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Statistical Determination of Optimal Baculovirus Infection Condition for Recombinant Protein Production in *Drosophila* S2 Cells

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Abstract Insect *Drosophila melanogaster* S2 cell was developed as plasmid-based and, therefore, a nonlytic expression system for functional foreign proteins. To achieve multiple protein expressions, it was suggested that baculovirus be used on S2 cell system because baculovirus can infect S2 cells but cannot replicate inside the cells. Therefore, establishment of baculovirus infection conditions is the first important step and this should be properly optimized for production yield. We used statistical methodology to optimize the baculovirus infection conditions using green fluorescent protein (GFP) as a reporter protein. Consequently, we arrived at optimal infection conditions through a statistical regression method. The secreted GFP yield from *v*MT-GFP baculovirus-infected wild-type S2 cells under optimal infection conditions was >15-fold higher than that under nonoptimal conditions and comparable to that from stably transfected recombinant S2 cells.

Keywords *Drosophila* S2 cells · Baculovirus · Infection condition · Statistical regression

Introduction

Among the insect cells introduced as high eukaryotic expression systems to overcome some deficiencies in prokaryotic and mammalian cell systems, Schneider line 2 (S2) cell derived from *Drosophila melanogaster* has been developed as a plasmid-based, nonlytic integration system [1, 2]. High copy numbers of recombinant plasmid vector are inserted into the S2 cell genome, with the advantage that foreign proteins are expressed stably without destroying cells [3, 4]. The nonlytic process is more efficient for secreted proteins. Therefore, the S2 cell system is suitable for the efficient expression and secretion of functional heterologous eukaryotic gene products [5–8]. However, despite the advantages of the S2 cell system, simultaneous stable expression of multiple types of foreign proteins

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might not also be simple because of the size limitation of an expression vector; construction of expression vector that has large foreign DNA inserts is difficult because of its genetic instability [9]. Although cotransfection of multiple expression vectors can be used [10], this strategy needs additional selection markers such as *neo* (resistance to antibiotic G418) and *hph* (resistance to hygromycin B) genes and continuous expressions of these markers can cause considerable metabolic burden on host cells. Also, modulating expression of target proteins is not easy in the stably transfected cell lines [11].

Baculovirus has been widely used for foreign protein expression in Lepidopteran insect cells such as *Spodoptera frugiperda* and *Trichoplusia ni* cells [12–15]. Recombinant baculovirus can infect these insect cell lines and facilitate expression of foreign protein. Also, expressions of multiple foreign proteins at different levels are possible using a coinfection strategy, and it was reported that up to three target proteins were coexpressed [16–19]. However, this baculovirus system causes cell lysis, which can result in severe contamination of target protein with many cellular proteins including proteases, and is therefore not a "clean" secretion system. Interestingly, it has been revealed that baculovirus infection does not lead to gene expression with its original promoters such as polyhedrin in mammalian and *Drosophila* cells, which are nonpermissive for viral replication although the virus can integrate into the host chromosome [20–24]. However, a modified baculovirus-containing promoter that can work in mammalian or *Drosophila* cells was able to express a target protein in each expression system [20, 22, 25]. Therefore, an advantage of *Drosophila* S2/baculovirus expression system is that baculovirus infection does not cause cell lysis, resulting in "clean" secretion of target protein. In addition, baculovirus enables to deliver multiple exogenous genes into S2 cells, resulting in simultaneous or successive expressions of multiple target proteins depending on infection strategies (co-, super-, or successive infection) [24, 26]. Furthermore, modulating target protein expression is possible by changing the infection doses of recombinant baculoviruses.

Optimization of infection condition will be the first and important step for maximum production of foreign protein in virus system. Usually, the method to search optimal infection is based on a single factor search (SFS). Although the SFS methodology might not require many measurements, this approach cannot explore the experimental space efficiently and also does not reflect interactions of several factors that should be considered in optimization. Therefore, SFS might miss the best possible solution. In the present work, we used statistical regression approach to determine optimal conditions for recombinant baculovirus infection on S2 cells. Target foreign protein was green fluorescent protein (GFP) from the jellyfish *Aequorea victoria*. Facile monitoring of green fluorescence makes it a widely used reporter protein, and it is a unique tool in that its fluorescence does not require any substrates, cofactors, or enzymes and can be detected in living cells in real time [14, 15, 27].

Materials and Methods

Expression Vectors and Recombinant Baculovirus

pMT/BiP/V5-His/GFP vector (Invitrogen, USA), which contains the *Drosophila* metallothionein (MT) promoter that is activated by Cu^{2+}, a BiP signal sequence to facilitate secretion, and GFP cDNA, was used for stable transfection of wild-type S2 cells [28]. pAc-MT-GFP, which contains the MT promoter, the BiP signal sequence, and GFP gene, was used for construction of recombinant baculovirus *v*MT-GFP [26]. The titer of recombinant baculovirus *v*MT-GFP was 3.57×10^8 pfu/ml.

Infection of S2 Cells using Recombinant Baculovirus and Induction of Protein

Wild-type S2 cells in 100-mm culture dishes were grown to 9×10^6 cells/ml in 4 ml M3 medium (Shields and Sang M3 insect medium; Sigma, USA) containing 10% IMS and 300 μg/ml hygromycin B (Sigma). Culture medium was then replaced with fresh medium containing a determined volume of *v*MT-GFP viral solution and incubated with gentle shaking. Baculovirus-containing medium was then replaced with fresh medium. To produce secreted GFP from infected S2 cells, cells in 100-mm culture dishes were grown at 27°C for 1 day in 4 ml M3 medium/10% IMS/hygromycin and then induced with copper sulfate to a final concentration of 500 μM.

Analytical Assays

Cell number was counted from each sample using a hemacytometer (Fisher Scientific, USA) and viability was determined by trypan blue (Sigma) exclusion using a 0.4% (*w*/*v*) solution. Because the secretion efficiency of GFP was more than 95% during the entire culture (data not shown), secreted medium fraction was only considered for GFP fluorescence intensity measurement. Fluorescence intensity of supernatant centrifuged at 5,000 rpm for 5 min was measured from the light emission at 509 nm with excitation at 395 nm using a fluorescence spectrophotometer (Shimadzu, Japan). Because of interference of medium components in measurement of GFP fluorescence intensity, we subtracted fluorescence intensity of the nontransfected sample (nontransfected S2 cells were grown in the same condition) from that of the transfected one.

Western Blot Analysis

Because GFP was secreted into the extracellular media, cellular and medium fractions of the culture samples were separated by centrifugation at 5,000 rpm for 10 min. Medium fraction was mixed with SDS sample buffer (10% sodium dodecyl sulfate (SDS), 10% β-mercaptanol, 0.3 M Tris–HCl (pH 6.8), 0.05% bromophenol blue, 50% glycerol), boiled for 5 min, and resolved by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to Hybond™-PVDF membranes (Amersham Pharmacia Biotech, England). After blocking for 1 h in tris-buffered saline (TBS) buffer (20 mM Tris–HCl, 500 mM NaCl, pH 7.5) containing 5% nonfat dry milk, the membrane was then incubated for 1 h at room temperature in antibody solution (1% nonfat dry milk in TTBS [TBS with 0.05% Tween-2]) containing rabbit anti-GFP polyclonal antibodies (1:1,000 *v*/*v*; Santa Cruz Biotechnology, USA) and probed with goat anti-rabbit monoclonal antibodies conjugated with alkaline phosphatase (1:1,000 *v*/*v*; Sigma). After successive washing with TTBS and TBS, alkaline phosphatase substrate (fast red tr/naphthol as-mx tablet sets; Sigma) was added to detect and the reaction was quenched with distilled water. The detected membrane was scanned, and the digitized images were stored and analyzed by Gel-Pro Analyzer software (Media Cybernetics, USA).

Regression Analysis

Regression analysis is a statistical technique for investigating and modeling relationship between variables. A regression model does not imply a cause–effect relationship. Although a strong empirical relationship may exist between two or more variables, this cannot be considered as the evidence that regressor variables and response are related in a

cause–effect manner. In almost all applications of regression, the regression equation is only an approximation to the true relationship between variables. An important objective of regression analysis is to estimate unknown parameters in the regression model. This process is also called parameter fitting in the model to the data. One of the parameter estimation techniques is least squared method, which analyzes continuous data. We used Minitab statistical program to find the least squared regression of our experiments because this program has been successfully used in many fields such as engineering, sociology, psychology, business administration, and quality control [29].

We defined a residual as

$$e_i = y_i - \widehat{y}_i, \quad i = 1, 2, \ldots, n,$$

where y_i is an observation and $\widehat{y}_i$ is a corresponding fitted value. Because the residual may be viewed as the deviation between the data and the fit, it can measure the variability that cannot be explained by the regression model. It is also convenient to consider the residuals as the realized or observed values of the errors [28]. Therefore, this residual was used to analyze property of regression.

Results and Discussion

We selected seven variables that have influence on baculovirus infection efficiency and foreign protein expression in S2 cells: multiplicity of infection (MOI), initial cell number, baculovirus incubation time, baculovirus incubation temperature, baculovirus total volume, postinfection time, and postinduction time. These experimental variables are generally regarded as important conditions for expression of heterologous protein in baculovirus/insect cell culture [26, 30–32]. MOI is a critical variable for baculovirus-mediated protein expression in nonpermissive cell lines such as S2 cells because infected baculovirus cannot be replicated inside the cells. It was already known that baculovirus infection efficiency is about 0.04 for flask cultures of permissive Sf9 cells although its efficiency can be increased by modulations of culture and infection conditions [33]. From these results, we can easily surmise that baculovirus infection efficiency might be much less in nonpermissive S2 cells than that in permissive Sf9 cells. Initial cell density in adherent culture should be approximately 50% confluent to allow maximal cell surface area to contact with virus and for subsequent infection. The degree of virus uptake is dependent on time and temperature of incubation [20, 34]. Ratio between virus total volume and cell culture dish area is also an important factor for infection. Postinfection time is a period of time for stabilization of cells after baculovirus infection and postinduction time is a period for expression of target protein after induction.

Twenty independent culture experiments were performed for statistical determination of optimal infection conditions using *v*MT-GFP baculovirus (Table 1). Based on the scatter diagrams for GFP fluorescence intensity according to change of each variable, we found that distribution of each scatter diagram had nonlinear pattern (Fig. 1). Therefore, we performed regression analysis for GFP fluorescence intensity as a function of seven variables using second- or third-order polynomial equation. Based on these equations, we compared experimental and simulated GFP fluorescence intensity values and found that summation of value differences was the smallest in the third-order equation, indicating that this equation can describe experimental results more effectively. Through the pareto chart of seven variables and interactions of these variables, we obtained the best suitable third-order equation as shown below.

Table 1 Experimental sets for *v*MT-GFP baculovirus infection on S2 cells.

MOI	Initial cell number ($\times 10^6$)	Baculovirus incubation time (h)	Baculovirus incubation temperature (°C)	Baculovirus total volume (%)	Postinfection time (h)	Postinduction time (h)	GFP fluorescence intensity (arbitrary value)
4	9	1	37	2.4	24	96	0.06
4	9	1	27	2.4	24	72	0.071
4	9	2	15	5.4	48	48	0.087
4	9	2	27	5.4	48	72	0.094
4	9	5	27	2.4	60	48	0.121
4	9	10	15	3.8	76	24	0.109
4	9	15	15	3.8	85	72	0.122
4	9	20	15	5.4	30	96	0.115
0.11	9	1	27	4.0	24	48	0.443
0.11	18	1	27	4.0	24	48	0.505
5.55	13.5	1	27	4.0	24	48	0.513
8.85	13.5	1	27	4.0	24	48	0.526
11.06	9	1	27	4.0	24	48	0.507
11.06	18	1	27	4.0	24	48	0.535
24.5	1.6	2	15	18.0	24	48	0.538
49	1.6	2	15	18.0	24	48	0.563
50	3.2	2	15	37.5	24	48	0.522
100	1.6	2	15	37.5	24	48	0.668
150	1.6	2	15	56.0	24	48	0.522
200	1.6	2	15	75.0	24	48	0.654

$$Y = 0.653 + 0.000030\,X_3X_3X_6 + 0.000151\,X_1X_2X_3 - 0.000382\,X_4X_4 + 0.000143\,X_4X_7 + 0.000047\,X_4X_4X_2 - 0.000001\,X_7X_7X_7 - 0.00129\,X_2X_6$$

Here, Y is GFP fluorescence intensity, X_1 is MOI, X_2 is initial cell number, X_3 is baculovirus incubation time, X_4 is baculovirus incubation temperature, X_5 is baculovirus total volume, X_6 is postinfection time, and X_7 is postinduction time. Regression can examine the relationship between a response and predictors. The p value of each coefficient of the predictor can tell us that the observed relationship between the response and the predictors is statistically significant or not. Because the p value of each coefficient was smaller than α-level (0.10), we convinced that each relationship had statistical meaning (Table 2). However, biological meanings of the interaction coefficients cannot be obtained from the optimization method based on regression because these coefficients are just meaningful for important terms in the combination equation. To obtain biological meaning for interaction of each parameter, we would need to employ other optimization techniques with consideration of viral infection progress in insect cells.

A normal probability plot of residuals is commonly used to check whether data are normally distributed or not. The residual, which is a difference between experimental results and fitness of regression model, plays an important role; if residual is zero, regression model is ideal. However, the residual is not generally zero. From the normal probability plot, we found that the residual points formed a rough linear line (Fig. 2a).

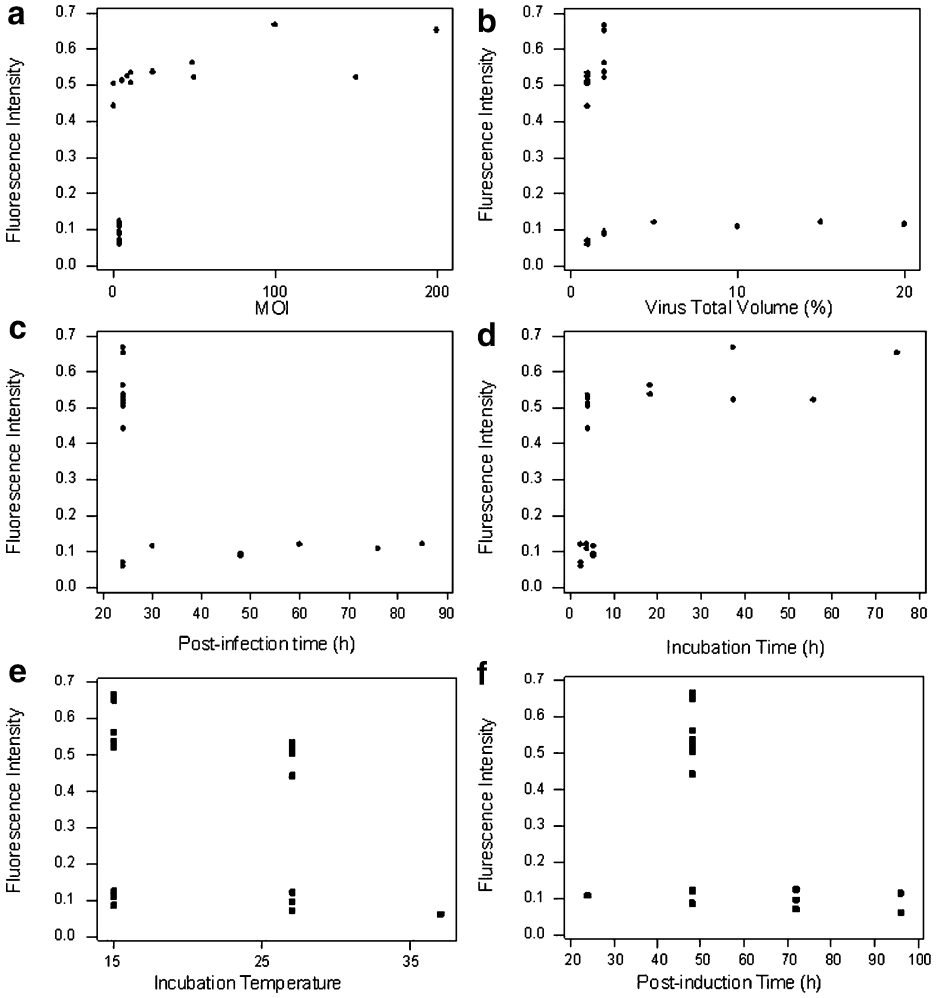

Fig. 1 Scatter diagrams for GFP fluorescence intensities according to (**a**) MOI, (**b**) baculovirus total volume, (**c**) postinfection time, (**d**) baculovirus incubation time, (**e**) baculovirus incubation temperature, and (f) postinduction time

Therefore, we convinced that the proposed third-order equation from regression analysis is reasonable and can be successfully used for subsequent simulation and optimization. We also plotted the residuals against the observation order to detect the correlation that may exist between random errors (Fig. 2b). According to a regression theory, random errors in the response measurements should be independent. The residuals versus other data showed randomly pointed pattern with adjacency to zero. However, because our experiments did not include upper range of variables, the results showed that the residuals were going away from zero point. Histogram of residuals can examine normal distribution of random errors, and a normal histogram should be symmetric and bell-shaped. We found that the histogram of residuals in our experiments had a bell-shaped pattern and had normal distribution (Fig. 2c). A residual plot against fitted values can be used to test model assumptions and identify unusual observations. This plot should be randomly distributed against zero and

Table 2 Coefficient analysis for third-order regression equation.

Predictor	Coefficient	SE coefficient	T	P
Constant	0.65259	17.30	0.03773	0.000
X3 X3 X6	0.00003011	0.00000478	6.31	0.000
X1 X2 X3	0.00015090	0.00007576	1.99	0.070
X4 X4	−0.00038213	0.00009753	−3.92	0.002
X4 X7	0.00014311	0.00002649	5.40	0.000
X4 X4 X2	0.00004668	0.00000508	9.19	0.000
X7 X7 X7	−0.00000098	0.00000007	−13.30	0.000
X2 X6	−0.00128747	0.00008661	−14.87	0.000
S=0.04151, R^2=97.9 %, R^2(adj)=96.7%				
F	P			
80.65	0.000			

[a] Abbreviations: X_1=MOI, X_2=initial cell number, X_3=baculovirus incubation time, X_4=baculovirus incubation temperature, X_5=baculovirus total volume; X_6=postinfection time, X_7=postinduction time, SE=standard error for estimated coefficient, T=t value, P=p value, S=estimate of standard deviation, R^2 =coefficient of determination, R^2 (adj)=adjusted R^2 , F=f value.

can be less concentrated as residuals deviate from zero. The residual plot showed the spread increases or decreases in conjunction with the fitted values; this might indicate that our experimental data had nonconstant variance (Fig. 2d).

Based on these statistical analyses and simulations using the proposed regression model, we determined optimal infection conditions for recombinant baculovirus on nonpermissive

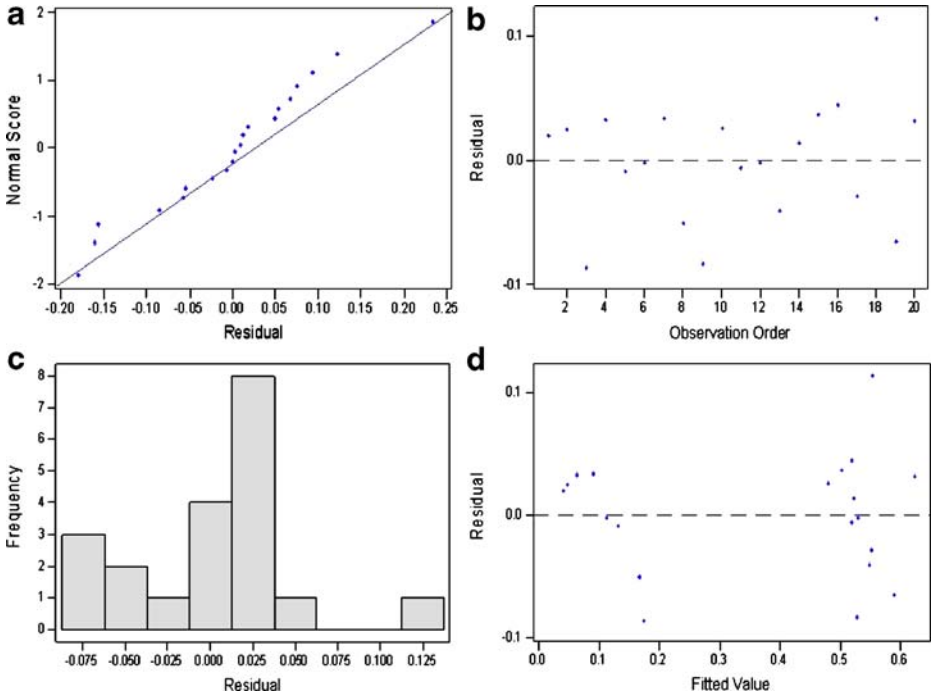

Fig. 2 Analyses of residuals for GFP fluorescence intensity. (**a**) Normal probability plot of residuals, (**b**) plot between residuals and observation orders, (**c**) histogram of residuals, and (**d**) plot between residuals and fitted values

Table 3 Statistically determined optimal infection condition for *v*MT-GFP baculovirus on S2 cells.

Variables	Optimal value
MOI	200
Initial cell number (10^6 cells)	18
Baculovirus incubation time (h)	20
Baculovirus incubation temperature (°C)	27
Baculovirus total volume (%)	2.4
Postinfection time (h)	24
Postinduction time (h)	42

S2 cells (Table 3). In the present work, optimal initial cell number was determined as 18×10^6 cells, that is our upper experimental range. Initial cell number should be more than a certain level because it significantly influences on medium consumption and cell growth kinetics [35]. Generally, higher initial cell number leads to higher expression level of foreign protein, but preparation of too high initial cell number might be practically difficult. MOI and initial cell number are correlated to each other, and higher MOI showed higher expression levels of recombinant proteins [26, 32, 35]. Therefore, optimal MOI value was also determined as the highest experimental value (200) in this work. Because a high virus incubation temperature (about 37°C) resulted in significant decay of virus titer [36], our determined optimal baculovirus incubation temperature (27°C) is reasonable. Also, 20 h baculovirus incubation time was enough to internalize baculovirus [36–38]. In the case of postinfection time, 24 h resulted in the highest protein expression levels [26, 39]. After 24 h, cell lysis was started and thus cell number and expression levels were both decreased. When a metallothionein promoter was used for expression of foreign protein in S2 cell/baculovirus system, about 40~48 h was reported as optimal postinduction time [25, 26, 40]. Using the determined optimal conditions, we performed *v*MT-GFP baculovirus infection on wild-type S2 cells and compared GFP production levels with stably transfected S2 cells using recombinant plasmid pMT/BiP/V5-His/GFP. Western blot analysis showed that the determined infection conditions conferred high and comparable (almost similar) expression level of target GFP (Fig. 3). The fluorescence spectrophotometric analysis also showed a consistent result (both arbitrary fluorescence intensity values were about 10) with Western blot. Therefore, the secreted GFP yield under optimal infection conditions was >15-fold higher than that under nonoptimal conditions. Note that this S2 cell/baculovirus is a kind of transient expression system because it was impossible to maintain production for a long time because of gradual decreases of cell viability and genetic stability during culture.

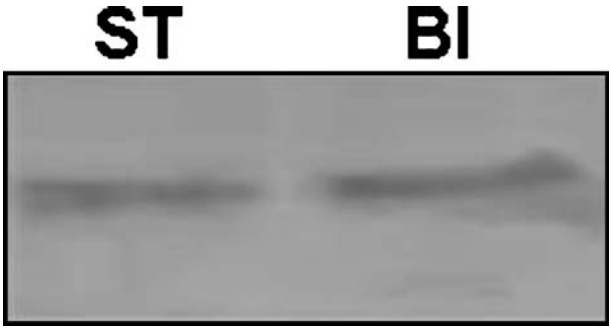

Fig. 3 Western blot analysis of GFP production in S2 cells. Lane ST, stably transfected S2 cells using pMT/BiP/V5-His/GFP; lane BI, vMT-GFP baculovirus-infected S2 cells using statistically determined infection condition

In summary, we performed establishment of optimal baculovirus infection conditions for nonpermissive S2 cells with statistical approach to obtain maximum production of target protein. Using the determined optimal *v*MT-GFP baculovirus infection conditions, the secreted GFP yield was comparable to that from stably transfected recombinant S2 cells.

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