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A Flow Cytometric Assay for Rapid, Accurate Determination of Baculovirus Titers

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Recombinant protein expression using the Baculovirus Expression Vector System (BEVS) is a powerful tool for the production of therapeutics, diagnostics, and reagents. To maximize efficiency of protein production, and thereby reduce costs, it is important to optimize the production parameters. A crucial step in optimization is determining the best multiplicity of infection (MOI) for the system in use. Factors that can affect the MOI include the recombinant baculovirus itself as well as cell line type and media composition. Typically the titer of a viral stock is determined in a standard manner, and then that titer is applied to each and every parameter tested; for instance, titering the virus on a *Spodoptera* cell line in a serum-containing media, and then using those data to determine the MOI used to infect *Trichoplusia* cells in a serum-free media formulation. The results may suggest that either the *Trichoplusia* cell line or the media formulation is inadequate for protein expression when, in fact, the MOI was incorrect for that particular combination.

The current "gold standard" for baculovirus titration, the plaque assay, is prohibitively time and labor intensive when comparing a number of cell lines and media formulations. The plaque assay is also technically difficult; requiring that the cells are not plated too thinly or thickly, and that the agarose overlay is not too hot (to protect the cells from being killed) or too cold (or the overlay will pour unevenly and disrupt the monolayer). Humidity in the incubator must be sufficient so that the plates do not crack during the 4–10 day incubation period required before plaques can be visualized. Once plaques are present, the accuracy of the titer is greatly dependent on the operator's skill and experience.[1] Additionally, the one-hour virus incubation step has been shown to lead to an underestimation of the virus titer as infectious virus is being removed, a step not usually performed during protein production.[2]

Other titration methods are available such as endpoint dilution, immunohistochemical staining, fluorescent markers and real-time PCR. The endpoint dilution method determines the titer within a range of one log when using a modified 12-well plate assay. The assay also requires a minimum of three days to complete, and can be challenging to interpret in the absence of a fluorescent marker or polyhedron protein. The 96-well endpoint dilution gives a more accurate titer determination when applying the $TCID_{50}$ adjustment to pfu/ml but also suffers from the interpretation limitations described for the modified assay.[3]

The available immunohistochemical staining methods have the advantage of timeliness. Titer determination can be performed in less than a 24-hour period. Limitations include the one-hour virus incubation time, the labor intensity of the staining steps, and the operator's skill in identifying stained foci.[4] Disadvantages of both the endpoint dilution assay and the immunohistochemical staining assay are the time and reagents required to determine the viral titer for a variety of cell lines and media. Fluorescent markers, that are the result of a fusion protein engineered into the vector, have the advantage of directly measuring infected cells that are actively producing the recombinant protein.[5] This is a powerful technique that allows the operator to quickly determine whether it is a high-titer virus stock. However, it requires the use of specialized equipment, such as a fluorescent microscope, and the fusion protein can be troublesome downstream when purifying the recombinant protein.

The real-time PCR assay has the advantage of speed and accuracy. Baculovirus specific primers are used to quantitate the number of viral transcripts present using a standard curve generated by a control of a known titer.[6] This assay has the disadvantages of being technically difficult to set up, requiring specialized equipment, measuring viral transcripts that may be defective, and it does not address the different susceptibilities to infection of different insect cell lines.

A desirable titer assay would have the following characteristics: short turnaround time, high-throughput capacity, a detection method compatible with any recombinant baculovirus, ease of use, direct measurement of infected cells, and a quantitation method that reduces operator bias. Here we present a high-throughput, flow cytometric assay to quantitate infectious baculovirus particles produced in insect cell culture, allowing for accurate virus titer determination for multiple conditions in less than 24 hours. The flow cytometric

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assay was compared to more traditional titering assays (such as real-time PCR, end-point dilution assay, and the "gold standard" plaque assay), and was found to be of equal or greater accuracy. The power of this assay lies not only in the user-friendly time-to-titer data, and the determination of infectious particles versus viral transcript, but the high-throughput format allows for direct comparisons of virus titer specific to target cell as well as media composition.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Cell lines used were either *Spodoptera frugiperda* cells (Sf9, Sf21) or *Trichoplusia ni* cells (Tni PRO™). Cells were routinely passaged in ESF 921 (Expression Systems LLC, Woodland, CA), a serum-free, protein-free insect cell media. Cultures were grown in 125 ml shake flasks in a New Brunswick shaker incubator at 27° C shaking at 120 rpm. For comparisons of media formulation effects on virus titer, cells were alternatively grown in ESF AF (Expression Systems), an animal and animal-derived component free variation of ESF 921, or in TNM-FH (Sigma, St. Louis, MO) a serum-containing formulation.

Virus Titer Assays

Modified end-point dilution assays were performed as described in the Pharmingen handbook.[7] Briefly, Sf9 cells were plated at 3 x 10^5 cells per well and infected with 10-fold dilutions of the test virus. wt *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) was plated in parallel as a positive control. Three days of culture at 27° C was followed by scoring the wells for the presence of infected cells. In the case of wt AcMNPV, polyhedron positive cells were counted in order to provide more accurate virus titer estimation. Plaque assays were performed on the wt AcMNPV as previously described.[2] Plaques were visualized by the addition of neutral red and by the presence of occlusion bodies. QC-PCR assays were performed following standard methods described previously.[6] Virus titer was determined by comparing the samples to a standard curve generated with wt AcMNPV.

Flow Cytometric Titer Assay

Insect cells were plated in a 96-well format, and 10-fold dilutions of the sample virus stock were used to infect the cells for a period of 18 hours. Cells were suspended at a concentration of 2 x 10^6 cells/ml and 100 µl were plated into each well. Virus stock dilutions were made: undiluted, 1:5, 1:50, 1:500 and 1:5000. One hundred µl of each dilution was added in duplicate to the cells for a final dilution of 1:2, 1:10, 1:100, 1:1000 and 1:10000. The culture plate was incubated in a New Brunswick shaker incubator at 27° C and a rotation of 270 rpm for 18 hours. Samples were washed twice with cold phosphate-buffered saline (PBS), then stained with 50 µl of a 1:250 dilution of anti-gp64 (clone AcV1, eBioscience, San Diego, CA) or an isotype control for 15 minutes at 4° C in the dark. Cultures were washed twice with PBS followed by a secondary immunostain for 15 minutes at 4° C with a PE-labeled antibody directed against mouse IgG antibody (Guava Technolo-gies, Hayward, CA) diluted 1:250. All antibodies were diluted in PBS containing 0.05% Bovine Serum Albumin (PBS-BSA). Samples were washed again and resuspended in 200 µl PBS-BSA. Fluorescent-labeled cells were acquired using a Guava Personal Cell Analysis (PCA) System, and data was collected and analyzed using the Guava Cytosoft Express software module.

RESULTS

Selection of and Optimization of Antibody Concentration

Selection of commercially-available antibodies against baculovirus gene products was considered for the flow cytometric assay. An antibody directed against gp64, the baculovirus envelope protein, was chosen as the target antigen because it is an essential gene product for virus infectivity and spread, and it had been validated for use in flow cytometry.[8] gp64 is also expressed on the cell surface of the infected insect cell starting at six hours post infection and peaking in expression at 12 hours p.i.[9]

Sf9 cells at a density of 10^6 cells/ml were infected with wt AcMNPV at an MOI of 0.1 or 5 in order to test the utility of the antibody under conditions of high and low viral expression. Cultures were incubated for 18 hours at 27° C with shaking at 120 rpm. Two hundred µl aliquots of the cell culture, along

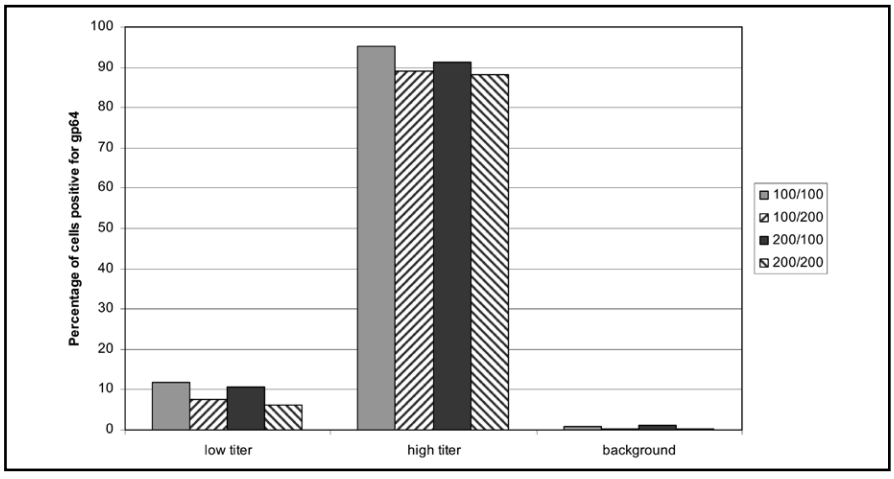

Figure 1. 50 ml shake-flask cultures of Sf9 cells at a density of 10^6 cells/ml were infected with wt AcMNPV at an MOI of 0.1 (low titer) or 5 (high titer) and cultured overnight. 200 µl aliquots of cultures were transferred to duplicate wells of a 96-well plate along with uninfected Sf9 cells as a negative control (data not shown). All conditions were stained with anti-gp64 or an isotype control for 15 minutes. After washing, all samples were stained with a PE-labeled anti-mouse IgG. Anti-gp64 and isotype control antibodies were used at either a 1:100; 1:200 or 1:400 dilution (1:400 not shown). Secondary staining was performed in a checkerboard fashion at 1:100 and 1:200 dilutions. Background staining shown above is the isotype control staining high titer samples followed by secondary staining.

with uninfected control Sf9 cells, were transferred to a round bottom 96-well plate and were washed twice with cold PBS. Anti-gp64 antibody, or an isotype control, were diluted 1:100 and 1:200 in PBS-BSA. Twenty µl of the primary antibody were added in duplicate to the appropriate wells and incubated at 4° C in the dark for 15 minutes. Cells were washed twice and the secondary antibody was added. The secondary antibody, which was an anti-mouse IgG antibody labeled with phycoerythrin (PE), was diluted 1:100 and 1:200 in PBS-BSA. The cells were incubated 4° C in the dark for 15 minutes, washed twice in cold PBS and resuspended in 200 µl PBS-BSA. Samples were then analyzed for fluorescence using the Guava PCA-96.

The percentage of fluorescently labeled cells indicated the percentage of infected cells expressing gp64 protein. The various combinations of dilutions were compared with the background staining to determine the strongest signal with minimal background staining (Fig. 1). The 1:100 dilution of anti-gp64 antibody was the equivalent of 0.01 µg Ab per $2x10^5$ cells. Later uses of the assay increase the staining volume to 50 µl per well, yet keep the amount of antibody consistent, leading to a 1:250 final dilution.

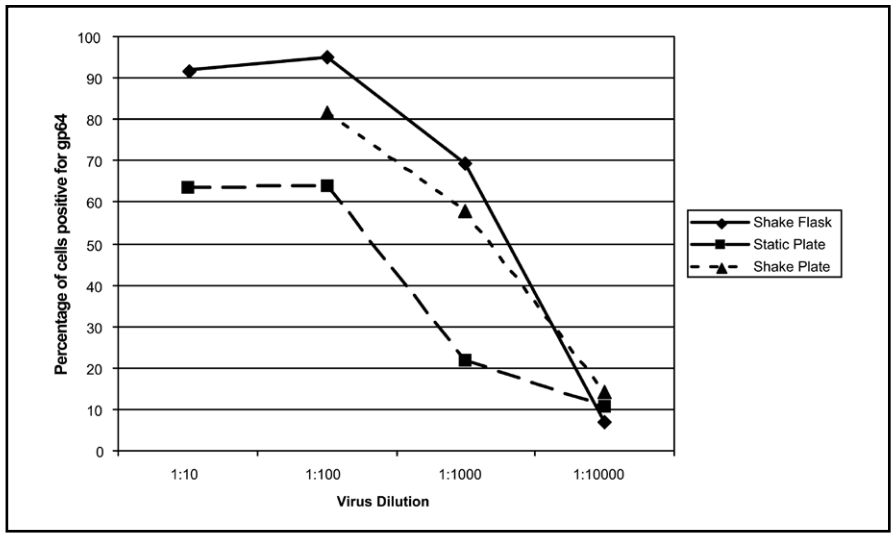

Figure 2. Shake-flask cultures (solid lines) of Sf9 cells at a density of 10^6 cells/ml were infected with wt AcMNPV at dilutions of 1:10, 1:100, 1:1000, and 1:10000 and incubated overnight. Static plate cultures (dashed lines) were infected as for shake cultures with 2 ml volumes per well of a 12-well plate. The plate culture was rocked for the first four hours and then placed in a stationary incubator for the remaining 14 hours. Shake plate cultures were infected as above with 200 µl volumes in a 96-well low attachment plate. Shake plates were incubated with shaking at 270 rpm overnight. Cells were stained for the presence of gp64 as described.

Optimization of Culturing Conditions

Fifty ml shake-flask cultures were used to test for the optimal antibody concentration. One goal in the development of the assay was to create a high-throughput assay that would utilize the 96-well plate capacity of the Guava PCA-96 and allow for the determination of multiple virus titers at one time. Cells were plated in a 12-well plate to mimic the stationary culture conditions planned for use with the 96-well plate but also to provide sufficient cell numbers for multiple tests of single wells. Two ml of Sf9 cells in a logarithmic phase of growth were plated at a concentration of 10^6 cells per ml. Fifty ml shake cultures at 10^6 cells/ml were seeded in parallel. Wells and flasks were infected with 10-fold dilutions of wt AcMNPV and rocked for four hours then transferred to a stationary incubator and cultured overnight at 27° C. Two hundred µl cell suspensions were transferred to a 96-well plate in duplicate and stained as described above.

The results presented in Figure 2 indicate that the percentage of positive cells was significantly lower than the same infection conditions as the shake flasks. Cells were then plated as above and placed in a shaking incubator during the incubation period in an attempt to reproduce the conditions in the shake flask. Removing the cells from the culture plate for staining was

Table 1. Determination of virus titer using the "normalization" method. Stock concentrations are considered to be saturating and therefore represent the maximum percentage of cells that can be infected for that assay. 10-fold dilutions are performed in duplicate and the average taken. To normalize the data, averaged percentages are divided by the stock, or maximum, percentage (Corrected Percentage). The corrected percentage is multiplied by the dilution factor and those values are again averaged for a total of eight measurements per virus. The averaged value is multiplied by the number of cells per ml to determine the viral titer.

Dilution	*Sample 1*	*Sample 2*	*Average*	*Corrected Percentage (CP)*	*Dilution Factor (DF)*	*CP x DF*	*Average x Cell Number*
Stock (1:1)	77.5	78.2	77.85				
1:10	72.9	72.4	72.65	0.93320488	10	9.3320488	
1:100	49.1	44.7	46.9	0.60244059	100	60.244059	
1:1000	10.9	9.5	10.2	0.13102119	1000	131.02119	
1:10000	1.8	1.4	1.6	0.02055234	10000	205.52344	
					Average→	132.26289	**2.65×10^8**

Table 2. Comparison of data analysis methods and two known standard curves. Sample A is a wt AcMNPV stock generated by Expression Systems and known to have a titer of 5 x 10^8 infectious units per ml. Sample B was obtained from Pharmingen and had a known titer of 10^8 pfu/ml. Unknown sample was generated by Expression Systems. The first data column presents the titer determined by the "normalization" method described above. The second data column uses Sample A to generate the standard curve and the third column uses sample B to generate the standard curve.

	"Normalization" Method	Sample A Standard Curve	Sample B Standard Curve
Sample A Titer	4.09 x 10^8	5.53 x 10^8	3.80 x 10^8
Sample B Titer	2.58 x 10^8	1.42 x 10^8	1.02 x 10^8
Unknown Sample Titer	2.04 x 10^9	3.40 x 10^9	2.37 x 10^9

difficult due to tight adherence of the cells, and the staining results were not improved over the stationary culture (data not shown). Costar UltraLow Attachment plates were obtained and cells were plated in triplicate in a 96-well format. One hundred µl of Sf9 cells at 2 x 10^6/ml were plated per well and 100 µl of virus at 10-fold dilutions was added. The culture was incubated overnight at 27° C while shaking at 270 rpm. Cells were stained the next day as described above. It was determined that shaking in a plate treated to prevent attachment more closely mimicked the situation seen in shake flasks (Fig. 2).

Data Analysis

Determination of viral titer was performed by one of two ways, one of which requires a standard curve. To determine the titer without a standard curve, the replicate wells were averaged and the percentage of infected cells was determined for each dilution. The stock virus dilution (a 1:2 dilution) is considered to be in excess of the amount of virus required to infect all the susceptible cells in the culture. This number will vary depending on the state of the Sf9 cells used in the assay. To normalize the data and compensate for the cells in the culture that are not able to be infected, the percentage of infected cells for all other dilutions is divided by the stock virus percentage. The average of the dilutions is determined by multiplying the normalized percentage for each dilution by the dilution factor. This average is multiplied by the number of cells per ml in the wells when seeded, to determine the number of infectious virions per ml of virus stock (Table 1). This method will not work for low titer virus stocks as the stock dilution will not be in excess.

Using a standard curve provides both a direct comparison to a known virus stock as well as a reproducible positive control. Data generated from a known sample are plotted on a logarithmic scale. A trend-line is generated using an exponential curve and an equation determined using the data analysis feature of Microsoft Excel. Data from the unknown virus samples are applied to the equation and the average of the results from each dilution is used to determine the viral titer. This method provides a more accurate determination of virus titer as long as there is confidence in the stock used for the standard curve. To determine the relative sensitivities of the two data analysis methods, virus titer determination for two known and one unknown viral stocks was compared using the "normalization" method, using a standard curve generated from the data from sample A and using the standard curve generated from Sample B. The data presented in Table 2 demonstrates that either method can determine virus titer within a 2-fold range.

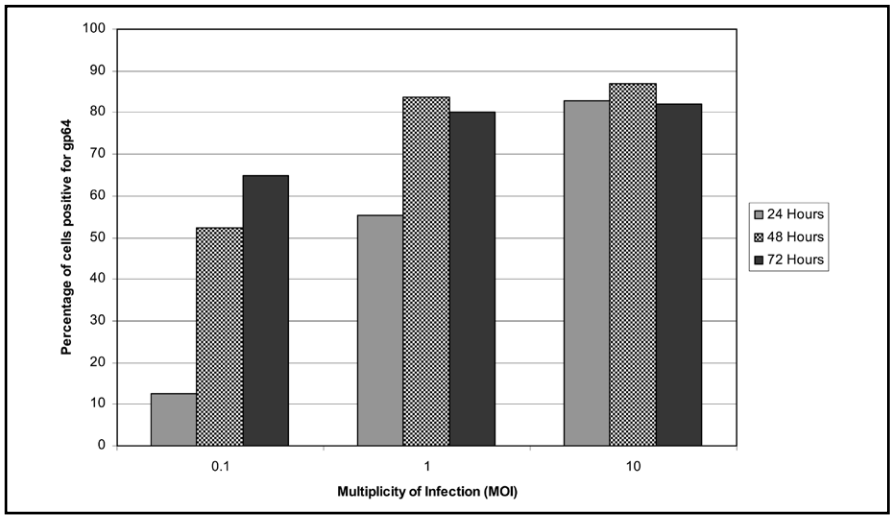

Figure 3. Sf9 cells at a density of 2 x 10^6 cells/ml were infected at an MOI of 0.1, 1, and 10 to confirm validity of titer assay. Cultures were incubated as described previously. Titer of this recombinant baculovirus was determined by the flow cytometric assay using a standard curve. Three replicate plates were infected and one plate each was stained for gp64 expression at 24 hours (gray bars), 48 hours (checkered bars) and 72 hours (black bars).

Confirmation of Flow Cytometric Titering Method

A virus stock determined to have a titer of 1.28 x 10^9 was used to infect Sf9 cells at three different MOI to confirm the accuracy of the titering method. Sf9 cells were infected at an MOI of 0.1, 1, and 10, and the percentage of infected cells was determined at 24, 48 and 72

hours post infection (Fig. 3). As expected, at 24 hours post infection there was a clear effect of the different MOIs on the percentage of infected cells. The culture infected with an MOI of 10 reached maximum infection rates at 24 hours as seen by the lack of change in the percentage of infected cells over the course of the experiment. The culture that was infected with an MOI of 1 resulted in 55 percent of the cells expressing gp64 24 hours later, thus achieving comparable levels as those seen with the MOI of 10 at 48 hours post infection. The culture infected at an MOI of 0.1 had a dramatically lower number of infected cells 24 hours post infection, and the secondary rounds of infection were not sufficient to infect the culture at levels seen with higher MOIs.

Data that was generated using the flow cytometric assay was compared to data from either a real-time PCR assay or modified end-point dilution assay. Measurements determined by the flow cytometric method were performed as described above and the titer was determined by comparison to a standard curve. The real-time PCR assay was performed using standard methods and primers specific for sequences present in the common transfer vector.[6] Quenching was compared to a standard curve generated from a known wt AcMNPV. Samples A and F were also titered using a combination of the modified end-point dilution method along with scoring for occlusion bodies as A and F were both wt AcMNPV. Titers are reported as infectious particles per ml rather than the standard plaque forming units per ml. Sample A was also titered by the standard plaque assay and was determined to have a titer of 5 x 10^8 pfu/ml (data not shown). All the tested viruses were shown to have equivalent titers by two or more methods, confirming that the flow cytometric method is an accurate and reproducible method for baculovirus titering.

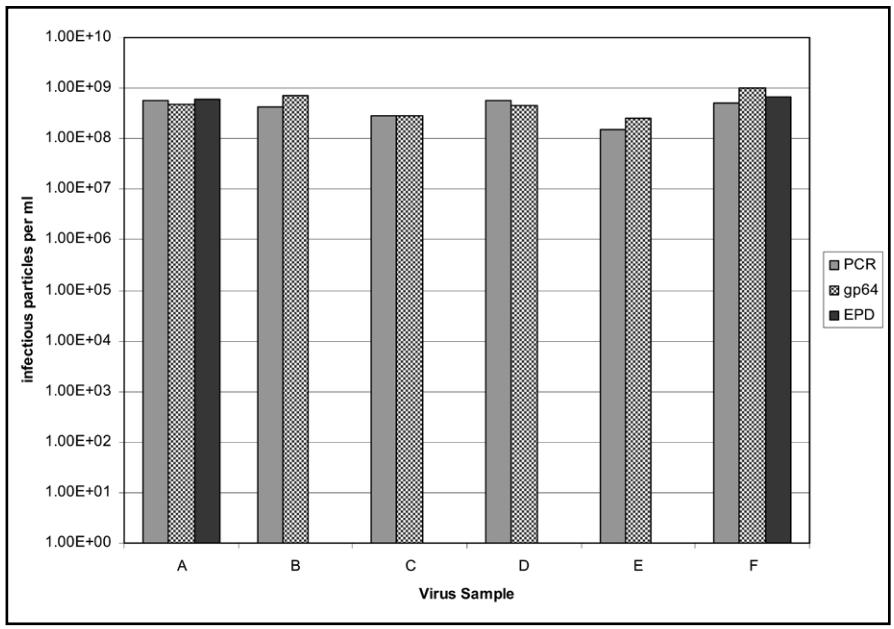

Figure 4. Six virus samples (A–F) were tested for virus titer using the flow cytometric assay as described. The same six samples were titered by real-time PCR method using primers specific for the transfer vector. Titer determination was made by comparison to a standard curve for both the flow cytometric assay and the PCR assay. Samples A and F are wt AcMNPV stocks generated in ESF 921 (A) or ESF AF (F). End-point dilution assays were performed in ESF 921 and titer confirmed by counting the number of occlusion body positive cells. Sample A was also titered by plaque assay, which gave a titer of 5 x 10^8 pfu/ml.

Table 3. Comparison of titer determined using different cell lines and different media formulations was performed for two viruses. All cell and media conditions for each virus were contained on a single plate ensuring the same culture conditions. Sf9, PRO™, or Sf21 cells were seeded as described previously in either ESF 921, ESF AF or TNM-FH. Virus dilutions were made in the same culture media as the cells it would be added to. Cultures were incubated overnight and stained as described. Titer columns show the titers determined by the flow cytometric assay using a standard curve. MOI columns demonstrate the volume of virus stock needed to infect a 1L culture of cells at 10^6/ml at an MOI of 1.

Cell Type / Media	Virus A	Virus A	Virus B	Virus B
	Titer	MOI of 1 (ml)	Titer	MOI of 1 (ml)
Sf9 / ESF 921	4.14 x 10^8	2.42	8.34 x 10^8	1.20
Sf9 / ESF AF	2.72 x 10^8	3.68	5.82 x 10^8	1.72
Sf9 / TNM FH	8.27 x 10^7	12.09	1.59 x 10^8	6.29
PRO™ / ESF 921	1.06 x 10^8	9.43	5.09 x 10^8	1.96
PRO™ / ESF 921	1.02 x 10^8	9.80	3.63 x 10^8	2.75
Sf21 / ESF 921	9.40 x 10^7	10.64	1.82 x 10^8	5.49

Comparison of Titer Using Different Cell Lines and Media Formulations.

The power of the high-throughput flow cytometric assay is demonstrated by comparing the virus titer that is determined when using different cell lines and media formulations in a simultaneous assay. Two virus stocks were examined for the ability to infect Sf9, Sf21, and PRO™ cells. The tested media formulations were ESF 921, ESF AF, and TNM-FH. Cells were plated in the different media, as previously described, and virus dilutions were made in the corresponding media. Incubations and staining were performed as described. The resulting titer of the virus varied depending on both the cell line and the media formulation that was used (Table 3). The effect of this variation is apparent when determining the volume of virus stock that is necessary to infect a 1L culture of cells at a density of 10^6 cells/ml at an MOI of 1.

DISCUSSION

The flow cytometric assay presented here detects expression of a viral protein on the cell surface of an infected cell and is an easy and accurate method of baculovirus titer determination. gp64 was chosen as the target antigen because it is required for viral binding to the insect cell surface.[8] This feature makes this assay applicable to all recombinant AcMNPV regardless of the method of generation. Cell surface expression of the gp64 protein also indicates a productive infection and ensures that the assay is only detecting infectious virus. The gp64 protein is synthesized within six hours of infection with accumulation at the membrane surface occurring some hours later.[9] Limiting the incubation to a maximum of 18 hours prevents an overestimation of viral titer as a consequence of viral spread while still allowing the sufficient protein expression needed for detection. Overnight incubation is also technically convenient as the assay can be set up on one day and stained the following day, with results in less than 24 hours.

The strategy of adding the virus and then shaking the plate during incubation has several advantages. Eliminating the virus removal step saves time and reagents. This also eliminates the problem of titer underestimation as observed in assays that require virus removal.[10] Cells grown in suspension are more sensitive to viral infection than cells grown in a monolayer, which are susceptible to cell-cell contact inhibition.[10] Shaking the plate during incubation and using a low attachment plate allows for more efficient viral infection and more closely simulates the culture conditions the virus will be subjected to during protein expression cultures. Therefore, more accurate viral titers are determined in a manner closer to the final application.

The state of the cells that are used to determine the virus titer is of great importance because the stage of the cell cycle at the time of virus addition greatly affects the percentage of infected cells. Cells in an exponential phase of growth are more likely to become infected than cells that are nearing the end of the cell cycle.[11] Therefore, it is important to seed the working stock of cells at a low density prior to using the cells in the assay to ensure that a maximal number of them are in an exponential growth phase. The cell cycle phase distribution can vary from culture to culture. This variation is addressed both by "normalizing" the data as described above, and by the use of a standard curve. Different cell lines have different virus attachment rates, and media components can affect virus binding.[10–12] The ability to determine viral titers for a number of cells and media formulations simultaneously allows for more accurate optimization of production parameters.

There are a number of virus titering methods available. Here we describe a method that is accurate, rapid and user friendly. For analysis of the fluorescently stained cells, the Guava PCA-96 was used due to its 96-well plate capacity and its affordability. However, this assay will work with any flow cytometer and has the potential to work with any fluorescence-based plate reader.

Whatever method is used, it is of great importance to clearly describe that method when stating a virus titer or multiplicity of infection. The viral dynamics of a culture will vary greatly between cultures infected with an MOI of 1 determined by plaque assay versus that determined by PCR. What appears to be a high MOI of 50 required to infect an Sf21 culture may in fact be an MOI of 5, but the titer was determined in Sf9 cells using a different media formulation. Reporting about the methodology used allows other investigators to more easily interpret results and apply those data to their own applications. The high-throughput nature of the flow cytometric assay described allows for the elimination of some of the potential confusion.

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