers were quantified from various incubation times following infection. To date, the shortest time of incubation required for baculovirus plaque assay by immunostaining is 48 h.<sup>25</sup> We observed plaques on day 1 post-infection, indicating that this method is highly sensitive and reliable. Furthermore, plaque titers were observed at longer incubation times with baculovirus and at various concentrations of Sf9 cells seeded.

In this study, we developed a simple and rapid protocol for baculovirus plaque assay with several advantages as described above. However, there are several limitations to this method. First and foremost, compared to the traditional plaque assay method, the method described here requires additional reagents such as DAB and antibodies, which may be considered expensive depending on the lab's financial status. Additional washing can disrupt cell integrity and lead to cellular detachment, which can alter the outcome of the experiment. Moreover, hazardous and toxic material build-up from formaldehyde fixation can be a detriment.

## Conclusion

In conclusion, we were able to develop a method of baculovirus titration that is simple and less time-consuming, allowing any novice researcher to easily and accurately perform this assay within a limited time frame. Visible baculovirus plaques were obtained on day 1 post-infection, but 3 days of incubation resulted in the highest plaque titer.

## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Ethics approval

Ethical approval for this study was obtained from \*NAME OF ETHICS COMMITTEE OR INSTITUTIONAL REVIEW BOARD (APPROVAL NUMBER/ID)\*, Or Ethical approval was not sought for the present study since human subjects were not involved.

## Animal welfare

All experimental protocols involving animals have been approved by the Kyung Hee University IACUC (Approval number: KHUASP-SE-18-024). All animals were housed in a specific-pathogen-free facility with 12 h day and night cycle. Prior to infection and bleeding, mice were anesthetized to ensure minimal pain infliction in accordance with the institutional IACUC guidelines.

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## Author biographies

Swarnendu Basak is currently working as a project assistant at the Indian Institute of Technology Kanpur. He has worked with baculoviruses and investigated their potential as effective vaccine candidates against the influenza virus.

Hae-Ji Kang is a graduate student at Kyung Hee University, Department of Biomedical Science. Her major research area of interest involves the use of virus-like particle vaccines against influenza virus and other protozoan parasites.

Ki-Back Chu is a graduate student at Kyung Hee University, Department of Biomedical Science. His research interest revolves around immunomodulation of respiratory viral pathogens and inflammatory response regulation.

Judy Oh is an undergraduate student at the Emory University and is interested in influenza vaccine studies.

Fu-Shi Quan is a full professor (M.D., Ph.D) at Kyung Hee University, Department of Medical Zoology. She is an expert in the field of vaccinology and researches virus-like particles as vaccine candidates for multiple respiratory and parasitic diseases of global importance.