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Establishment, Verification and Application of Rapid Detection of Baculovirus Infectious Titer by Flow Cytometry

Wu Qingsheng and Li Yuanyuan*

Department of Research Laboratory, National Vaccine and Serum Institute, Beijing, China

Abstract

Titer detection of baculovirus usually is time-consuming.It is important to establish a rapid detection method for baculovirus titer. In this report, Staining of cells with a fluorescently labeled anti-gp64 antibody allows for identification of infected insect cells. By inoculating cultures with a series of log dilutions of virus and staining of the cultures 13-22 hours post inoculation, the ratio of infected to un-infected insect cells can be determined by flow cytometry. Statistical analysis of the percentage of infected cells in the virus dilution series enables accurate infectious titer determination.

The culture time, cell growth state, the concentration of GP64-APC antibody and the concentration of inactivated Fasting blood sugar (FBS) in diluent were optimized. The generality, repeatability and intermediate precision of the method were verified. The Futures Commission Merchant (FCM) method has the advantages of simplicity, accuracy, low cost and good repeatability.

Keywords: Cytometry; Baculovirus; Cells; Titer

***Corresponding author:** Li Yuanyuan, Department of Research Laboratory, National Vaccine and Serum Institute, Beijing, China

lily13102@163.com

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Introduction

Baculovirus expression vector system Baculovirus Expression Vector System (BEVS) can express foreign genes at a high level. The biological characteristics of the expression product are similar to those of natural products. It has good application prospects in vaccine and drug development. Seven products including cancer vaccines and influenza vaccines have been approved, including vaccines and therapeutic products [1]. It is worth mentioning that on November 16, 2020, the new coronavirus recombinant protein vaccine developed by West China Hospital of Sichuan University using insect cells has entered the phase II clinical stage [2].

The determination of baculovirus titer is very important for the control and optimization of recombinant protein expression process parameters. Baculovirus determination methods mainly include plaque method, endpoint dilution method Terminology median tissue culture infectious dose (TCID50), live cell size determination method, fluorescent quantitative Polymerase Chain Reaction (PCR) method, flow cytometry detection method, immunostaining method, and β- Galactosidase activity assay. Lactosidase activity [3]. The plaque method is a classic method titer determination, but the detection cycle is long (6-10 days), and the operator's technical and experience requirements are high the end-point dilution method also requires at least 3 days. Currently, immunostaining and Quantitative Polymerase Chain Reaction (Q-PCR) are widely used. The Quantitative Polymerase Chain Reaction (Q-PCR) method has the advantage of a short detection cycle, but it requires a nucleic acid extraction step, which is to detect the number of gene copies, rather than the infectious virus titer value, which is not accurate enough. The immunostaining method also has a series of shortcomings: there are many operating steps and a long detection cycle; manual counting is required, which is more labor-intensive; insect cells are semi-adherent cells, which are not firmly attached during detection, and they may fall off in the multi-well plate. In the process of counting spots, the subjectivity is strong and requires certain experience or standards to distinguish.

The Flow CytoMetry (FCM) method is a technique commonly used to identify and isolate specific cells from mixed samples [4]. It can be used to detect viruses and cells infected by viruses [5]. Gp64 protein locates in the cytoplasm in the early stage of virus infection, then migrates to the plasma membrane, and can be expressed on the surface of infected insect cells

within 6 hours of infection [6,7]. Refer to the relevant literature, combined with the actual application, stain the cells with fluorescently labeled anti-gp64-APC antibody, and establish an Flow CytoMetry (FCM) method for rapid detectionof baculovirus titer Several factors that may influence the test results were optimized and validated, such as incubation time,concentration of gp64 antibody,cell growth status,etc.[8].

Materials and Methods

Viruses and cells

Recombinant H5N1-HA,H5N1-NA,H5N1-M1,H5N1-M2, H5N1-NP Baculovirus, Recombinant H1N1-HA Baculovirus, Recombinant HEV Baculovirus, Recombinant 2019-nCOV-N Baculovirus, 2019-nCOV-S Baculovirus, 2019 -nCOV-S1、RBD、RBD dimer baculovirus and empty baculovirus were prepared by the No.7 Research Laboratory, National Vaccine and Serum Institute; Sf9 insect cells were purchased from ATCC; ExpiSf9 insect cells were purchased from Thermo Fisher Scientific.

Reagents and instruments

SFX-Insect medium was purchased from GE; Baculovirus Envelope gp64 Monoclonal Antibody (AcV1), APC, and eBioscience™ were purchased from ThermoFisher Scientific; TC Plate 24 Well, Suspension, F were purchased from Sarstedt; fetal bovine serum was purchased from Thermo Scientific; PBS and resuspension (including 2% FBS) buffer, self-prepared. FACSCalibur™ flow cytometer and Falcon flow cytometry tube were purchased from BD; 3-18K refrigerated centrifuges was purchased from Sigma; QT-2 vortex mixer was purchased from Shanghai Qite Analytical Instrument Co., Ltd.; ZWY -211C constant temperature culture oscillator was purchased from Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd.; Countstat® BioTech automatic cell counter was purchased from Shanghai Ruiyu Biotechnology Co., Ltd.;

Method establishment

Add 1 ml logarithmically diluted HEV virus solution and 800 µl to a 24-well suspension culture plate with a density of 1.25×10^6 cells logarithmic phase insect cells, set up multiple wells and negative control wells. 27°C 225 rpm, suspension culture for 14- 16 hours, aspirate the liquid from each well to the flow cytometrytube and centrifuge to pellet the cells (300 × g, 5 minutes), discard the supernatant, and add 100 µl at a concentration of 0.15 µg/ ml Stain the cells with the gp64-APC antibody, vortex and mix for3 to 5 seconds, let stand at room temperature for 30 minutes, centrifuge and discard the supernatant, slowly add 1 ml PBS along the tube wall to wash, add 1 ml resuspension solution to resuspend the cells **(Figure 1)**. Flow cytometry detection; select the detection value of positive cells less than 10% to calculate the virus titer.

$$\text{Virus titer(ivp/ml)} = \frac{\text{Total number of cells} \times \text{Percentage of positive cells}}{\text{The dilution times of virus}} \times 0.01$$

Calculation formula (Infectious virus particle):

Virus titer(ivp/ml)=(Total number of cells×Percentage of positive cells)/(The dilution times of virus)×0.01

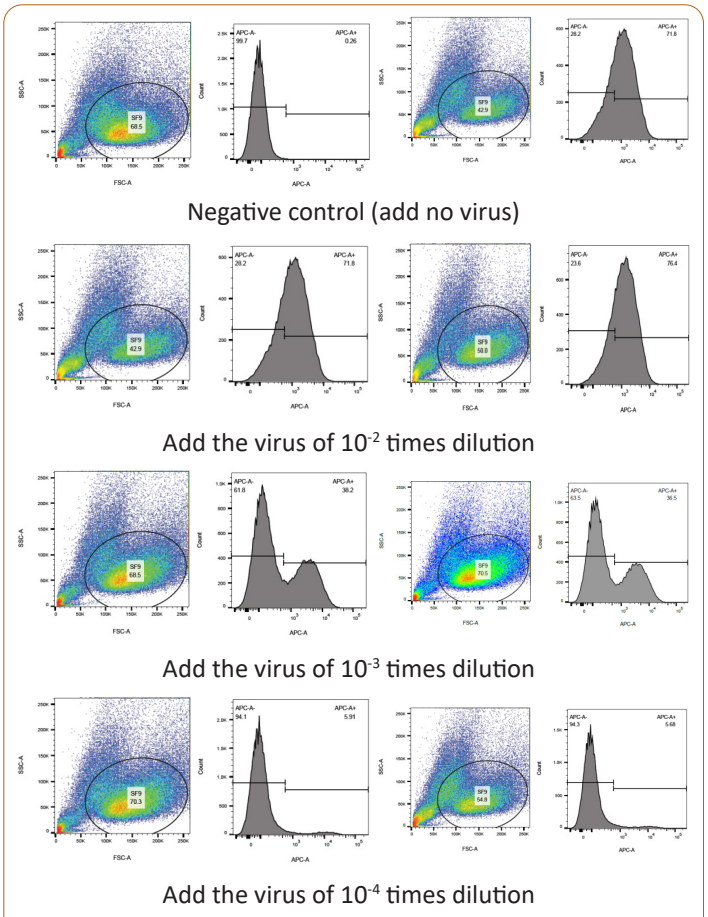

Figure 1: FCM scatter plots of Sf9 cells infected with different dilutions of virus.

Method optimization

Incubation time: After 6-48 hours of baculovirus inoculation and culture, test cell growth and titer change trends to determine the best culture time.

Concentration of gp64 antibody: Add 100 µl of gp64-APC antibody with concentrations of 0.05, 0.1, 0.15, 0.2, 0.4 µg/ml for detection. Observe the trend of titer changes and select the best antibody concentration.

Concentration of inactivated FBS in dilute diluent: Use HEV virus with 0%, 0.2%, 0.5%, 0.8%, 1%, 2%, 5%, 8%, 10% dilutions to resuspend stained cells washed with PBS Evaluate the effect of FBS concentration in resuspension buffer on virus titer detection values.

Cell growth status: Use cells of different growth periods (early, middle, late of logarithmic growth phase;plateau growth phase,decay growth phase) and different cell generations for titer detection.

1.4.4.2 Use another recombinant baculovirus: 2019-nCOV-RBD dimer-working virus library to verify the influence of cell growth status on the detection value.

Method verification

Versatility: Use the Futures Commission Merchant (FCM) method to detect baculoviruses carrying different foreign genes

(H5N1-HA, H5N1-NA, H5N1-M1, H5N1-M2, H5N1-NP, H1N1-HA, 2019-nCOV-N, 2019-nCOV-S) Perform testing to verify the versatility of the method.

Repeatability: For the same virus sample, repeat the test 6 times and calculate the Relative standard deviation (RSD) value.

Intermediate precision: The same sample was repeatedly tested by three different operators to calculate the RSD value.

Method application: The FCM method was used to the titer of 2019-nCOV-S1, 2019-nCOV-RBD, and 2019-nCOV-RBD dimer in the tertiary virus seed library. The results were compared with the detect value of the immune-fluorescence method.

Results

Method establishment

Use the Sf9 cells of P44 generation, 1 day after the passage, to detect the HEV virus, the detection values were 5.91×10^8 ivp/ml, 5.68×10^8 ivp/ml , the average value was 5.79×10^8 ivp/ml.

Method optimization

Incubation time: The virus is added to cells and culture. The Incubation time is less than 13 hours, the detection value is seriously low; between 13～22 hours, the detection value is not much different; between 26～32 hours, the detection value is reduced; between 36～40 hours, the detection value has a slight upward trend; At 42 hours, the detection signal decreased significantly and almost disappeared; between 44～48 hours, the detection value increased significantlyagain **(Figure 2)**.

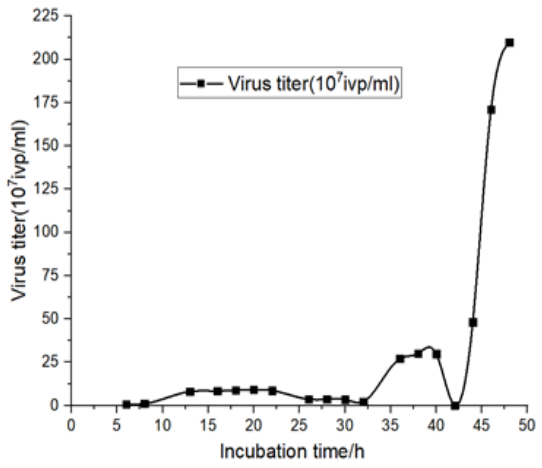

Figure 2: Changes of virus titer detection values at different incubation times.

As the culture time increases, the cells that are not infected by the virus will continue to amplification and the cell density will increase**(Figure 3)**.

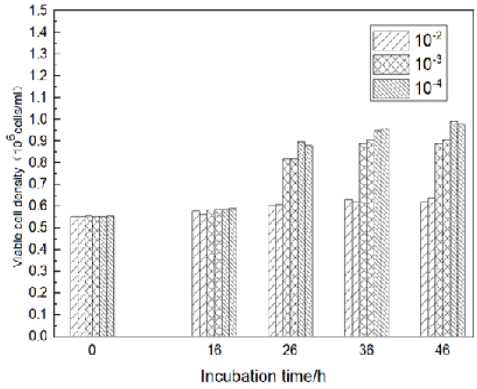

Figure 3: Changes of the total number of living cells infected with different culture time and different dilution ratio of virus.

GP64-APC antibody concentration: The concentration of 100 µl gp64-APC antibody for cell staining between 0.15～1.2 µg/ml has little effect on the detection value, and has no effect on the detection value of about 10% positive cell wells, so 0.15 µg/ml is selected as the detection antibody concentration **(Figure 4)** . If it is less than 0.15 µg/ml, it will seriously affect the detection value; if it is less than 0.0375 µg/ml, the proportion of positive cells in each dilution cannot be distinguished from the negative control.

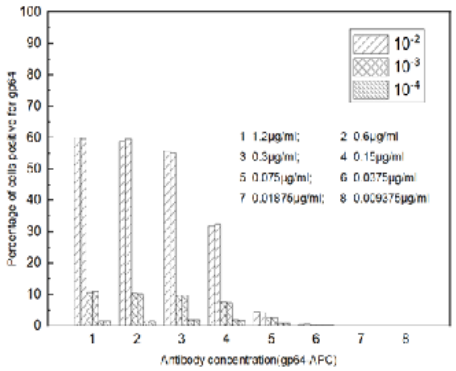

Figure 4: The effect of GP64-APC antibody concentration on virus titer detection value.

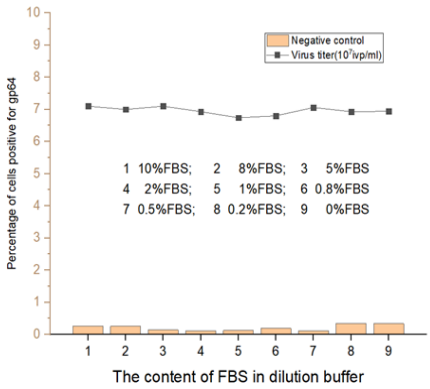

Figure 5: The effect of inactivated FBS content in diluents on detection value.

The content of inactivated FBS in the diluent: The content of inactivated Fetal Bovine Serum (FBS) in the dilution buffer has no significant effect on the detection value of HEV virus titer. The average detection value of 9 groups is 6.95, and the proportion of gp64 positive cells in the 2% Fetal Bovine Serum (FBS) group is6.93, which is close to the mean value; the negative control value((Detection background)) between each group is not obviously different,such as the 2% concentration group is 0.11, which is relatively low, the 2% concentration group is 0.11, which is relatively low. Therefore, the content of FBS in the dilution buffer was optimized to 2% **(Figure 5)**.

Cell growth status: According to the detection value of Sf9 cell titer of different cell cycle stages it can be seen that the detection value of the cells in the early stage of logarithmic growth is higher and the stability is better **(Table 1)**. Use the cells in the adaptive and plateau phase, the detection value is lower; The cells in the recession cannot be used for titer detection due to the extremely poor cell state or low viability; The cell generation had no significant effect on the detection value of virus titer. High-passage cells (119 generations) are not suitable for titer detection; but, after 3 passages (122 generations), they can be used for titer detection again **(Table 2)**.

Method verification

Versatility: The Flow CytoMetry (FCM) method can detect baculoviruses carrying different foreign genes, and the method has a good versatility.

Repeatability: The same virus sample was tested repeatedly for 6 times, and the calculated RSD=1.58%, with good repeatability.

Intermediate precision: Three testers repeated the test for the same virus sample three times, with RSD-average=1.41%, with good intermediate precision.

Method application: Flow cytometry was used to detect the titers of the seed bank, RBD and RBD dimer of the 2019-NCoV-S1 virus. There was no significant difference between the results of flow cytometry and immune-fluorescence.

Discussion and Conclusion

The commercial kit (Expression Systems) of Flow CytoMetry (FCM) method uses Gp64-PE antibody for cell staining, and the kit is restricted sales in some areas. In this article, Gp64- APC antibody is used for detection. The concentration of this antibody was 0.015 μg/test, which was lower than that of GP64-PE antibody.

The titer value is calculated according to the virus dilution well, which the positive cell proportion is about 10%, so there is no need to draw a standard curve, so it is more efficient.

Unlike the plaque method, the Flow CytoMetry (FCM) method uses suspension culture, closely reflect actual culturing conditions utilized for expression cultures, and can obtain titer results within 24 hours. By using a centrifuge and a flow cytometer equipped to handle 96-well plates, the Flow CytoMetry (FCM) method can easily achieve high- throughput detection. Since the Flow CytoMetry

(FCM) method detects the percentage of positive cells, it is not necessary to analyze all cells to get the correct detection value, during cell culture, centrifugation, cell staining, washing, etc. even if the cell count is inaccurate, or the total cell volume is lost due to cell shedding or other reasons, it will not affect the test results.

Using vortex shaking can better resuspend the cells. Vigorous vortex shaking will affect the binding betwwen cells and antibodies and reduce the detection value. But in the actual test, it was found that the short-term vortex oscillation would not have a significant impact on the test value. The reason may be that the affinity is high enough between Gp64 antibody and Gp64. FCM has the ability of accurate recognition of single cell signal and the specific binding of Gp64 antibody to Gp64, which are the reasons for the good repeatability of FCM method. Inactivated FBS in diluted buffer can reduce nonspecific adsorption of antibodies to cell surface proteins. However, in the actual experiment, the non-specific adsorption of Gp64 antibody was not serious, so the content of inactivated FBS in the dilution buffer had no significant effect on the detection results.

The cell growth status had a significant effect on the titer value. The operational process of cell passage and the different stages of the cell growth cycle have great influence on the cell growth state. In the process of passage, many factors will have adverse effects on the growth state of cells.The cell state decreased after several passages,and not suitable for titer detection. After more standardized multiple passages, the cell state could be restored to be suitable for virus titer detection **(Table 2)**.

The cell growth cycle can be divided into four stages adaptive phase, logarithmic growth phase (early, middle, and late), plateau phase, and recession phase. The density after passage has an effect on the time of the cells to enter the logarithm phase. Generally speaking for Sf9 cells, when the density is 1.0 ～1.2 × 10^6 cells/ml after passage, the cells can enter the logarithmic growth phase within 24 hours. When the passage density is 0.8 ～ 1.0 × 10^6 cells/ml, the adaptive phase is longer, and it will enter the logarithmic growth phase after more than 24 hours. The cell state in the early phase of the logarithm is the best, which is suitable for titer detection. The detection values were lower when using middle logarithmic and late logarithmic cells.

The cells of recession phase are not suitable for titer detection **(Table 2)**.

The assay is based on detection of the baculovirus gp64 fusion protein which is expressed on the surface of infected insect cells after infection . Ensure that the incubation time is between 13-22 hours, which is very important to obtain accurate titer detection values. If the incubation time is less than 13 hours, the GP64 protein cannot be adequately expressed and distributed on the surface of infected cells, resulting in low detection value If the incubation time is more than 22 hours, secondary infection will occur (the progeny virus produced first, which then infects healthy cells) **(Figure 2)**.

In addition,the multiplication time of insect cells is 16 ~ 24 hours.If the incubation time is much longer than the doubling time, then the uninfected cells will continue to grow and divide, which will cause the living cell number in the culture pore increased significantly, thus affecting the detection value.

Table 1: Comparison of titer values of Sf9 cells in different cell cycle stages.

Generation	Culture days	Viable cell density(10^6cells/ml)	Viability (%)	Diameter (μm)	Phase	Titer-1 (10^7 ivp/ml)	Titer-2 (10^7 ivp/ml)	Titer-average (10^7 ivp/ml)
40	2	2.97	97.51	16.98	Early-logarithmic	9	8.8	8.9
40	5	11.2	97.35	17.67	plateau	4.9	4.7	4.8
42	3	3.6	96.4	16.7	Early-logarithmic	8.3	8.1	8.2
44	1	1.4	95.35	16.53	Early-logarithmic	8.8	9	8.9
44	3	5.52	98.96	16.03	Middle-logarithmic	7.1	7.1	7.1
47	1	1.6	98.39	16.46	Adaptive	7.7	7.5	7.6
47	2	3.5	98.71	16.28	Early-logarithmic	8.1	8.3	8.2
48	1	1.3	98.83	16.5	Adaptive	7.5	7.1	7.3
54	7	3	29.8	15.55	Recession	Not-suitable	Not-suitable	Not-suitable
55	5	7.41	69.84	16.34	Recession	Not-suitable	Not-suitable	Not-suitable
60	2	3.7	98.72	16.06	Early-logarithmic	8.4	8.2	8.3
119	4	8.92	90.58	17.57	Later-logarithmic	Not-suitable	Not-suitable	Not-suitable
122	1	2.78	92.5	17.02	Early-logarithmic	8	8.7	8.4

Table 2: Comparison of titer detection values of high passage cells (199 passages) and the cells which after three passage (122 passages).

Sample	SF9/APC-A+ | Freq. of parent	
	Sf9-P119-4	Sf9-P122-1
Negative control	8.88%	1.55%
10^{-2} times dilution	25.40%	40.40%
10^{-3} times dilution	11.60%	8.01%
10^{-3} times dilution	12.40%	8.77%
10^{-4} times dilution	9.92%	2.23%
10^{-4} times dilution	9.15%	2.85%

Table 3: Another virus was used to verify the influence of cell status on titer detection values.

Sample	SF9/APC-A+ | Freq. of Parent	
	Sf9-P41-2 days	Sf9-P54-2 days
	Viable cell density:3.08×10^6 cells/ml	Viable cell density:5.71×10^6 cells/ml
Negative control	0.85%	0.80%
10^{-2} times dilution	71.40%	69.50%
10^{-3} times dilution	34.20%	21.20%
10^{-3} times dilution	34.40%	18.40%
10^{-4} times dilution	5.49%	2.76%
10^{-4} times dilution	5.91%	2.80%

Table 4: Universality verification.

Baculovirus	Titer-1 (10^7 ivp/ml)	Titer-2 (10^7 ivp/ml)	Titer-average (10^7 ivp/ml)
H5N1-HA	49.4	53.6	51.5
H5N1-NA	4.5	4.38	4.44
H5N1-M1	5.55	5.07	5.31
H5N1-M2	4.13	4.18	4.16
H5N1-NP	2.86	2.88	2.87
H1N1-HA	16.8	17.6	17.2
2019-nCOV-N	96.4	108	102.2
2019-nCOV-S	58.1	58.7	58.4
Empty baculovirus	193	198	195.5
5.91%	5.91%	5.91%	5.91%

Table 5: Repeatability verification.

Times of repetition	Titer-1 (10^7 ivp/ml)	Titer-2 (10^7 ivp/ml)	Titer-average (10^7 ivp/ml)
1	8.29	8.54	8.42
2	8.48	8.13	8.31
3	8.49	8.16	8.33
4	8.26	8.52	8.39
5	8.67	8.52	8.6
6	8.63	8.59	8.61
Average			8.44
RSD(%)			1.58

Table 6: The intermediate precision.

Times of repetition	The operator A-titer (10^7 ivp/ml)	The operator B-titer (10^7 ivp/ml)	The operator C-titer (10^7 ivp/ml)
1	8.29	8.22	8.32
2	8.16	8.37	8.35
3	8.4	8.08	8.12
Average	8.28	8.22	8.26
RSD (%)	1.45	1.76	1.51
RSD-Average (%)	1.41%		
5.91%	5.91%	5.91%	5.91%

Table 7: FCM method and fluorescence immunoassay were used to detect the titer of 2019-nCOV virus.

Virus bank	Titer-1 (10^7 ivp/ml)	Titer-2 (10^7 ivp/ml)	Titer average (10^7 ivp/ml)	fluorescence immunoassay (10^7 ivp/ml)
S1-primary	6.99	7.18	7.09	7.51
S1-secondary	28.7	26.1	27.4	38.6
S1-working	45.4	46.5	45.95	32.85
RBD-primary	16.6	13.3	14.95	18.42
RBD-secondary	26.8	27.2	27	24.57
RBD-working	36.6	33.3	34.95	28.62
RBD dimer-primary	47.5	48.2	47.85	42.4
RBD dimer-secondary	35.1	35.6	35.35	38.93
RBD dimer-working	54.9	59.1	57	58.76

References

1 Wu Qingsheng, Li Yuanyuan (2020) Research progress on key technologies and applications of insect baculovirus expression vector system. Inter J Virolo 27:348-352.

2 Yang J, WangW, Chen Z, Wei X, etal. (2020) A vaccine targeting the RBD of the S protein of SARS-CoV-2 induces protectiveimmunity.Nature 586:572-577.

3 Zhang Yuexia, Zhang Liying, Zhao Quan (2019) Current status and prospects of medical applications of flow cytometry. J Chinese Contemporary Medi 26:28-30.

4 Sivaraman Divya, Yeh Hsiao-Yun, Mulchandani Ashok, Marylynn V Yates, Wilfred Chen (2013) Use of flow cytometry for rapid, quantitative detection of poliovirus-infected cells via TAT peptide-delivered molecular beacons. Appli Enviro Microbiolo 79:696-700.

5 Long Yuyan, Guo Wang, Lan Huangli, Qu Dacai, Liang Xiang, et al. (2019) The research progress of silkworm nuclear polyhedrosis virus envelope glycoprotein GP64. J Guangxi Sericulture 56:25-32.

6 Lin, Chung, Hu (2014) Update on baculovirus as an expression and/or delivery vehicle for vaccine antigens. 13:1501-1521.

7 Mulvania T, Hayes B, Hedin D (2004) A Flow Cytometric Assay for Rapid, Accurate Determination of Baculovirus Titers. BioProcess J 3:47-53.