

## A Quick and Efficient Method to Quantify Baculo Virus by Quantitative Real Time PCR

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Recombinant protein expression using the Baculovirus Expression System is widely used for the production of therapeutics, diagnostics, and reagents. The current "gold standard" for Baculovirus titration, the plaque assay, is labor intensive and time consuming. The real-time PCR assay has the advantage of speed and accuracy. We determined Baculovirus titre by a novel quantitative PCR (qPCR) method, using Baculovirus gene gp64 (Coat protein) specific primers and a recombinant gene-specific primers. Virus specific amplification and gene specific amplification were compared, and both values were found to be very close, suggesting that most of the virus were expressing our gene of interest. This method is useful to determine the percentage of non-recombinant virus particles in virus solution. Further, we compared real-time PCR results with plaque assay, and found that the values were much similar. This method has the advantage of time saving as it completes in 2-3 hours, and not only quantifies the virus, but also quantify the virus, containing the gene of interest.

**Key words:** QPCR; *Baculovirus*, AcMNPV, MOI, gp64.

The Baculovirus expression vector system (BEVS), is widely used for the over expression of recombinant protein in insect cells, providing protein post-translational modifications, similar to those obtained when using higher eukaryotic cells. Furthermore, insect cells can be grown to a very high density in serum-free medium as a suspension culture, enabling the production of large quantities of recombinant protein<sup>1</sup>.

To maximize efficiency of recombinant protein production, determining the best multiplicity

of infection (MOI) is very crucial. Conventionally, Baculoviruses have been titrated by plaque assay<sup>2-4</sup> end-point dilution<sup>5</sup>, and antibody-based assays<sup>6,7</sup>. But, all of these methods are labor intensive, time consuming, and need a degree of expertise in virus handling and cell culture, sometimes producing results that can be hard to interpret<sup>8</sup>. Therefore, a quick, easy, and accurate quantification of Baculoviruses is required for the establishment of multiplicities of infection, which is critical for establishing a productive infection cycle.

Real Time quantitative PCR has been used widely for the quantification of DNA<sup>9</sup> and RNA<sup>10</sup>. It has been used for the quantification of several viruses<sup>11</sup> and has been very well demonstrated to be a sensitive and accurate method, for virus detection and quantification. Its main advantages are its rapidity and the accuracy of quantification<sup>12</sup>.

In this study, we describe an improved QPCR titration method with SYBR green chemistry,

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using two different Primers set for quantification. The quantification of accuracy obtained using QPCR, was verified by plaque assay.

## MATERIAL AND METHODS

### Reagents

CFX96 real time PCR machine, qPCR 96 well white plates from Bio-Rad. QiAMP DNA and blood mini kit from Qiagen MESA GREEN qPCR Master Mix Plus for SYBR Assay No ROX from Eurogentec. Cellfectine, Grace insect media, FBS, 4% Agarose, 2x Grace insect media from Invitrogen (Gibco), Cell culture flask and plates from Corning. Primers were synthesized at Eurofins genomics, India.

### Cell Culture and Recombinant Baculovirus Production

Sf9 cells ( $1 \times 10^6$ ) were removed from an exponentially growing culture, and seeded in 6 well cell culture plate. Enterokinase gene containing recombinant bacmids (Viral DNA) previously generated in our lab, sequence and insert presence, was confirmed by sequencing. Using Cellfectine (Invitrogen), Cells were transfected with recombinant bacmid DNA ( $1 \mu\text{g}$ ), and incubated at  $27^\circ\text{C}$ . Five days post-transfection, the culture medium containing recombinant virus was harvested, and 0.5 mL (P1 Virus) was used to inoculate T-25 flasks containing  $2.5 \times 10^6$  Sf9 cells, and were incubated at  $27^\circ\text{C}$  in non-humidified incubator. Three days post-infection, the culture medium containing recombinant virus was harvested, and 0.5 mL (P2 Virus) was used to inoculate T-75 flasks containing  $8 \times 10^6$  Sf9 cells, and were incubated at  $27^\circ\text{C}$  in non-humidified incubator. Recombinant virus stocks were harvested three days post-infection (P3 Virus), clarified by centrifugation at low speed, and titrated by plaque assay (in duplicates) in Sf9 Cells.

### Plaque Assay

Plaque assays were carried out as described by King and Possee [3]. Briefly, six well cell culture plates were seeded with Sf9 cells at a density of  $0.8 \times 10^6$  cells/well, in a final volume of 2 mL media and allowed to settle for 30 min. P3 Viral stocks was serially log diluted (1:10) to  $10^{-8}$  in Grace insect media supplemented with 10% fetal bovine serum (FBS). Media was removed from six well plates and diluted viral stock added in wells,

starting from  $10^{-4}$  to  $10^{-8}$ . In control well only media was added and incubated at room temperature. After 1 h, the virus inoculum was removed and 2 mL low gelling temperature agarose (4%) was overlaid onto the cells. After the agarose overlay had solidified, cells were incubated at  $27^\circ\text{C}$  for 8 days. The cells were then stained with Neutral Red (Sigma-Aldrich) to visualize plaques.

The following calculation yielded the virus titer in plaque-forming units (pfu) per milliliter:

$\text{Pfu/mL} = \text{average plaque count} \times \text{dilution factor} \times 10$

### Viral DNA Extraction

Under sterile conditions, 200  $\mu\text{L}$  virus solutions was removed from the viral stock to be titrated (P3 Virus stock) and used for DNA extraction. Viral DNA was extracted and purified using a Genomic DNA isolation Kit (Qiagen-India) as described by the manufacturer's Protocol. Briefly, 200  $\mu\text{L}$  of virus was lysed by addition of 20  $\mu\text{L}$  Proteinase K and 200 lysis buffer, immediately mixed by pulse vortexing for 15 sec, and incubated at  $56^\circ\text{C}$  for 10 min. 200  $\mu\text{L}$  ethanol (96-100%) was then added to the sample. The sample was then applied on the QiAamp Mini spin column and centrifuged at 8000 rpm for 1 min, followed by two washes with wash buffers and centrifugation (8,000 rpm for 1 min). A final spin at 13,000 rpm was carried out to remove residual buffers, and the DNA was then eluted from the column in a final volume of 200  $\mu\text{L}$  elution buffer.

### Primers

Primers (Table.1) for amplification of a 72-bp region of the *Autographa californica* nucleopolyhedrovirus (AcNPV) gp64, and 78-bp region of the Bovine Enterokinase gene, were designed by using Oligonucleotide Properties Calculator online software.

### Quantitative PCR Conditions

QPCR reactions were prepared in 96-well white plate in a final volume of 20  $\mu\text{L}$ , BioRad CFX96 qPCR machine, and 2x MESA GREEN qPCR Master Mix used for set up reaction. Using known concentration of viral DNA, standard curve was prepared by diluting a 10 fold dilution. qPCR reaction as follows: Initial denaturation  $95^\circ\text{C}$  for 3 Min,  $95^\circ\text{C}$  5sec,  $60^\circ\text{C}$  for 30sec followed by signal read (35 cycles), Melt curve  $55-95^\circ\text{C}$  for 0.05 sec with increment of 0.5 degree with every cycle.

### Data Analysis

After qPCR run was finished, Data analysis was done with the CFX software manager version 2.0 installed on the system computer. Unknown samples titer was calculated by extrapolating unknown samples values with standard curve.

## RESULTS AND DISCUSSION

Baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) envelope glycoprotein gp64 gene was selected as the target for amplification because it is specific to AcMNPV, and is responsible for coding a viral envelope fusion protein that is necessary for receptor binding and membrane fusion, in the event of viral infection. Along with gp64 gene,

we choose one more target, Enterokinase gene, for amplification, which gene was incorporated in bacmids. Then we compared both results and correlation between both gene amplification. Comparing both results of gp64 and EK gene amplification, we found that the results were very close - qPCR determined Virus titer with EK was  $0.94 \times 10^8$  pfu/ml, and for GP64 was  $1.1 \times 10^9$ . In another experiment, EK titer was  $0.994 \times 10^8$  and gp64 was  $1.22 \times 10^9$  these results indicate that there is no or very less non recombinant virus (i.e. virus without our gene of interest), and results were consistent from one assay to another. Further PCR results were analyzed, and we found results were matching with plaque assay (Table 2). So, this is a novel and useful method to determine functional viral titer in sample.

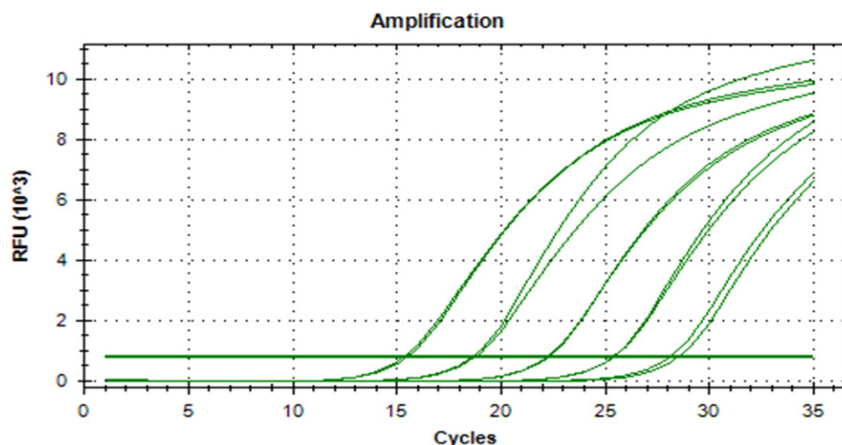
**Table 1.** Sequence of the primers

Primer name	Orientation	Sequence(5'-3')
GP64 sense	Forward	CGGCGTGAGTATGATTCTCAAA
GP64 anti sense	Reverse	ATGAGCAGACACGCAGCTTT
EK sense	Forward	GATGGCTCTGGCTGGCGTG
EK anti sense	Reverse	GGACCCGGGCATACACCCCT

**Table 2.** QPCR and Plaque assay titer comparison

Target	Cq mean	QPCR titer SQ mean (pfu/mL)	Plaque titer (pfu/mL)
Enterokinase	16.53	$9.44E+0.8$	$1.3 \times 10^9$
Gp64	16.25	$1.14E+0.9$	

Note: Cq-Quantification cycle; SQ-Starting quantity;Pfu-Plaque forming unit



**Fig.1.** Standards Amplification of gp64 target

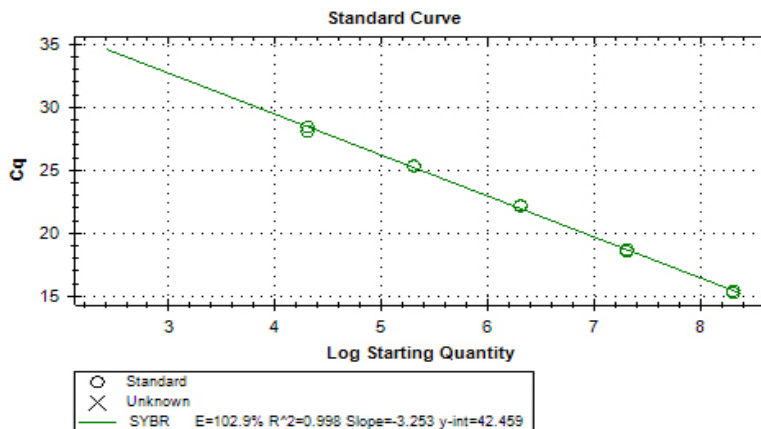


Fig. 2. Standard Curve

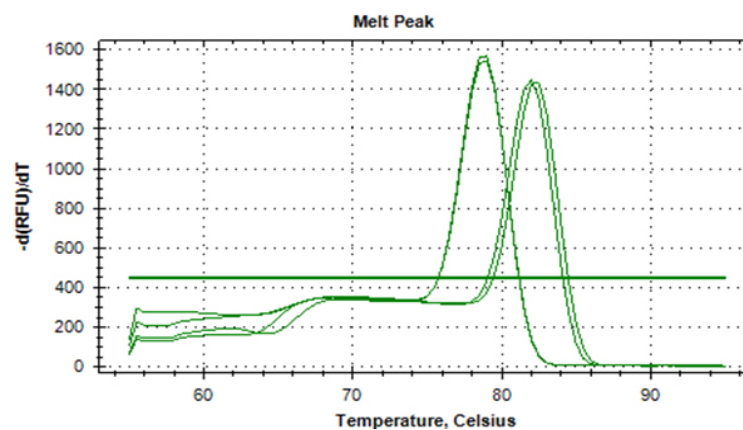


Fig. 3. Melt curve peaks of gp64 and EK

Standard curve was generated by using Baculovirus sample, which virus titer was previously determined by Plaque assay, and gp64 gene was the target for amplification in standards. Standards amplification duplicates were very close (Fig. 1) and standard curve, all dilutions were correlating with amplification, its regression square was 0.99 (Fig. 2). This emphasizes that, much care needs to be taken while setting up experiments in q PCR, to get reliable results.

In this experiment, we used SYBR green chemistry for detection of amplified product and Melt curve generated two separate peaks, each specific to gp64 and EK (Fig. 3). Looking at this data, we found that results were much reliable and cost-effective, compared to probe-based assay, to determine viral titer.

## CONCLUSION

In this study, it was established that quantitative PCR can be applied for precise and quick quantification of recombinant Baculovirus. It is apparent especially when this method is employed for quantification of huge number of recombinant Baculovirus samples.

Moreover, the time required is reduced from 8-12 days for plaque assay, to 2-3 hours for q PCR, to determine titer of virus stocks. Henceforth, quantitative PCR can be employed in future applications for convenience, accurate and rapid virus titer determination.

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