A rapid method for titrating baculovirus stocks using the Sf-9 Easy Titer cell line

Ralph F. Hopkins and Dominic Esposito

Protein Expression Laboratory, Advanced Technology Program, SAIC-Frederick, National Cancer Institute at Frederick, Frederick, MD, USA

BioTechniques 47:785-788 (September 2009) doi 10.2144/000113238

Keywords: baculovirus; titrating; green fluorescent protein; GFP; Sf-9 cell line; method

Supplementary material for this article is available at www.BioTechniques.com/article/113238.

A new rapid method for titrating baculovirus stocks has been developed using a novel cell line Sf-9 Easy Titer (Sf-9ET). The Sf-9ET cell line has been transfected with plasmid DNA containing the enhanced green fluorescent protein (eGFP) gene under the control of the baculovirus polyhedrin promoter. When used in the titration assay, the Sf-9ET cells turn green when they are infected with baculovirus due to the activation of the polyhedrin promoter/eGFP complex by baculovirus gene products expressed during the infection. Using a 96-well plate format end-point dilution assay, baculovirus titers can be determined in three days using a fluorescence microscope.

The baculovirus expression vector system (BEVS) has proven to be a powerful technology for expression of recombinant proteins in eukaryotic cells (1,2). The BEVS technology has many advantages that include the capacity for large DNA insertions (3), the capacity for simultaneous expression of multiple genes, high expression levels for many proteins (4), and posttranslational modifications (5–7) that often resemble those found in mammalian cells.

We have used the BEVS technology to express hundreds of proteins and routinely titer the recombinant baculovirus stocks to ensure reproducible expression. Over the years, many different assay systems have been developed for quantitating baculoviruses. They include the original plaque assay (8), an end-point dilution assay with various improvements (1,9,10), an anti-gp64 antibody-based assay (11) and a PCR-based assay (12). The plaque and end-point dilution assays are lengthy and laborious, and the antibody and PCR assays measure total—as opposed to infectious—viral particles. Here we describe a GFP-based baculovirus titrating method that is convenient, direct, and accurate.

Baculovirus replication and gene expression occur in an ordered cascade of events, regulated by early, late, and very

late gene promoters (13). Early viral gene products act as activators for promoters of late genes such as the very powerful baculovirus polyhedrin promoter. The Sf-9ET cell line was created by transfecting normal Sf-9 cells with plasmid DNA containing the enhanced green fluorescent protein (eGFP) gene under the control of the baculovirus polyhedrin promoter. The plasmid also

contained the neomycin resistance gene that allowed selection of transfectants when grown in medium containing Geneticin (G418) (Invitrogen, Carlsbad, CA, USA) (14,15). Uninfected Sf-9ET cells do not express detectable GFP, while infection with baculovirus particles results in production of early viral proteins which turn on GFP expression from the integrated polyhedrin promoter. Release of viral particles and infection of adjacent cells results in small foci of green cells that are easily detected using a fluorescence microscope (Model no. CKX41; Olympus, Center Valley, PA, USA). Virus titers are determined in three days using a statistical method for calculating median tissue culture infectious dose (TCID₅₀) (16). We tried another method to score GFP-positive wells using a BMG Labtech Omega FLUOstar microplate reader (Durham, NC, USA), but this method was not as sensitive as the human eye in detecting small foci and required additional steps to remove the highly auto-fluorescent culture medium.

To perform the transfection, exponentially growing Sf-9 cells were placed in a 125-mL polycarbonate Erlenmeyer flask (Corning, Corning, NY, USA) in 25 mL HyClone SFX-Insect medium (HyClone Laboratories, Logan, UT, USA) at a density of 1×10^6 cells/mL. To prepare the DNA/lipid complex, 50 µg DNA (DNA preparation is described in the Supplementary Materials) were added to 1 mL normal saline (Quality Biologicals Inc., Gaithersburg, MD, USA) in a 5-mL round-bottomed test tube (Cat. no. 352063; Becton Dickinson Labware, Franklin Lakes, NJ, USA). To a separate tube, 125 µL Insect Gene Juice (Novagen, Madison, WI,

Figure 1. Sf-9ET cells infected with a recombinant baculovirus. Three days following infection, a high frequency of eGFP-positive plaques was observed.

Table 1. Comparison of virus stock titers determined using end-point dilution assays with the Sf-9 Easy Titer cell line vs. the standard Sf-9 cell line

Virus stock	Sf-9 Easy Titer cell line ^a	Standard Sf-9 cell line ^b
1	$2.0 \times 10^8/\text{mL}^c$	$2.4 \times 10^8/\text{mL}$
2	$7.5 \times 10^8/\text{mL}$	$7.5 \times 10^8/\text{mL}$
3	$8.8 \times 10^8/\text{mL}$	$8.8 \times 10^8/\text{mL}$
4	$1.1 \times 10^9/\text{mL}$	$9.7 \times 10^7/\text{mL}$
5	$2.1 \times 10^8/\text{mL}$	$2.1 \times 10^8/\text{mL}$

^aEnd-point assay was read at day three by scoring GFP-positive wells. ^bEnd-point assay was read at day seven by scoring wells positive for CPE. ^cTCID₅₀, median tissue culture infectious dose.

USA) was added to 1 mL saline. The two tubes were mixed, vortexed briefly, and then incubated at room temperature for 15 min to allow DNA-lipid complexes to form. Following the incubation step above, the DNA-lipid mixture was added to the 125-mL Erlenmeyer flask containing the 25-mL suspension of Sf-9 cells. The transfected cells were cultured at 27°C on a shaker at 100 rpm. After 24 h, G418 was added at a final concentration of 150 µg/mL to begin the selection process. Five days after starting the selection process, cell viability dropped to 54%, as non-transfected cells were killed by the G418. Sf-9ET cell viability improved to normal

levels (>95%) 12 days after starting the selection process. Routine culture transfers were performed twice a week using seeding densities of $3-4 \times 10^5$ cells/mL. The cell line was maintained in growth medium containing G418 at a concentration of 150 µg/mL. Since the transfection plasmid DNA was not constructed for episomal integration (no origin of replication) we believe that the genes have been integrated into the host cell genome; however, studies to determine the gene stability in the absence of G418 have not been performed. To verify that baculovirus infection of Sf-9ET cells would activate the polyhedrin promoter and induce expression of eGFP,

the following experiment was performed. Adherent cultures of Sf-9ET cells were infected with a recombinant baculovirus containing the gene for the recombinant protein cereblon. After three days of infection, the cultures were observed for expression of eGFP (Figure 1). Two rounds of single-cell cloning were performed to select a clone cell population that gave good eGFP expression following baculovirus infection. Sf-9ET cells were cultured for three months in the presence of G418 with no loss in the eGFP expression following infection. Recombination events do occur when the Sf-9ET cell line is infected with baculovirus. Progeny virus from the infection can result in GFP expression when normal Sf-9 cells are infected. The Sf-9ET cell line should not be used to propagate virus stocks.

End-point dilution assays were set using Sf-9ET cells grown to 1×10^6 cells/mL in SFX medium, as described in the transfection protocol above. Baculovirus titrations were carried out in black 96-well microtiter plates (Costar, Corning, NY, USA) to compare virus titer data determined by either scoring the eGFP-positive wells for each virus dilution or scoring wells

Eliminate Homogenizing Bottlenecks

Homogenize 6 samples in under a minute!

The NEW OMNI-PREP

Omni Tip™ Plastic Probes
completely eliminate
cross-contamination

1-800-776-4431
www.omni-inc.com

for cytopathic effect (CPE) of the virus. Starting at a dilution of 10^{-3} , the recombinant baculovirus was serially diluted $5\times$ in SFX medium, in replicates of eight, to a final dilution of $10^{-8.6}$. Duplicate titration plates were set and to one plate 7.5×10^4 Sf-9ET cells in 100 μ L SFX medium were added to each well. To the second plate, an equal number of Sf-9 cells in 100 μ L SFX medium were added to each well. Plates were incubated at 27°C without shaking. At three days post-infection, the Sf-9ET plate was scored by monitoring wells containing eGFP foci. Note that single green cells were not scored, since replication-competent virus should spread to adjacent cells and cause foci of green cells. Seven days post-transfection, the Sf-9 plate was scored for CPE-positive wells. The titers determined by the two methods were not significantly different, as shown in Table 1. The Sf-9ET assay was also scored at 7 days post-transfection for CPE and gave comparable results to the 3-day GFP assay (data not shown). In addition to the GFP cell line described here, another Sf-9ET cell line was developed using red fluorescent protein (mRFP1), which works equally well for titrating baculoviruses.

Other reporter proteins such as the β -galactosidase (β -gal) gene *lacZ* (9) and green fluorescent protein (10) have been cloned into recombinant baculoviruses under control of the polyhedrin or P10 promoters to aid in scoring the end-point dilution assays. These modifications have improved the ability to score end-point dilutions assays; however, they require additional cloning and the β -gal and GFP reporter proteins are expressed along with the protein of interest, which may not be desirable. Decreasing the time it takes to express GFP would be helpful to further shorten assay time. It has been reported that use of the baculovirus early-to-later (ETL) promoter instead of the P10 or polyhedrin promoter can shorten the time it takes to express GFP by 18 h (17).

In summary, we have created novel cell lines that improve the baculovirus end-point dilution assay in three important ways. First, scoring the assay plates for eGFP or RFP is much less ambiguous than scoring for cell death, especially for the novice. Secondly, assay results are obtained in only 3 days in contrast to the 6–8 days required for the CPE method. And thirdly, the Sf-9ET cell line allows easy quantitation of recombinant baculoviruses, thus avoiding the need to co-express other reporter genes along with the protein of interest. Compared with alternative assay systems for quantitating baculovirus

stocks, the Easy Titer method is sensitive, rapid, and specific.

It is intended that the cell line will be widely distributed for research purposes by the National Institutes of Health (NIH) consistent with the NIH Principles and Guidelines for Sharing of Biomedical Resources (http://ott.od.nih.gov/policy/research_tool.aspx).

Acknowledgments

We are grateful to Veronica Roberts and Cammi Bittner for their technical assistance. This research has been funded in whole or in part by federal funds from the National Cancer Institute (NCI; contract no. NO1-CO-12400). The content of this paper does not necessarily reflect the views or policies of the Department of Health and Human Services; nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

The authors declare no competing interests. This paper is subject to the NIH Public Access Policy.

References

- O'Reilly, D., L.K. Miller, and V.A. Luckow. 1992. *Baculovirus Expression Vectors: A Laboratory Manual*. W.H. Freeman and Company, New York.
- Kidd, I.M. and V.C. Emery. 1993. The use of baculoviruses as expression vectors. *Appl. Biochem. Biotechnol.* 42:137-159.
- King, L.A. and R.D. Possee. 1992. *The Baculovirus Expression System: A Laboratory Guide*. Chapman and Hall, London.
- Luckow, V.A. and M.D. Summers. 1988. Trends in the development of baculovirus expression vectors. *Bio/Technology* 6:47-55.
- Hoss, A., I. Moareft, K.H. Scheidtman, L.J. Cisek, J.L. Dorden, I. Dornreiter, A.K. Arthur, and E. Fanning. 1990. Altered phosphorylation pattern of simian virus 40 T antigen expressed in insect cell by using a baculovirus vector. *J. Virol.* 64:4799-4807.
- Kloc, M., B. Reddy, S. Crawford, and L.D. Etkin. 1991. A novel 110-kDa maternal CAAX box-containing protein from *Xenopus* is palmitoylated and isoprenylated when expressed in baculovirus. *J. Biol. Chem.* 266:8206-8212.
- Kuroda, K., M. Veit, and H.D. Klenk. 1991. Retarded processing of influenza virus hemagglutinin in insect cells. *Virology* 180:159-165.
- Hink, W.F. and P.V. Vail. 1973. A plaque assay for titration of alfalfa looper nuclear polyhedrosis virus in a cabbage looper (TN-368) cell line. *J. Invertebr. Pathol.* 22:168-174.
- Sussman, D.J. 1995. 24-hour assay for estimating the titer of β -galactosidase-expressing baculovirus. *BioTechniques* 18:50-51.
- Cha, H.J., T. Gotoh, and W.E. Bentley. 1997. Simplification of titer determination for

recombinant baculovirus by green fluorescent protein marker. *BioTechniques* 23:782-786.

- Kitts, P.A. and G. Green. 1999. An immunological assay for determination of baculovirus titers in 48 hours. *Anal. Biochem.* 268:173-178.
- Lo, H.R. and Y.C. Chao. 2004. Rapid titer determination of baculovirus by quantitative real-time polymerase chain reaction. *Biotechnol. Prog.* 20:354-360.
- Rohrmann, G. F. 2008. *Baculovirus Molecular Biology*. National Library of Medicine (US) National Center for Biotechnology Information. Bethesda, MD.
- Jarvis, D.L., J.G.W. Fleming, G.R. Kovacs, M.D. Summers, and L.A. Guarino. 1990. Use of early baculovirus promoters for continuous expression and efficient processing of foreign gene products in stably transformed lepidopteran cells. *Bio/Technology* 8:950-955.
- Shishido, T., M. Muraoka, M. Ueda, M. Seno, K. Tanizawa, S. Kuroda, H. Fukuda, and A. Kondo. 2006. Secretory production system of bionanocapsules using a stably transfected insect cell line. *Appl. Microbiol. Biotechnol.* 73:505-511.
- Reed, L. and H. Muench. 1938. A simple method for estimating fifty percent endpoints. *Am. J. Hyg.* 27:493-497.
- Dalal, N.G., W.E. Bentley, and H.J. Cha. 2005. Facile monitoring of baculovirus infection for foreign protein expression under very late polyhedrin promoter using green fluorescent protein reporter under early-to-late promoter. *Biochem. Eng. J.* 24:27-30.

Received 4 June 2009; accepted 30 July 2009.

Address correspondence to Ralph F. Hopkins, Protein Expression Laboratory, Advance Technology Program, SAIC-Frederick, National Cancer Institute at Frederick, Post Office Box B, Frederick, MD, 21702, USA. email: hopkinsrf@mail.nih.gov