Journal of Virological Methods 183 (2012) 201–209

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Suspension culture titration: A simple method for measuring baculovirus titers

Leila Matindoost[a,b,*], Leslie C.L. Chan[a], Ying Mei Qi[a], Lars K. Nielsen[a], Steven Reid[a]

[a] Australian Institute for Bioengineering and Nanotechnology, University of Queensland, St Lucia, QLD 4072, Australia
[b] Environmental Sciences Institute, Shahid Beheshti University, G. C., Tehran 19839, Iran

Article history:
Received 28 October 2011
Received in revised form 24 April 2012
Accepted 25 April 2012
Available online 3 May 2012

Keywords:
Virus titration
Baculovirus
Insect cells
Suspension culture

A B S T R A C T

The baculovirus-insect cell expression system is an important technology for the production of recombinant proteins and baculovirus-based biopesticides. Budded virus titration is critical when scaling up baculovirus production processes in suspension cultures, to ensure reproducible infections, especially when a low multiplicity of infection (MOI) is applied. In this study, a simple suspension culture titration (SCT) assay was developed that involves accurate measurements of the initial cell densities (ICDs) and peak cell densities (PCDs) of an infected culture, from which the MOI and hence the virus inoculum infectious titer can be estimated, using the established Power–Nielsen baculovirus infection model. The SCT assay was assessed in parallel with two adherent culture-based assays (MTT and AlamarBlue) for the Heliothine baculovirus HaSNPV, and was shown to be more objective, time-efficient and reproducible. The model predicted a linear correlation between log(PCD/ICD) and log(MOI), hence an alternative model-independent SCT assay was also developed, which relies on a well-replicated standard curve relating suspension culture-derived PCD/ICD ratios with plaque or endpoint assay-derived MOIs. Standard curves with excellent linearity were generated for HaSNPV and the industrially significant rAcMNPV, demonstrating the feasibility of this simple titration approach, especially in terms of its applicability to a wide range of virus infection kinetics.

1. Introduction

The baculovirus-insect cell expression system is a very valuable and widely used technology for the production of recombinant eukaryotic proteins, due to its potential for high-level heterologous gene expression, and ability to perform near-authentic post-translational modifications resulting in biologically active proteins (King and Possee, 1992; Kost et al., 2005). The applications of baculovirus expression are expanding and evolving, including virus-like particles (VLPs) for the Cervarix® human papilloma virus vaccine (Senger et al., 2009), hemagglutinin antigens for the FluBlok® influenza vaccine (Baxter et al., 2011), adeno-associated virus (AAV) vectors for gene therapy (Mena et al., 2010), baculovirus display (fusing peptides/proteins on baculovirus particles for use as immunogens), humanized N-glycoproteins, and gene delivery vectors for mammalian cells (Kost et al., 2005). Baculoviruses are also increasingly being reconsidered as viable biopesticides for the control of insect pests of agriculture and forestry (Moscardi, 1999; Moscardi et al., 2011; Szewczyk et al., 2006).

The scale-up of baculovirus production processes is most efficiently carried out using suspension insect cell cultures, and one of the key prerequisites of reproducible baculovirus infections in such cultures is the ability to apply an accurate and consistent multiplicity of infection (MOI). This is especially important when a low MOI process is prescribed, in order to reduce the amount of virus inoculum required (Wong et al., 1996), and to discourage the selection for non-productive defective interfering particles (DIPs) (Kool et al., 1991). The key measurements required for calculation of the MOI are the cell density at the time of infection or initial cell density (ICD), and the infectious budded virus (BV) titer of the virus inoculum. The BV titer is by far the more challenging one to measure, and much research has been devoted to developing accurate and reproducible methodologies for BV titration (Janakiraman et al., 2006; Kitts and Green, 1999; Lo and Chao, 2004; Mena et al., 2003; Nguyen, 2007; Pouliquen et al., 2006; Roldao et al., 2009; Shen et al., 2002).

Adherent culture titration (ACT) assays are the most common approach for quantifying infectious BV. The most established of these is the plaque assay ('the gold standard'), which provides a direct measurement of the concentration of infectious BV or plaque forming units (PFUs), by counting well-isolated virus plaques formed on an agarose-immobilized cell monolayer (King and Possee, 1992). However, plaque assays are complex to perform, and are difficult to get right without considerable operator experience, hence compromising reproducibility. For example, the

* Corresponding author at: Australian Institute for Bioengineering and Nanotechnology, University of Queensland, St Lucia, QLD 4072, Australia. Tel.: +61 733463147; fax: +61 733463973.
E-mail addresses: leila.matindoost@uqconnect.edu.au (L. Matindoost), l.chan1@uq.edu.au (L.C.L. Chan), steven.reid@uq.edu.au (S. Reid).

0166-0934/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jviromet.2012.04.015

condition of stock cells, the confluency of the cell monolayer, the quality and temperature of the agarose overlay, and the efficiency of the virus adsorption step, will greatly impact on the number of plaques formed (Dee and Shuler, 1997b; King and Possee, 1992; O'Reilly et al., 1994). Other titration assays only provide an indirect measurement of infectious virus titers, but these are generally less complex to perform. For example, the well-established endpoint dilution ACT assay is conducted in multi-well plates, and depends on the scoring of a large number of negative and positive-infected wells (O'Reilly et al., 1994). However, the endpoint assay is statistically more complex to analyze, as it follows a binomial rather than a Poisson distribution (like plaque assays), but a maximum likelihood estimator can be used to improve its accuracy (Nielsen et al., 1992). The virus titer unit of an endpoint assay, the median tissue culture infectious dose ($TCID_{50}$), can be theoretically converted to the PFU by using the Poisson distribution-derived conversion factor, $-\ln(0.5) = 0.693$ (O'Reilly et al., 1994).

Another multi-well plate ACT assay is the immunological assay (Kitts and Green, 1999), which combines the features of plaque and endpoint assays, and involves the use of an antibody to a viral envelope glycoprotein to score foci of infection in each well. Two other multi-well plate ACT assays were developed recently, involving the use of cell viability dyes (MTT or AlamarBlue) to score negative and positive-infected wells (Mena et al., 2003; Pouliquen et al., 2006). Unlike the previous assays, the MTT and AlamarBlue assays rely on the generation of dose–response (sigmoidal) curves to obtain the median tissue culture lethal dose ($TCLD_{50}$), which is then converted to a $TCID_{50}$ or PFU basis via correlation with endpoint or plaque assays.

Suspension culture titration (SCT) assays have also been developed recently, which rely on the measurement of cell size change (Janakiraman et al., 2006), cell growth cessation (Roldao et al., 2009), or cell expression of a reporter gene via flow cytometry (Roldao et al., 2009), to indicate the extent of virus infection.

Apart from infectious BV assays, there are other assays that measure the total concentration of virus particles using techniques such as quantitative real-time PCR (Lo and Chao, 2004) and flow cytometry (Ferris et al., 2011; Shen et al., 2002). However, these assays are of limited use for calculating the MOI, if the proportion of non-infectious virus particles in the virus stock is undefined. Unfortunately, the total vs infectious virus particles (T/I) ratio is a difficult number to pinpoint. For example, the T/I ratio was found to be as low as 4 for recombinant *Autographa californica* nucleopolyhedrovirus (rAcMNPV) (Rosinski et al., 2002) and 3 for *Helicoverpa armigera* nucleopolyhedrovirus (HaSNPV) (Pedrini et al., 2011) for freshly harvested virus stocks (40–60 h post infection). However, this ratio is temporally dynamic and will increase with increasing time post infection, and is likely to reach very high (>100) levels upon storage (Pedrini et al., 2011; Rosinski et al., 2002), due to virus particle aggregation, which leads to a progressive loss of infectivity (Jorio et al., 2006).

Hence there are a wide variety of infectious BV titration assays to choose from, but each has its own specific strengths and weaknesses. In terms of assay duration, the well-established plaque and endpoint assays are quite lengthy (up to 7 days required) (O'Reilly et al., 1994), while the immunological (48 h), AlamarBlue (24 h) and SCT (18–24 h) assays have a speed advantage (Kitts and Green, 1999; Pouliquen et al., 2006; Roldao et al., 2009). A reporter gene will increase the objectivity of the plaque and endpoint assays (although not necessarily essential), while it is not required for most of the other assays, which have their own objective means of identifying an infection event. One major disadvantage of the existing titration assays is procedural complexity, involving the analysis of multiple virus dilutions, and the setup of a large number of well (microplate) or shaker infections just to obtain the titer of one virus sample. Another disadvantage is the fact that these assays

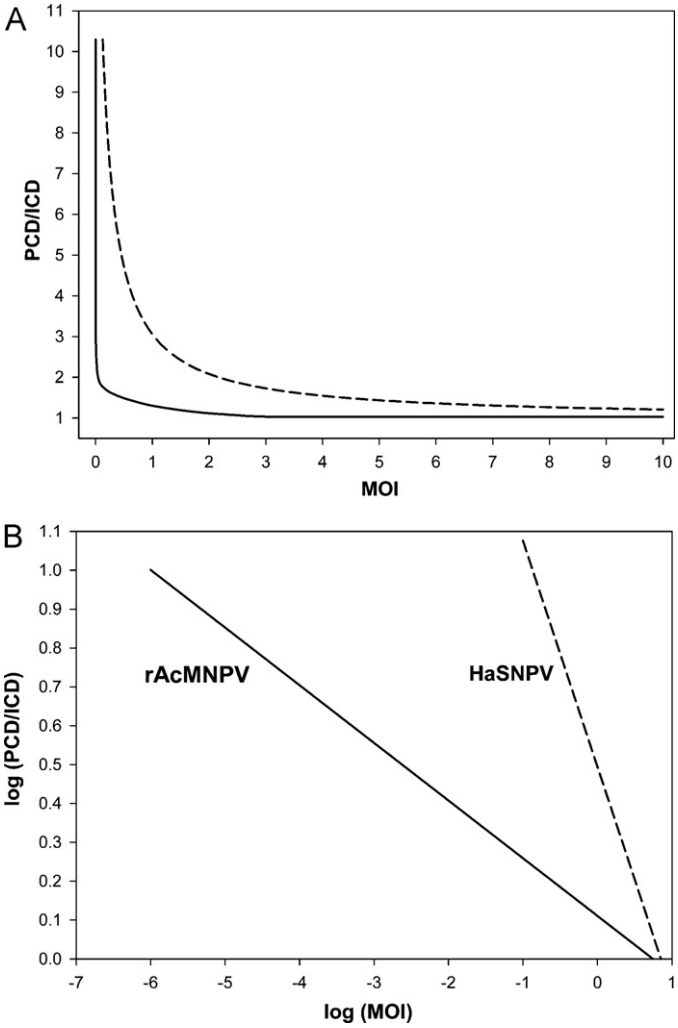

Fig. 1. Correlation between the peak cell density/initial cell density (PCD/ICD) ratio and the multiplicity of infection (MOI) for rAcMNPV–Sf9 and wild-type HaSNPV–HzAM1 infected systems, as predicted by the Power–Nielsen baculovirus infection model. (A) Non-transformed data and (B) Log-transformed data.

are primarily optimized for the titration of rAcMNPV, the most prominent baculovirus expression vector (Jarvis, 2009; Roldao et al., 2009). rAcMNPV exhibits fast infection kinetics (Pedrini et al., 2011; Power et al., 1994), hence assays designed for its titration may not work as efficiently for baculoviruses exhibiting slower kinetics such as HaSNPV (Pedrini et al., 2011).

In this study, a new type of SCT assay for infectious BV titration has been developed, with the objective of delivering a less complex, more robust, and more objective methodology that is consistent with a suspension culture-based baculovirus production process, and that is equally effective for baculoviruses with slow or fast kinetics. This assay is informed by the Power–Nielsen baculovirus infection model (Power et al., 1994), a powerful tool that allowed insights to be made on the most efficient means of devising the new methodology. The model predicted that the peak cell density (PCD)/ICD ratio of an infected culture will decline linearly with increasing MOI (after log transformation), which indicated the dose-dependency of cell growth suppression on the amount of infectious virus added. The model showed that such a linear correlation can be obtained from baculovirus-insect cell systems, whether the infection kinetics are slow (HaSNPV–HzAM1) or fast (rAcMNPV–Sf9) (Fig. 1B). Hence, a titration assay can be devised by first generating a one-off standard curve relating suspension culture-derived PCD/ICD ratios with adherent culture-derived