Rapid determination of baculovirus titers an antibody-based assay

M. S. Kwon, T. Dojima1 , and Enoch Y. Park*

Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529; Japan, 1Production Technology Laboratory, Kaken Pharmaceutical Co. Ltd., 301 Gensuke, Fujieda 426-8646,

*Phone: (81)-54-238-4887. Fax: (81)-54-238-4887. E-mail: yspark@agr.shizuoka.ac.jp.

Abstract

A novel method is developed to yield virus titers in 10 h, is easy to perform using 96-well plates, and applicable to both any *Autographa californica* nucleopolyhyderovirus (AcNPV) and *Bombyx mori* nucleopolyhedrovirus (BmNPV)-based recombinant baculovirus. This assay uses an antibody to a DNA-binding protein to detect the infected cells via immuno-staining. The titer is determined by counting foci produced due to infection of virus under a fluorescent microscopy. The required incubation period was shortened considerably because infected cells expressed viral antigens at the post infection time of 4 h. Therefore, 10 hours were enough to estimate the virus titer including virus infection time, insect cell culture, and estimation of virus titer.

Key words: DNA-binding protein, DBP, Anti-DBP, AcNPV, BmNPV, virus titer

Introduction

Insect cells are an attractive host for recombinant protein production via infection with a genetically modified baculovirus expression vector system (BEVS) allows for high production level of proteins that are functionally similar to the native one (1). However, to accomplish successful protein expression, it is necessary to determine titers of the recombinant baculovirus preparations at every step of virus preparation or amplification. The plaque assay (2) is one of potential method for exact determination of virus titer, but it is difficult to judge the formed plaque due to virus infection and it requires a long processing time of about 1 week.

In another approach to shorten the assay time end point dilution methodby introducing green fluorescent protein (GFP) gene (3), LacZ gene was introduced and the plaques were visualized (4) or the b-galactosidase activity was used (5) for determination of virus titer. Although it reduced

shortening the assay time, but, those methods are limited to recombinant baculovirus using introduced either GFP or b-galactosidase genes as reporter genes.

To avoid time consuming step it needs to judge whether cells are infected or not as possible as soon after baculovirus infection. Handling and measuring processes should be simple, and moreover, if the new titration method is to be applicable in both cell lines of *Spodoptera frugiperda* and *Bombyx mori,* it will be better. We noticed DNA-binding proteins, DBP and LEF-3, from nuclear lysates of BmNPV-infected BmN cells (6). DBP was found to be an early gene product during viral replication, so that the DBP was appeared at 4 to 6 h postinfection.In this report, the DBP was used for simple, rapid, and universally applicable titration method. Furthermore, we developed an antibody-based baculovirus titration method and adapted it possible for use in 96-well plates. This titer determination allows to obtain titers of baculovirus stocks in 10 hours, with very convenient and simple procedures.

Materials and Methods

Cell lines and Media. Sf9 cells (B825-01; Invitrogen, San Diego, CA, USA) derived from *Spodoptera frugiperda* were maintained as monolayer cultures in 25 cm2 tissue culture flask (353014; Falcon) at 28oC in TNM-FH medium (Sigma, St. Louis, MO, USA) supplemented with 0.35 g/L NaHCO3, 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and 1% antibiotic-antimycotic (Gibco BRL, Rockville, MD, USA).

Recombinant Baculovirus. *Autographa californica*nuclear polyhedrosis virus (AcNPV) inserted downstream of the polyhedral promoter with GFPuv gene (nGFPuv) was used.

Titer determination for recombinant baculovirus using Green fluorescent protein (GFP) marker. For performing end-point dilution, the virus stock sample were diluted serially 10-2 to 10-7. The titer of nGFPuv was calculated using the standard method (50% tissue-culture infectious dose: TCID50).

Titer determination for recombinant baculovirus by antibody-based titration assay using expression of DNA-binding protein (DBP). The method for dilution of virus stock sample was the same method as the titer determination using GFP. After mixing 200 ml of early logarithmic growth phase Sf9 cells in 96-well plates were infected by virus to be titered, and then the 96-well plates were incubated for 6 h. The medium was removed, and then 100 ml of 2% formalin in phosphate-buffered saline (PBS) was added to fix infected Sf9 cells. After 1% of bovine serum albumin (BSA) diluted in PBS was added to each well and the plate was incubated

for 1 h. Since the DNA-binding protein is produced for 6 h culture, the 96-well plates were followed to next assay procedure.The expressed DNA-binding protein was allowed to bind to anti-DBP for 1 h reaction. At last cells were washed and incubated with rodamine-conjugated goat anti-rabbit IgG for 1 h reaction. Wells were examined using a fluorescent microscope (IX-70, Olympus, Tokyo) and the foci formed due to infection were counted. The titer was calculated using the standard method (TCID50).

Results

Development of a 96-well plate rapid titer assay. We made the assay more convenient by adapting the procedure to use 96-well plates for standard tissue culture which required no preparation. The use of 96-well plates allows a multichannel pipettor to be used for addition, aspiration, and wash steps, thereby facilitating the titration of multiple virus stocks at the same time. Total reaction time was 9 h, and even though handling, pipetting times, and calculating time were included, ten hours would be enough to determine a virus titer. The Sf9 cells are not infected by virus, the fluorescence was not shown. However, at the postinfection time of 6 h, red foci could be detected and the fluorescent intensity was stronger and stronger with the increase in the DBP accumulation, therefore it is enough to judge whether the Sf9 cells were infected or not (Fig. 1.). This indicates that this antibody-based titration assay using DBP is useful to determine the virus titer and is a promising method to shorten virus titration time.

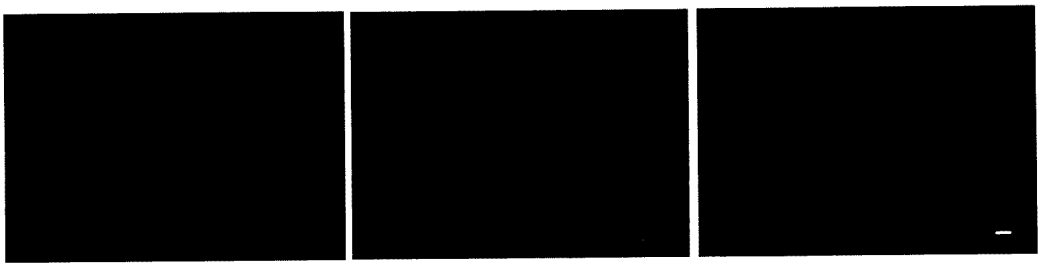

Figure 1. Identification of foci of infected Sf9 cells following the antibody-based titering procedure. Cells were infected with the nGFPuvstock (MOI 10) and antibody-based titration as described. Infected cells appeared at a magnification of 400x using fluorescent microscope. A, B, C denote mock-infected cells, cells at post infection time of 4 h, 6 h, and 10 h, respectively. The bar indicates 25 mm.

Validity of the assay. To test the validity of the pfu and compare the reproducibility of the antibody-based assay to that of the well-established end-point dilution method, we repeatedly

measured using the same virus stock in each method for at least ten times. The variability in titers obtained by the antibody-based assay and the end-point dilution assay using GFP were compared. The average titer of antibody-based assay was little higher than the end-point dilution assay using GFP, but both standard deviation and standard error were 51% lower than those of GFP, respectively. Confidence test for 95% showed 3.88x105 and 2.83x105 pfu/ml for two methods, respectively. Therefore, the antibody-based assay confirmed to be more accuracy than the end-point dilution method in the determination of baculovirus titers.

We tested the validity betweentwo methods of titer assay in the range of 103 to 107pfu/ml. In the case of the antibody-based assay using DBP the measured titer was well consistent with the estimated titer and its correlation coefficient was 0.99. However, the end-point dilution method using GFP the measured titers were deviated from the estimated titers and its correlation coefficient was 0.97.

Application of the assay to virus stocks of different titers. To test the validity of the virus titer using this antibody-based titration assay the virus titer were compared with those using end-point dilution assay using GFP. Virus titer of three unknown virus stock samples using this antibody-based titration assay was little higher or negligible than that of using GFP. The FTEST between two methods were 0.18. This result demonstrates that this antibody-based titration assay can be replaced with the conventional end-point dilution assay using GFP.

Discussion

It is very important to know the titer of a recombinant baculovirus stock, in plaque-forming units (pfu/ml), when new viral stocks or when achieving optimizing protein yields from the baculovirus expression system. The plaque assay (2) is one of potential method for exact determination of virus titer, but it is difficult to judge the formed plaque due to virus infection and it requires a long processing time of about 1 week. To overcome these difficulties end-point dilution method incorporating GFP or b-gal marker (3,4,5) was developed for simplification of the determination of virus stock titer, and the plaques are more easily visualized due to the expression of GFP or Lac Z gene. Although it reduced shortening the assay time, but, those methods are limited to recombinant baculovirus using introduced either GFP or b-galactosidase genes as reporter genes. Kitts and Green (7) shorten the assay time in 48 h with reliability. However, this assay is applicable to only *Autographa californica* nucleopoly hedrovirus-based recombinant baculovirus.

The assay we describe here can be used to titer any recombinant AcNPV virus and BmNPV virus, regardless of how the recombinant virus was generated. Furthermore, it is likely that this assay could also beused to titer baculovirus that have DNA-binding protein, which have been purified and characterized as DBP, from nuclear lysates of BmNPV-infected BmN cells (6). The DBP expression in insect cells begins from post infection of 4 h, but the assay of virus stock titer with accurate estimation need 6 hours. The reliability of this assay was demonstrated in the region of 103to 107 pfu/ml. Although the titers were deviated from those of end-point dilution using GFP, both standard deviation and standard error were 50% lower than those using GFP marker. This antibody-based titration assay could easily be adapted to work with other baculovirus, BmNPV simply by substituting another antibody. However the antibody is still expense, but if the antibody-based titration assay becomes widespread, the antibody will be used on the cheap. Theoretically, it should even be possible to develop similar assays for any virus that can be grown in tissue culture.

References

(1) Luckow, V. A. Protein production and processing from baculovirus expression vectors. In *Baculovirus Expression System and Biopesticides* Shuler, M. L., Wood, H. A., Granados, R. R., Hammer, D. A., Eds.; Wiley-Liss: New York, 1995; pp 51-90.

(2) Hink, W. F.; Vail, P. V. A plaque assay for titration of alfalfa loopernuclear polyhedrosis virus in a cabbage looper TN-368 cell line. *J. Invertebr. Pathol.* **1973**, *22*, 168-174.

(3) Cha, H. J.; Gotoh, T.; Bentley, W. E. Simplification of titer determination for recombinant baculovirus by Green fluorescent protein marker. *BioTechniques*, **1997**, *23*, 782-786.

(4) Sussman, D. J. 24-hour assay for estimating the titer of b-galactosidase expressing baculovirus. **1995**, *18*, 50-51.

(5) Yahata, T.; Andriole, S.; Isselbacher, K. J.; Shioda, T. Estimation of baculovirus titer by b-galactosidase activity assay of virus preparations. *BioTechniques*, **2000**, *29*, 214-215.

(6) Mikhailov, V. S.; Mikhailova, A. L.; Iwanaaga, M.; Gomi, S.; Maeda, S. *Bombyx mori*nucleopolyhedrovirus encodes a DNA-binding protein capable of destabilizing duplex DNA. *J. of Virol.*, **1998**, *72*, 3107-3116.

(7) Kitts, P. A.; Green, G. An immunological assay for determination of baculovirus titer in 48 hours. *Anal. Biochem.* **1999**, *268*, 173-178