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Identification of recombinant baculovirus and determination of virus titer with fluorescence quantitative PCR assay

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[**ABSTRACT**] **AIM**: To develop a real - time PCR assay based on TaqMan technology for the identification of recombinant baculovirus and determination of virus physical titers in Bac - to - Bac system. **METHODS**: The recombinant baculovirus containing human IL - 18 gene was produced using Bac - to - Bac system. A 10 - fold serially diluted primary viral stock was used for plaque assay and DNA extraction. Bacmid (baculovirus plasmid) was 10 - fold serially diluted and served as standards. Real - time PCR amplification of the IL - 18 gene was performed in triplicate for each diluted recombinant virus. At the same time, plaque assays were performed using overlay agarose method. **RESULTS**: The standard linear range (10^1 to 10^8 copies) for quantitation was achieved with the standard curve. We also find that the "vg/mL" titer value is generally about 10 times than "pfu/mL" titer of the same recombinant virus stock. **CONCLUSION**: A TaqMan real - time PCR method is established to identify the recombinant baculovirus and determine the "vg/mL" titer of virus. The method is rapid and quantitative over a wide range of virus titers.

[**KEY WORDS**] Fluorescence quantitative PCR; Identification of recombinant baculovirus; Virus titer

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Recombinant baculoviruses have been extensively used as vectors for abundant expression of a large variety of foreign proteins in insect cell cultures. The appeal of the system lies essentially in easy and quick cloning techniques in *E. coli* strain and virus propagation combined with the eukaryotic post - translational modification machinery of the insect cell[1]. Baculovirus vectors offer many advantages over other gene delivery systems and have a potential to employ *in vivo* and *ex vivo* gene therapy for the treatment of a diverse array of human diseases[2]. In the past few years, there have been substantial efforts to employ baculovirus vector systems in gene therapy and many study groups had a good progress.

No matter how to employ baculovirus in genetic engineering or gene therapy, it is necessary to identify proper clone of foreign gene in recombinant baculovirus and determine virus titers. Generally, plaque purification or observation of GFP gene expression is still needed for titer determination. These procedures are time consuming, laborious and inaccurate. In addition, for use in human clinical trials, preparations must meet a Food and Drug Administration (FDA) requirement of a particle to infectious unit ratio of less than 100, so a rapid and accurate titer determination will become an essential step in the thorough assessment of viral gene therapy vectors prior to their use in clinical trials[6]. To simplify titer determination, a method for quantitative estimate physical titers of recombinant baculovirus was developed with real - time PCR, which is also a novel way to identify proper clone of a foreign gene[3].

METHODS

1 Generation of recombinant baculovirus plasmid (bacmid)

The recombinant bacmid was produced using the Bac - to - Bac[TM] vectors system (Invitrogen) according to the manufacturer's manual. Briefly, pFastBace - 18 plasmid containing human IL - 18 gene[4] was transformed into component *E. coli* DH10Bac, which

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contained baculovirus genomic DNA (bacmid) as well as a helper plasmid. In transformed cells the helper plasmid facilitated a recombination event in which the IL-18 gene was inserted into the bacmid DNA. Recombinant bacmid were purified and confirmed by DNA sequence. The purified recombinant bacmid were used for transfection and served as standard.

2 Production of recombinant baculovirus

Approximately 2.0×10^6 sf9 cells (maintained in Grace 1s medium) (Gibco), supplemented with 10% fetal bovine serum from Gibco, were seeded in 25 cm^2 flasks for 24 h before transfection, at that time they reached 50% -70% confluency. The transfection mixture containing recombinant bacmid DNA and Cell FECTION (Invitrogen) were prepared according to the manufacturer's instructions and then added to the cells. After 5 h at 37 ℃, the Grace media containing the trasfection mix was removed, and 6 mL growth media was added. 3 days after transfection all culture supernatant was collected by microcentrifugation and used for plaque assay and DNA extraction.

3 Plaque development assay[5]

Plate sf9 cells at a density of 5×10^5 per well of 6-well tissue culture plates and incubate overnight at 37 ℃. Dilute viral stocks in 1 mL volumes over a 10-fold series from 10^1 to 10^{10} in growth medium. Add 1 mL of each dilution to a separate well of the 6-well plate and incubate at 37 ℃ for 2 hours. Prepare a solution of 5% agarose and add 30 mL of growth medium previously equilibrated to 37 ℃. Completely remove the growth medium from the wells and spread on the overlaid agarose. After incubated at 37 ℃, plaques which have the appearance of small white spots could be visible to the naked eye within 21 days. To determine titer, count plaques from wells where isolated plaques are clearly visible and countable. Average the counts from duplicate wells and multiply that number by the dilution factor to estimate pfu/mL.

4 Viral DNA extraction

After digestion with *DNaseI* at 37 ℃ for 1 h, 200 μL volumes of each dilution of virus stock were used to prepare baculovirus genome DNA using the QIAamp Blood Mini kit. In brief, the virus stock were lysed with proteinase K at first and then were purified on QIAamp spin columns. At last, the pure DNA was eluted as a PCR template in 50 μL TE buffer.

5 Quantitative PCR

PCR primers were designed to amplify a conserved region of human IL-18 gene[4]. The forward primer (primer F) was 5'-TGGCTACACTGGTATTCG-3', and the reverse primer (primer R) was 5'-ATAAAGATAGCCAGCCTA-3'. PCR amplification yields a 253 bp product. The probe sequence is 5'-(FAM) ATTCGTTGCCCAAGCAGTAGTT-(TAMRA) 3'. FAM is the reporter dye used and TAMRA is the quencher. The bacmid were diluted from 10^1 to 10^8 copies per 5 μL and served as standards to determine the sensitivities of the assays. 2 μL DNA template of standards or recombinant baculovirus were added to a PCR amplification system from TaqMan Gold PCR Kit (PE applied biosystems). The 20 μL total reaction volume consisted of 150 nm each of forward and reverse primer, 200 nm probe, 200 mmol/L each dNTP, 4.0 mmol/L $MgCl_2$, 1.5 units/test AmpliTaq Gold, 1 × TaqMan Buffer. A PCR amplification was performed in a thermocycler (light cycler, Roche) using a program of 94 ℃ at 5 min followed 40 cycles of 92 ℃ at 5 s, 60 ℃ at 30 s. The intensities of the fluorescent dyes in each reaction were read automatically during PCR cycling. Each sample was run in triplicate. The software determines the standard curve, which is then used to calculate the precise quantities of starting template molecules for the unknown sample.

RESULTS

1 Real-time PCR assay

Initially, the sensitivity of the TaqMan PCR was evaluated to detect purified recombinant bacmid DNA. A linear quantitation was achieved with each of the 1-log dilutions of sample, the standard deviation for each curve was less than 1% for each dilution. The assay was able to detect as few as 10 copies of bacmid. Fig 1 demonstrates that the linear range of the assay was from 10 to 10^8 copies; the assay variability was less than 3%. A linear relationship was noted over the range tested with a correlation coefficient of 0.985. Next, a series of experiments was to evaluate the triplicate: PCR reactions were performed for each diluted recombinant virus and compared with a standard curve derived from serial dilutions of the bacmid. The determination of

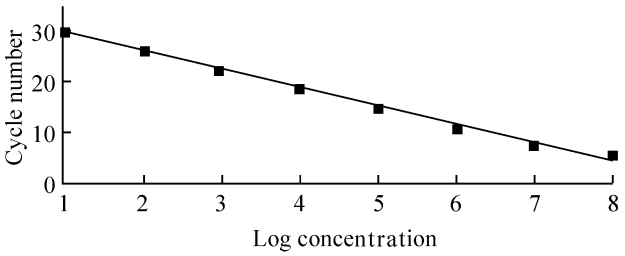

Fig 1 Standard curve generated from PCR assay, each triplicated assay is presented as one dot.

copy number was done by comparison of the number of PCR cycles at which fluorescence was detected to the number of PCR cycles necessary for known amounts of DNA to cross a fluorescent threshold on a standard curve. The viral genome copies in per milliliter of viral stock determined by this method was called "vg/mL". The values were given a mean ± standard deviation, all samples were evaluated in triplicate.

2 Comparison of biological titer

At last, a biological titer was determined in triplicate using the plaque assay. As shown in Tab 1, there was a close correlation between the biologically determined titers and those with TaqMan PCR method: $\gamma = 0.985$, the "vg/mL" titer value was generally about 10 times than "pfu/mL" titer of the same recombinant virus stock. The FQ – PCR method greatly shortens the time required to determine titer. The presented data demonstrated that the utility of our primers and probe at viral concentrations suitable for experimental and clinical work.

Tab 1 Comparison of the "vg/mL" titer with the "pfu/mL" titer of the same virus stock($\bar{x} \pm s$. $n = 30$)

Method	Titer value				
Pfu/mL	$(3.1 \pm 1.0) \times 10^3$	$(3.8 \pm 0.8) \times 10^4$	$(3.5 \pm 1.0) \times 10^5$	$(3.2 \pm 0.7) \times 10^6$	$(4.1 \pm 0.9) \times 10^7$
Vg/mL	$(1.9 \pm 0.9) \times 10^{4\,\triangle}$	$(3.4 \pm 0.9) \times 10^{5\,\triangle}$	$(3.1 \pm 0.8) \times 10^{6\,\triangle}$	$(2.7 \pm 0.8) \times 10^{7\,\triangle}$	$(3.2 \pm 0.5) \times 10^{8\,\triangle}$

$^{\triangle}P < 0.05$ *vs* "pfu/mL" titer of the same virus stock.

DISCUSSION

The identification of recombination baculovirus and determination of virus titer is necessary for gene dilivery, a simplified and accurate method is urgent. Traditionally, virus titers are measured in many different units including VP (virus particle), GTU (gene transduction unit), PFU (plaque forming unit), CFU (clone forming unit) and $TCID_{50}$[6]. Among them, VP is a physical titer and others are biological titer. In gene delivery test, PFU, CFU and GTU are most widely used. However, these methods are time consuming and inaccurate. The results are variant in different labs and deeply depended on skills of operators. In addition, PFU, CFU and GTU reflect the efficiency and ability of virus infection. Generally PFU, CFU and GTU values are less than the actual virion numbers because only parts of the recombinant virus have the ability of infection. When it is need to evaluate the titer of stocking virus and to monitor the changes of viral vector after *in vivo* administration, the physical titer is more suitable. So, more and more researchers selected VP units. The VP was detected by measuring the A_{260} of the virions and one A is generally equal to 1.1×10^{12} VP. However, this method has some disadvantages. Firstly, the VP can be calculated only after thorough purification (nearly 100%). The procedure is also very complex. Secondly, when recombinant virus is produced, some of the viral genome can not be packaged into the virus particle and shaped the hollow virion at last. The VP methods can not identify them. As a result, "vg/mL" has been gradually accepted by researchers[7]. The vg/mL describes the viral genome copies contained in per millilitre of viral stock. Because baculovirus is a kind of single copy DNA genome virus, we believe that the vg/mL is an ideal substitute unit to describe the titer of baculovirus.

In this study, the TaqMan real – time PCR method has been established to determine the "vg/mL" titer of the recombinant baculovirus, meanwhile, a proper clone of foreign gene in recombinant virus was identified. The method was rapid and quantitative over a wide range of vector titers (between 10 to 10^8 vector copies per reaction). We also find that the "vg/mL" titer value is generally about 10 times than "pfu/mL" titer of the same recombinant virus stock. This phenomenon may be due to the "plating efficiency" of virus infection. Theoretically there is no wild or chimerical baculovirus produced from Bac – to – Bac system, so the "vg/mL" unit determined by quantitative PCR is more accurate than "pfu/mL" unit to describe the actual numbers of recombinant baculovirus particles.

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荧光定量 PCR 用于重组杆状病毒鉴定及病毒滴度检测的研究

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[摘 要] **目的**：建立一种高效、简便的荧光实时定量 PCR 方法，用于重组杆状病毒鉴定及病毒滴度的检测。**方法**：利用 Bac - to - Bac 载体系统在昆虫细胞中构建含人 IL - 18 基因的重组杆状病毒，收获的病毒母液以 10 倍梯度系列稀释后，提取病毒基因组 DNA。以 10 倍梯度稀释的重组杆状病毒穿梭质粒(bacmid)作为标准模板，进行荧光定量 PCR 反应扩增 IL - 18 基因片段并绘制标准曲线，然后以上述的重组杆状病毒基因组 DNA 作为模板，采用同样体系进行实时 PCR 反应检测，同时用琼脂糖空斑法测定病毒母液的滴度。**结果**：成功构建了重组杆状病毒并建立了病毒滴度的实时荧光 PCR 检测方法。运用标准模板进行的 PCR 反应显示该方法的线形范围为 $10^1 - 10^8$ 拷贝，病毒母液的 DNA 拷贝浓度(vg/mL)值约为空斑检测的滴度 pfu/mL 值的 10 倍。**结论**：荧光定量 PCR 方法可灵敏快速地鉴定重组杆状病毒，并在较大的线形范围内检测重组杆状病毒滴度，较之空斑法更准确地反映了重组杆状病毒的实际数量。

[关键词] 荧光定量 PCR；重组病毒鉴定；病毒滴度

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