Automated baculovirus titration assay based on viable cell growth monitoring using a colorimetric indicator

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Baculoviruses have become powerful tools for a growing number of applications, such as the overproduction of recombinant proteins in insect larvae, insect cells, and mammalian cells, the surface display of peptides or proteins, and the potential for use as safe vectors for gene therapy (1,2). The precise titration of virus stocks is a prerequisite for the optimization of protein expression in insect and mammalian cells (3). The accuracy of the resulting titer is determined by the precision and the reproducibility of the method employed. Unfortunately, commonly applied methods (plaque assay and end-point dilution assay) are known to produce variable results, are laborious to perform, and require long incubation times (4). Recently, the variability of virus titer determinations has been shown to be reduced by coexpression of reporter proteins, such as β -galactosidase (5) or green fluorescent protein (6–8) to differentiate infected from noninfected cells. However, the coexpression of a reporter protein is not always desirable for target protein expression because it may reduce the expression levels of the protein of interest. Other methods have been developed that use antibodies against baculoviral proteins (9), and commercial immunostaining kits are available [e.g., the FastPlax™ Titer Kit (EMD Biosciences, Madison, WI, USA) and the BD BacPAK™ Baculovirus Rapid Titer Kit (Clontech, Mountain View, CA, USA)]. However, these methods necessitate laborious data collection processes and, in our hands, are subject to high user-to-user variation for baculovirus titer determination.

The fastest methods reported are based on viral DNA quantitation using either flow cytometry (10) or real-time PCR (11), which allow titer determination within two hours. The drawbacks of these approaches are the costs of equipment and staining reagents, and the fact that the total number of particles and not the number of infectious particles is determined.

An alternative approach is based on the lytic nature of the viral system (12), and more specifically on the fact that cell growth is attenuated upon virus infection. This growth reduction is dose-dependent and can be estimated by measuring the viable cell concentration and subsequently correlating this to the virus titer. Indeed, a new method was recently developed for virus titration by spectrophotometrically monitoring the cell viability with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (4). The accuracy of the method was clearly demonstrated; however, the number of preparation steps and the overall duration (6 days) are not compatible with a fast and automated high-throughput process.

In this work, we demonstrate that AlamarBlue™ (Serotec, NC, USA) is a powerful substitute for MTT to monitor the early cell growth arrest induced by a 24-h baculovirus infection cycle. AlamarBlue is a sensitive oxidation-reduction indicator; the fluorescent color change upon reduction by living cells is believed to be mediated by mitochondrial enzymes (13). AlamarBlue has been shown to be useful for measuring the proliferation of several eukaryotic cell lines (14–17). However, to our knowledge, no attempt

was made to use AlamarBlue to assess insect cell proliferation.

In the first step, we investigated the correlation between viable insect cell number and fluorescence intensity. A suspension culture of *Spodoptera frugiperda* Sf21 cells (Invitrogen, Carlsbad, CA, USA) in mid-exponential growth phase was used to set up the assay. The cells were grown in ExCell 401™ cultivation medium (JRH Biosciences, Lenexa, KS, USA) supplemented with 1% fetal calf serum (FCS) and 2 mM glutamine, ensuring a cell doubling time of 22 ± 2 h and viability higher than 94%. From 3.75×10^2 to 6.0×10^3 viable cells per well were seeded in a flat-bottomed 96-well plate (BD Falcon™; BD Biosciences, San Jose, CA, USA) in 200 μ L culture volume. To avoid the variations inherent to the plate side effects, wells at the edges of the plate were not used for measurements. After the addition of 10% AlamarBlue (v/v), the plate was incubated at 28°C, and fluorescence was measured after 3–24 h using a fluorescence plate reader (CytoFluor® II; Applied Biosystems, Foster City, CA, USA). The excitation wavelength was set to 530 nm and the emission wavelength to 590 nm. This correlation proved to be linear for cell concentrations up to 4.5×10^3 cells per well with a correlation coefficient of $R = 0.9995$. A slight decrease of linearity is observed at cell concentrations above 4.5×10^3 cells per well ($R = 0.9743$), indicating that a saturation response is reached above the tested cell concentrations. We therefore chose to seed 3×10^3 cells per well and a measurement of fluorescence 5 h after the addition of AlamarBlue because these conditions ensure a reliable linear response even if the cells are doubling during the incubation time.

For virus titration, all pipeting steps of the AlamarBlue assay described below were automated on the liquid-handling workstation epMotion® 5070 (Vaudaux-Eppendorf, Schönenbuch, Switzerland). This includes the dispensing of medium and cells, the serial virus dilutions, and the addition of AlamarBlue to each well. Details on the automation of the protocol are available online as supplementary material at www.BioTechniques.com.

Table 1. Titration of Three Different Virus Stocks with AlamarBlu Fluorescent Assay (TCLD₅₀/mL) and Standard Plaque Assay (pfu/mL)

	Titration Assay	Experiment 1	Experiment 2	Experiment 3	Mean	SD	Pfu/mL (calculated or measured)
V3	TCLD ₅₀ /mL	6.457×10^2	5.248×10^2	6.026×10^2	5.910×10^2	0.500×10^2 (8.5%)	4.45×10^7 (calc.)
	Pfu/mL	3.8×10^7	4.2×10^7	4.8×10^7	4.27×10^7	5.0×10^6 (11.8%)	4.27×10^7 (meas.)
V8	TCLD ₅₀ /mL	6.052×10^1	8.259×10^1	N.D.	7.156×10^1	1.10×10^1 (15.4%)	1.1×10^7 (calc.)
	Pfu/mL	8.3×10^6	2.3×10^7	N.D.	1.6×10^7	7.4×10^6 (46.9%)	1.6×10^7 (meas.)
V9	TCLD ₅₀ /mL	4.333×10^3	5.325×10^3	N.D.	4.829×10^3	4.96×10^2 (10.3%)	1.71×10^8 (calc.)
	Pfu/mL	1.4×10^8	2.8×10^8	N.D.	2.1×10^8	7.0×10^7 (33.3%)	2.1×10^8 (meas.)

This table shows the comparison of mean values and standard deviations (SDs) of data obtained from two or three independent titration experiments. N.D., not determined; TCLD₅₀, tissue culture lethal dose 50.

For the titration of each virus stock, a single 96-well plate was used for the virus dilution and for the cell-based assay. One hundred microliters of 2-fold serial dilutions of the virus stock (ranging from 1/2 to 1/1024) were prepared in a 96-well plate, using up to 4 wells per dilution and 20 wells with medium as virus-free cell growth control. Assessment of the fluorescence background and residual reducing enzyme activity in virus stocks revealed no significant difference as compared with fresh culture medium (data not shown). Therefore fresh culture medium was used for t_0 value determination. Subsequently, 100 μ L of cell suspension at 3×10^4 cells/mL were added per well and gently mixed. After the addition of cells, t_0 staining was performed by adding 20 μ L AlamarBlue solution to the first 10 wells of the virus-free medium control (10% final AlamarBlue concentration). The plate was subsequently stored at 28°C for 5 h, followed by fluorescence reading to obtain the t_0 values. The plate was then incubated at 28°C for an additional 19 h to complete the 24 h infection cycle. The resulting t_{24} values were determined by adding 10% AlamarBlue to the remainder of the wells and reading its fluorescence 5 h later. $t_{24}-t_0$ values are calculated for each dilution and for the virus-free medium control. This virus-free

medium control value represents 100% growth performance (i.e., undisturbed cell growth). Next, the percentage of growth inhibition (GI) for each virus dilution is determined as follows:

$$GI(\%) = \frac{t_{24}^{\text{virus}} - t_0^{\text{control}}}{t_{24}^{\text{control}} - t_0^{\text{control}}} * 100 \quad [\text{Eq. 1}]$$

To determine the virus dilution corresponding to 50% growth inhibition (TCLD₅₀) (4), data were analyzed with the Origin 7 software (OriginLab, Northampton, MA, USA) using the following sigmoidal equation:

$$y = A_1 + \frac{A_2 - A_1}{1 + e^{-(D-D_0)p}} \quad [\text{Eq. 2}]$$

where y is the growth inhibition, A_1 is the minimum growth inhibition (undisturbed cell growth), A_2 is the maximum growth inhibition (100% infected cells), D is the dilution factor, D_0 is the dilution at which the growth inhibition was 50% (1/TCLD₅₀), and p is a slope factor.

Figure 1 shows mean values and standard deviations (SDs) of data obtained from three independent titration assays of the same viral stock. The average inter-assay SD is 4% for this example, and a maximum of 20% was revealed with other virus stocks (data not shown), proving the reliability and robustness of the assay.

The obtained D_0 value was then converted into TCLD₅₀/mL by applying the following equation (4):

$$TCLD_{50}/mL = \frac{1}{(D_0 V)} \quad [\text{Eq. 3}]$$

where V is the volume of virus solution added per well.

Representative titration results for three virus stocks are described in Table 1 using the standard plaque assay (18) and the AlamarBlue assay. The data show that the AlamarBlue fluorescent titration assay has an accuracy and reproducibility that is comparable or higher to the standard plaque assay. Comparison of our TCLD₅₀/mL values with data from Mena et al. (4) is not possible because our determination is not based on the end-point dilution method (18). Additional titration experiments with other virus stocks revealed standard deviations ranging from 9% to 35%, confirming published standard deviations from other assays (4–6,10,11).

The correlation between the TCLD₅₀ values from the fluorescent assay and the plaque-forming units (pfus) from the standard plaque assay is shown in Figure 2 for several different virus stocks. This correlation between Log(TCLD₅₀/mL) and Log(pfu/mL) was established by linear regression

Figure 1. Fluorescence measurement of cell growth inhibition as a result of virus infection. Each point represents the mean value of three independent experiments. Error bars represent the standard deviations. Sigmoidal curve fitting using the least-squares method was performed using Origin 7 ($R^2 = 0.999$). The values of parameters in Equation 2 were $A1 = -1.76$; $A2 = 100$, 76; $\log D_0 = -1.76$, and $p = 1.83$.

Figure 2. Correlation between AlamarBlue assay and the standard plaque assay. Correlation between $TCLD_{50}$ values using the AlamarBlue assay and plaque-forming units (pfus) from the standard plaque assay for $n = 7$. $TCLD_{50}$, tissue culture lethal dose 50.

analysis ($n = 7$). The resulting equation is shown below (correlation coefficient is $R = 0.98924$):

[Eq. 4]

$$\text{Log}(\text{pfu/mL}) = 0.643 (\text{Log}TCLD_{50}/\text{mL}) + 5.867$$

The good correlation between the new fluorescent assay and the standard plaque assay allows a rapid conversion of $TCLD_{50}$ values into pfus. The titer range determined with the

AlamarBlue assay is restricted to the dilution window used to set up the assay, 5.1×10^6 pfu/mL as a lower limit and 2.8×10^8 pfu/mL as an upper limit. Based on the literature, this range comprises normal titers obtained for baculovirus working stocks (5,6,9–11). Titer determination should be repeated for virus stocks exhibiting titers outside this range, with a pre-dilution for higher titers, and with an alternative assay for the lower titers.

The automated titration assay described here has several advantages. First, it is based on the infectivity and cytopathic effect of the virus, resulting in a realistic titer determination because it is not capturing the noninfectious viral particles as do flow cytometry and real-time PCR assays (10,11). Because results are obtained after a 24-h infection cycle, the AlamarBlue assay does not overestimate the virus titers caused by progeny virus from a secondary infection cycle, as described in other methods (7). Second, data collection from the fluorescent plate reader is fast, convenient, and is not subject to user-specific interpretation, as is the case for viral plaque or focus counting.

Automation of the protocol further improved the assay performance: an increase in accuracy intra-assay was observed by reducing the variations inherent to manual pipeting, and the SD of triplicates was below 15% (data not shown). Considering these observations and by testing duplicates instead of triplicates, it is possible to titrate two virus stocks per 96-well plate. Thus, within 3 h, four 96-well plates can be set up for the assay on the epMotion 5070, allowing the titration of eight virus stocks in parallel.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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