A new method for the calculation of baculovirus titre using the Agilent 2100 bioanalyzer and the flow cytometry set

Application Note

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Abstract

This Application Note provides an extended discussion of results presented in a recent publication in BioTechniques[1] and describes the accurate and rapid determination of baculovirus titre using the Agilent 2100 bioaanlyzer with the flow cytometry set. The high reproducibility of the microfluidic chip-based approach, coupled with the low cell consumption, ease of use and automated data collection make this method an extremely attractive alternative for the calculation of baculovirus titre compared with other standard viral titre determination methods. Detailed protocols and reagent recommendations for the calculation of viral titre using the 2100 bioanalyzer are given.

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Introduction

There are a variety of expression systems for higher eukaryotic cells – one of the best known is the baculovirus system for over expression of a protein in insect cells. This method allows very high levels of expression and proper post-translational modification of the protein. The baculovirus system requires homologous recombination inside a transfected insect cell. Recombination takes place between a linearized version of the baculovirus genome that has part of an essential gene missing and a transfer vector carrying the needed missing piece and the gene of interest. The gene to be over expressed has to be cloned into the transfer vector, where it is under the control of a strong promoter that normally controls formation of a major baculovirus protein. The cell combines the linear baculovirus and transfer vector to create a complete baculovirus genome containing the gene of interest.

To maximise protein expression it is often necessary to perform a series of optimization experiments. Such optimization experiments often include varying the time of infection (48-96 hrs), or the volume of virus added to a viable cell number (expressed empirically as the multiplicity of infection). The multiplicity of infection (MOI) is the ratio of input virus to number of cells. The average MOI in a culture of cells is simply the number of infectious units (IU) divided by the number of cells in the culture. Calculating the MOI can prove extremely time consuming and laborious. Optimization studies can be run without calculating the viral titre by analyzing recombinant protein expression levels in a given volume of insect cells infected with different volumes of recombinant virus. Whilst this is a useful shortcut when working with a large viral stock that is to be used for both optimization and final expression studies, the calculation of a value for the viral titre of a recombinant baculovirus stock is recommended as a point of reference in case subsequent further protein expression studies require the use of different viral stocks for the generation of the same recombinant protein.

The titre of a recombinant baculovirus stock can be determined by plaque assay[2], end point dilution[3] and immunoassay[4,5]. The advantage of the plaque assay is the potential for an exact determination of viral titre. However, the disadvantage of this method is that it is difficult to perform and that it requires a long processing time (approximately 1 week). Furthermore, visualizing plaques can be difficult and requires an expert, well-trained eye. On the other hand, the end-point dilution method is very simple. However, results from this method are often more difficult to interpret than plaques assays. A number of vectors that carry an infection marker have therefore been developed to overcome problems associated with the identification of recombinant plaques and infection. Typically these incorporate the β-galactosidase gene and require the addition of substances such as X-gal [6,7], although green fluorescent protein (GFP) has also been used with some success in this and other laboratories [8,9,10]. An immunoassay procedure is marketed by BD Biosciences & Novagen under the trade names BacPAK™ & FastPlax™. The assay uses a primary monoclonal antibody raised to an Autographa californica nuclear polyhedrosis virus (AcNPV) envelope glycoprotein, gp64. A secondary HRP-conjugated antibody facilitates visualization of infected cells by light microscopy and thus determination of the viral titre. The main advantage of this approach is that since viral antigens are expressed long before plaques are formed, the assay can be completed in a much shorter time period than the traditional methods described above. The disadvantage is that the commercial kits are very expensive and typically over-estimate viral titre several-fold. In addition, they can, as with plaque assays and end-point dilutions, be difficult to interpret giving rise to high variability between users. Therefore, despite the popularity of the baculovirus expression system in protein production laboratories worldwide, there is no fast, reliable and inexpensive method of virus titre determination.

We have therefore developed an automated method for the determination of baculovirus titre that uses GFP-linked co-expression plasmids similar to those recently published[11] and the Agilent 2100 bioanalyzer (Stockport UK) to generate quick, highly reproducible viral titre estimates.

Materials and methods

Cell culture

Suspension cultures of *Sf*21 cells were grown in 5-mL flasks at 27 ºC.

Construction of expression vectors

A *Xho*I - *Kpn*I flanking GFP fragment was generated by standard PCR methodology and sub-cloned into the p10 multiple cloning site (MCS) of pFastBac Dual (Invitrogen) to create a GFP co-expression plasmid compatible with the Bac-to-Bac Expression System. GFP pFastBac Dual was transposed into DH10Bac (Invitrogen) competent cells and recombinant bacmid isolated following the manufacturer's instructions (Invitrogen). The bacmid was then transfected into *Sf*21 and high titre recombinant virus produced. Viral titre was calculated using standard viral plaque assay (Invitrogen), BacPak™ immunoassay kit (BD Biosciences) or 2100 bioanalyzer (Agilent Technologies) following the manufacturer's instructions or as described in the figure legends.

Transfection

A 50-mL culture of *Sf*21 cells was infected with 1 mL of GFP virus standard Cellfectin ™ transfection methodology as described by the manufactures (Invitrogen).

CBNF staining

Following incubation for 48 hrs at 27 °C. Cells were then stained for 15 min at room temperature with 0.5 µM live cell dye carboxy-naphthofluorescin diacetate [CBNF] (Molecular Probes), pelleted by centrifugation (500 g, 5 min), and resuspended in cell buffer ($2x10^6$ cells/mL). 10 µL of viral sample was then loaded onto a cell fluorescence LabChip and analyzed on the Agilent 2100 bioanalyzer [12].

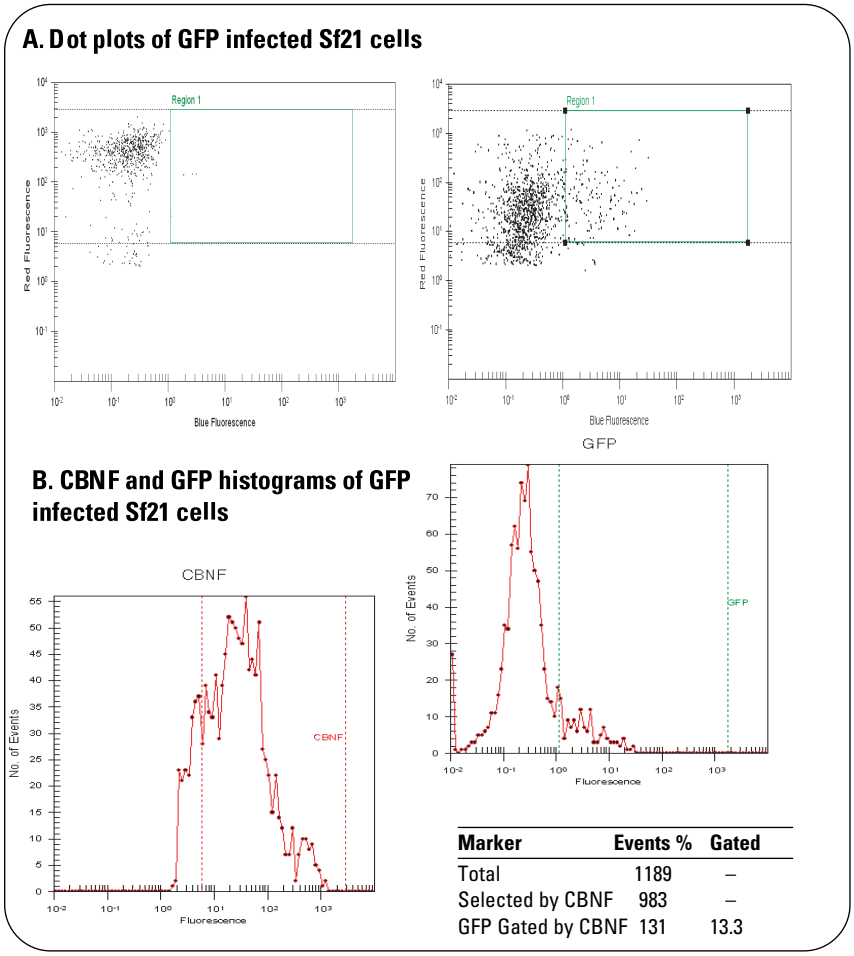


Figure 1

GFP infected *Sf*21 cells analyzed by the Agilent 2100 bioanalyzer. Cell culture volumes of 50 mL ($0.5x10^6$ cells/mL) were infected with 1 mL of GFP virus and incubated for 48 hr, at 90 rpm, 27 °C. Following incubation, cells were counted, harvested and resuspended at $1x10^6$ cells/mL in HBSS with 0.05 % (v/v) pluronic acid (Molecular Probes). Cells were then stained for 15 minutes at room temperature with 0.5-µM live cell dye carboxy-naphthofluorescin diacetate [CBNF] (Molecular Probes), pelleted by centrifugation (500, 5 minutes), and resuspended in cell buffer ($2x10^6$ cells/mL). 10 µL of viral sample was then loaded onto a cell fluorescence LabChip and analyzed on the Agilent 2100 bioanalyzer[12].

Results and discussion

In an initial experiment to ascertain whether recombinant baculovirus expressing GFP could be detected using the Agilent 2100 bioanalyzer a 50-mL culture of *Sf*21 cells was infected with 1-mL of GFP virus. Following incubation for 48 hrs at 27 °C, cells were harvested and stained with CBNF. The stained cells were then analyzed on the 2100 bioanalyzer, each run taking less than 25 minutes to complete. Figure 1A shows fluorescent dot blots (CBNF versus GFP) of cells that have been infected with a 1-mL volume of 10^{-1} diluted GFP-viral stock. The population within the rectangular region represents live (CBNF-positive) and GFP expressing *Sf*21 cells. The data can also be displayed as frequency histograms, as depicted in figure 1B.

In order to determine the percentage of GFP expressing cells, live cells in the CBNF-positive population were cross-gated onto the GFP histogram. Figure 1B shows that of 983 CBNF-positive cells, 13.3 % can be cross-gated with GFP fluorescence. Knowing the number of CBNF-positive cells and the number of GFP expressing cells also allows the calculation of the viral titre using the equation described by Berns and Giraud[12].

From Berns and Giraud, 1996:

$$\text{viral titre (IU / mL)} = \frac{\text{\#GFP gated cells x dilution factor x \#CNBF-positive cells}}{\text{volume of virus added (mL)}}$$

From Figure 1B:

viral titre = 131/1 x 10 x 983 = 1.26 x 106 IU/mL

Table 1
Calculation of viral titre using the Agilent 2100 bioanalyzer

The calculated value is 1.26 x 10^6 IU/mL (table 1) and compares favourably with the viral titre calculated using the traditional plaque assay (4.1 x 10^6 pfu/mL) or the BacPAK immunoassay (6.4 x 10^6 IU/mL) respectively. Figure 2 shows a photograph of the same 50 mL volume of infected *Sf*21 cells when viewed under a fluorescence microscope. The infected cells can clearly be seen as bright green foci.

It is noteworthy that since *Sf*21 cells are maintained in suspension culture throughout the course of the experiment (and are not immobilized as is the case in plaque and immunological methodologies) there is a chance that secondary infections may occur, thereby giving rise to over-

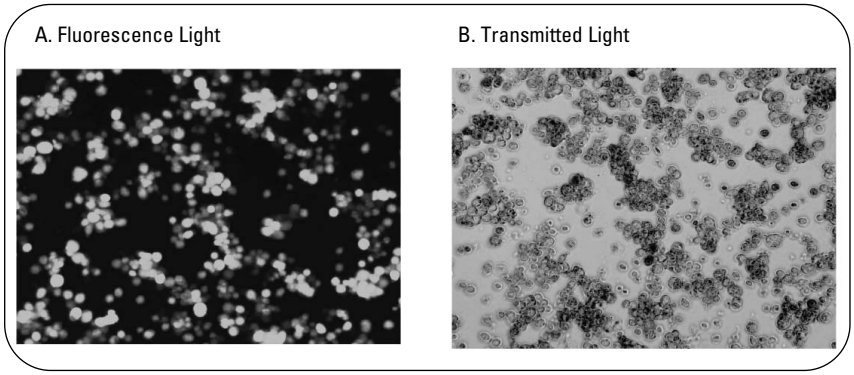


Figure 2
Analysis of GFP infected *Sf*21 cells by fluorescence microscopy
***Sf*21 cells infected with recombinant GFP expressing baculovirus were photographed using fluorescence microscopy.**

estimates of viral titre. However, based on the estimates given above the bioanalyzer assay appears to provide a value that is actually slightly lower than the other approaches. This suggests that such concerns are not a major issue, although further study is required before a definitive conclusion can be drawn.

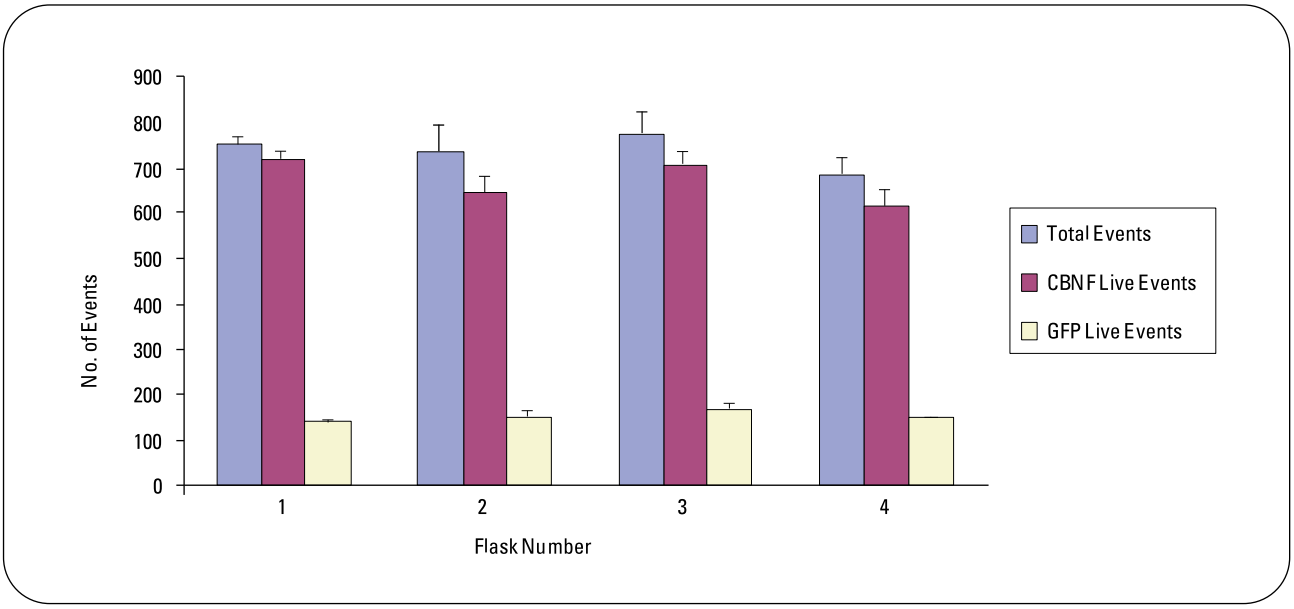
In a separate experiment we also tested the sensitivity of the method. Table 2 shows after 48 hours the number of GFP-gated events in *Sf*21 cells infected with GFP containing baculovirus drops from 86 (9.8 % gated) to 4 (1.4 % gated) when the virus added is diluted from 10^{-1} through to 10^{-3}. Conversely, *Sf*21 cells when infected with the GFP-minus baculovirus consistently show only 3-4 GFP-gated events (0.5 – 1 %

Flask	Dilution	#Total events	# CBNF events	#GFP events	% Gated
GFP infected cells					
1	10^{-1}	965	879	86	9.8
2	10^{-2}	708	633	9	1.4
3	10^{-3}	747	654	4	0.6
4	10^{-4}	802	598	5	0.8
5	10^{-5}	751	518	3	0.6
6	10^{-6}	762	478	4	0.8
Mock infected cells					
1	10^{-1}	934	913	3	0.3
2	10^{-2}	833	802	4	0.5
3	10^{-3}	789	754	5	0.6
4	10^{-4}	694	613	3	0.5
5	10^{-5}	716	624	3	0.5
6	10^{-6}	656	574	5	0.9

Table 2
Sensitivity of detection of GFP and non-GFP-containing baculovirus measured using the Agilent 2100 bioanalyzer.
6 x 50 mL volumes of Sf21 cells (0.5x106 cells/mL) were infected with a 1-mL volume of viral stock (GFP-expressing and non GFP-expressing) diluted to 10-1, 10-2, 10-3, 10-4, 10-5, and 10-6 and incubated for 48 hrs at 27 °C. Cells were then harvested and samples prepared for analysis as described in figure 1. Prior to dilution, the titres of both GFP-minus and GFP-containing baculovirus stocks were determined by plaque assay and normalized to 0.64x107 pfu/mL by dilution.

gated). This small number of events presumably represents background fluorescence, since no GFP is present in any of these samples. Taken together this suggests that the GFP-containing virus is effectively removed from the cells by dilutions at 10^{-3} and beyond.

It must be stressed however that for each recombinant virus studied, the point at which the virus is effectively neutralized by dilution will vary considerably. For example, in the case of a stock with a viral titre of 10^{8} /mL the use of a 10^{-1} dilution to calculate titre could cause significant secondary infections, giving rise to erroneous results. We therefore recommend that a serial dilution profile is performed on each viral stock to ascertain the most appropriate conditions for the calculation of viral titre when using this method. Finally, to ascertain the reproducibility of the assay and to identify any variability between cell fluorescence LabChips we infected 4 flasks with GFP and analyzed them on multiple chips. Figure 3 shows that for each flask infected with GFP-containing baculovirus, the number of total cell events, the number of live CBNF events, and the number of GFP-gated events calculated are in extremely close agreement. Similarly this pattern is also observed between different chips suggesting high reproducibility of the system.



Chip #	Flask #	Av. Total Events	SD	SError	Av. CBNF	SD	SError	Av.GFP	SD	SError
1	1	751	24	14	719	27	16	145	4	2
	2	738	62	36	646	58	33	147	15	8
2	3	774	61	35	705	52	30	169	17	10
	4	685	40	23	616	45	26	149	0	0

Figure 3
Reproducibility of baculovirus infection using the Agilent 2100 bioanalyzer.
4 x 50 mL volumes of *Sf*21 cells (0.5 x 10^{6} cells/mL) were infected with 1 mL of viral stock diluted 10^{-1}. Following incubation for 48 hrs at 27 °C, cells were harvested and samples prepared for analysis as described in figure 1. For each 50 mL *Sf*21 flask infected with GFP-containing virus, samples were prepared and loaded in triplicate onto a single cell fluorescence chip for data capture. Two chips were analyzed, each containing 2 flasks loaded in triplicate.

Conclusions

In summary, we report the development of a fast and highly reproducible method of calculating the viral titre of a baculovirus stock using the Agilent 2100 bioanalyzer and cell fluorescence LabChip kit in concert with GFP baculovirus co-expression plasmids. The method described offers several advantages over alternative approaches for viral titre calculation. Firstly, since the 2100 bioanalyzer automates data collection, it does not rely upon the operator to differentiate between infected and non-infected cells. Automation of data collection therefore removes user-to-user variability and thereby one of the largest sources of error associated with other viral titre determination methods. Secondly, the method is very quick and simple to perform. 48 hours post-infection, insect cells infected with GFP-containing virus can be harvested and within 90 minutes a value for viral titre determined. This compares favourably with the more time-consuming and labor intensive plaque assay and immunoassay which both require extensive washing and fixing of cells. The cell chips have been designed to have individual, isolated micro-fluidic channels for each of the samples so there is no possibility of cross contamination between samples. The cell chips are consumables, intended for single use only. This makes disposal easy and prevents contamination of equipment.

These protocols could also be used in concert with other flow cytometer systems such as FACS or MoFlo, (although these methods do require larger quantities of cells) making this approach applicable to many laboratories. The methods could also be adapted for use on adherent insect cell cultures. Using standard 6-well plates, cells could be infected with viral stock and following incubation simply detached from the plate and resupended ready for analysis using standard techniques. Indeed, this approach may be more favourable in many laboratories where suspension cultures are not typical. A second approach would be the use of 24-deep-well blocks for the culture of insect cells and concomitant infection with virus. Both these approaches have the advantage of requiring much smaller volumes of cells and virus.

To further facilitate baculovirus mediated expression using this system we have adapted the GFP co-expression plasmid described above to make use of GATEWAY™ technology (Invitrogen). Thus, the use of the GATEWAY™ rapid cloning in conjunction with the enhanced speed and reproducibility of the Agilent viral titre method described in this report have enabled us to make significant savings in time and effort in our protein optimization studies. We have also extended this further by generating a series of plasmids that allow the co-expression of up to 4 proteins in concert with GFP. These plasmids are particularly useful when expressing a GPCR and the concomitant G-proteins or when proteins require the expression of adaptor or stabilising co-factors.

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