baculoQuant™

one-step titration kit

1st Edition, January 2007

Catalog No. GWB-100600
GWB-100601

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Fee	shall mean the fee invoiced for the Materials by the Licensor to the Licensee;
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Licensor	shall mean Oxford Brookes University, a higher education corporation Gipsy Lane, Oxford OX3 0BP;
Material	shall mean the Licensor's product known as the BaculoQuant kit comprising either or both an agreed quantity of DNA, primers and probe and the relevant User Guide;
Purpose	shall mean the use by the Licensee of the Materials for the titration of recombinant viruses for Research purposes only. Use for diagnostic purposes is specifically prohibited;
Research	shall mean the Licensor's systematic search or investigation towards increasing the sum of its knowledge in the titration of recombinant viruses;
User Guide	shall mean the instructions provided with BaculoQuant to enable the Licensee to titre recombinant viruses from the BaculoQuant kit.

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Kit contents

All reagents and materials provided and referred to in this User Guide are for research purposes only:

1 DNA standard. Store at -20°C.
2 Dual labelled probe. Store at -20°C in the dark.
3 Forward and Reverse primers. Store at -20°C.
4 baculoQuant User Guide

NOTE: Viral DNA extraction kit and Quantitative PCR Master Mix are ***NOT*** supplied with this kit.

NOTE: You will also be supplied with a username and password for use with the online titration facility. This will be on the front of the User Guide.

Essential Information

The information given in this User Guide is accurate to the best of our knowledge. It is a practical guide to allow researchers to determine baculovirus titres via quantitative PCR (QPCR) using primers and probe to the viral gene, gp64. It is not intended as a comprehensive guide to QPCR or other virus titration methods.

Ordering Information

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Technical Assistance and Further Information

For additional help or guidance, please contact us at techline@genwaybio.com. All technical assistance is provided without charge and is given in good faith; we cannot take any responsibility whatsoever for any results you obtain by relying on our assistance. We make no warranties of any kind with respect to technical assistance or information we provide.

Safety Requirements

The research products have not been approved for human or animal diagnostic or therapeutic use.

Procedures described within this User Guide should only be carried out by qualified person trained in appropriate laboratory safety procedures.

Always use good laboratory practice when handling this product.

WARNING: SAFETY PRECAUTIONS MAY BE NECESSARY WHEN HANDLING SOME OF THE PRODUCTS DESCRIBED IN THIS USER GUIDE. PLEASE REFER TO THE MATERIAL AND SAFETY DATA SHEET SUPPLIED BY THE APPROPRIATE MANUFACTURER.

Quantitative PCR for Titration of Recombinant Baculovirus Particles

Introduction

The baculovirus expression system is widely used for the production of foreign proteins in insect cells and recombinant baculoviruses can be amplified to very high titres in suspension cell culture. However, virus amplification can be variable and is dependent on a number of factors such as the health of cells, multiplicity of infection and culture conditions, for example temperature and oxygenation of cultures amongst others. A prime cause of failure to detect recombinant protein production is reliance on un-titrated recombinant virus stocks that on subsequent analysis are found to have unexpectedly low titres. It is therefore essential that an accurate titre of virus be obtained to ensure optimal recombinant protein production.

Traditionally, baculoviruses have been titrated by plaque assay[1,2,3], end-point dilution[4] and antibody-based assays[5,6], with plaque assay regarded as the most reliable titration method. These methods require 4-6 days to yield a virus titre and involve procedures which require a degree of expertise in cell culture and virus handling. Alternative titration methods have included flow cytometry[7,8], colorimetric indicators[9], and measurement of cell-diameter changes of infected cells using a cell counter[10]. Despite these recent innovations, titration of baculovirus is still considerably time consuming and can frequently delay the protein production process.

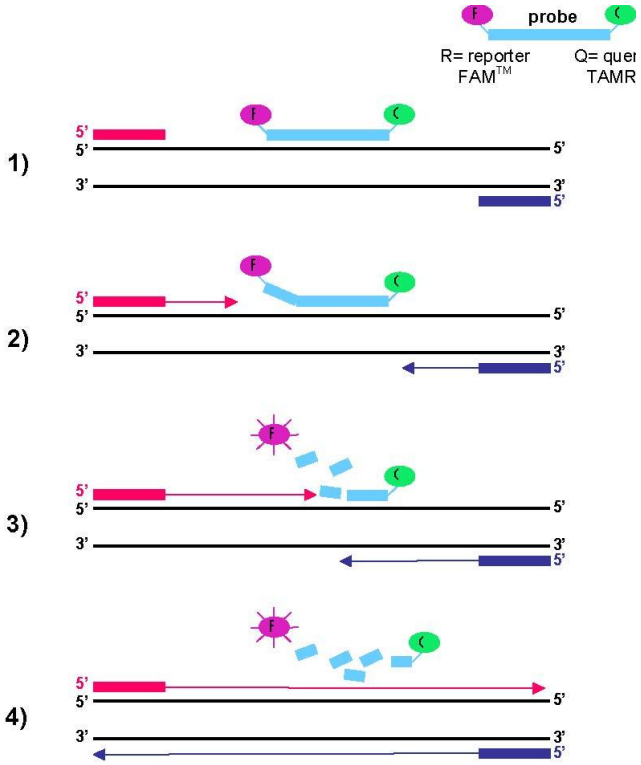
We have developed a rapid and accurate method for titration of baculoviruses based on QPCR. By utilising QPCR, it is possible to establish viral titres from budded virus

stocks in a number of hours, compared to 4-6 days to obtain a titre via plaque assay. This ability to rapidly and accurately quantify large numbers of viruses ultimately results in a fast and reliable titration system that will eliminate the need for plaque assays.

The principle of QCPR

Quantitative real-time PCR was first developed by Higuchi et al.[13] who determined PCR kinetics by developing a system capable of detecting PCR products as they accumulate during a PCR cycling reaction. This system was further advanced by the 5' nuclease assay[14] which utilises the 5' exonuclease activity of Taq polymerase to digest a dual-labelled probe hybridized between flanking PCR primer. The target-specific probe consists of an oligonucleotide tagged with a reporter fluorescent dye (FAM) at the 5' end and a quencher dye (TAMRA) at the 3' end. Whilst the probe is intact, the proximity of the two dyes results in fluorescent resonance energy transfer (FRET), with the quencher greatly reducing the fluorescence of the reporter dye.

During the QPCR reaction, the target-specific probe anneals downstream from one of the primer sites (Figure 1). During PCR amplification, the 5'-3' exonuclease activity of Taq polymerase cleaves the dual-labelled probe as the primer extends, resulting in a release of the reporter dye and interruption of the FRET, and therefore increasing the reporter dye fluorescence signal. This cleavage removes the probe from the target DNA strand permitting primer extension. Subsequent rounds of PCR amplification result in additional cleavage of reporter dye molecules from their respective dual-labelled probes, culminating in a successive increase in fluorescence intensity proportional to the amount of PCR product produced. This increase in fluorescence can be quantified in real-time and permits quantification during the exponential phase of the reaction as opposed to end-point accumulation of a product by conventional PCR.



Dual labelled probe

Q= quencher TAMRA™

igure 1. 5' nuclease assay using dual labelled probes. (1) Forward andverse primers anneal to target DNA sequences and are extended as perstandard PCR. The probe, dual-labelled with FAM™ and TAMRA™ fluorescentdyes, anneals to the gene sequence between flanking

PCR primers. (2) As theTaq polymerase extends the primer, the probe is displaced. (3) The 5'-3'nuclease activity of the polymerase cleaves the reporter dye (FAM™) from theprobe, interrupting the FRET between the reporter and quencher dyes and thusgenerating a fluorescent signal (4). Quantitative analysis is represented by means of an amplification plot, illustrating the fluorescence signal versus the Cycle threshold (C_t) of the reaction. During the initial cycles of the QPCR reaction, there is minimal change in the levels of fluorescence intensity, thus defining the baseline levels of fluorescence for the assay. The cycle number at which point the fluorescence intensity rises above the background-noise baseline is referred to as the C_t value and is determined at the most exponential phase of the PCR reaction. This value is inversely proportional to the amount of target DNA molecules and therefore permits quantification of the template DNA using a standard curve. Therefore, the higher the concentration of target DNA, the more rapidly a significant increase in fluorescent intensity will be detected resulting in a lower C_t value.

Utilising QPCR to analyse viral titre

Virus titration by QPCR is carried out as described by Hitchman et al.[14,15] using probes and primers specific to gp64, a gene that encodes an essential virus envelope protein. GP64 is involved in membrane fusion during viral entry and incorporated into budded virus particles during virus egress[16]. Following extraction of virus DNA from freshly amplified low passage budded virus samples, it is possible to determine the number of virus particles via QPCR amplification using the gp64 probe and primers. The greater the number of virus particles within a sample, the higher the concentration of virus DNA that will be present and correspondingly, the lower their C_t value. By comparing this Ct value to a known standard curve, it is possible to establish an accurate titre which can be converted to equivalent plaque forming units per ml (Qpfu/ml), using an equation derived from linear regression analysis.

Advantages of *baculo*Quant™ titration system:

- Rapid and accurate titration method
- Reduces the amount of cell handling required
- Amenable to high throughput and automated systems
- Back-compatible with all recombinant baculoviruses containing complete native gp64 sequence

Limitations of *baculo*Quant™ titration system:

- BaculoQuant™ titration system must be used on DNA extracted from fresh budded virus stocks (less than 3 months old). Aggregation and degradation of virus particles over a period of time may occur resulting in misleading Qpfu/ml values.

Protocol: Rapid baculovirus titration using *baculo*Quant™

Supplied in *baculo*Quant™ kit:

- Forward primer 2.5 µM
- Reverse primer 2.5 µM
- Dual labelled probe 2.5 µM
- DNA standard (1 µg at 25 ng/µl)
- baculoQuant™ User Guide

Required:

- Recombinant budded virus stock to be analysed

  NB: Prior to viral DNA extraction, ensure cell culture media has been clarified by centrifugation to remove all traces of cellular DNA and debris. This is normally a routine procedure when producing budded virus stocks.
- High Pure Viral Nucleic Acid Kit (Roche Molecular Biochemicals)

  NB: The protocol described below was optimised using the above kit and we strongly recommend using this method for viral DNA extraction.
- Quantitative PCR MasterMix e.g. ABsolute Blue QPCR ROX Mix (ABgene)

- Real-Time PCR system, such as Applied Biosystems 7500 Real-Time PCR Sequence Detection System
- Optical 96-well plates and adhesive plate seals (ABI)
- 96-well plate rotor for low-speed centrifuge
- Sterile water
- Ice box

Optional

- Robot, such as Baculoworkstation™ (NextGen Sciences Ltd) or similar
- Liquid level sensing tips

NOTE

This kit provides sufficient reagents to titre up to 26 viruses, either in a single QPCR run filling a 96 well plate or in up to 5 runs, with a maximum overall analysis of 26 recombinant viruses. Sufficient standard DNA is provided for up to 5 independent standard curves.

The protocol described below has been developed using Applied Biosystems 7500 SDS. We recommend following the manufacturers instructions when using alternative QPCR systems.

Procedure

1. Following the manufacturers instructions, isolate viral DNA from 200µl of recombinant budded virus stocks using the viral nucleic acid isolation kit (Roche Molecular Biochemicals). Elute the virus DNA in elution buffer to a final volume of 50 µl. Store at 4°C until required.
2. Prepare a log series of 10-fold dilutions of the supplied DNA standard in final volumes of 10 µl (25, 2.5, 0.25, 0.025, 0.0025 ng/µl).

Note 1: Supplied DNA is at a concentration of 25 ng/µl.

Note 2: 2 µl of each of the diluted standards will then be used in each DNA standard reaction, see section 5.

3. Prepare QPCR reactions on ice, as a master mix (See Table 1), according to how many viruses you need to titrate. Multiply the amounts shown in Table 1 (1 QPCR reaction column) by the number of reactions required. Each virus sample, along with the control sample and each of the five standard DNA samples (step 2), should be analysed in triplicate. Control reactions contain water in the place of DNA.

NB: The probe is light sensitive and therefore should be stored in the dark as much as possible.

Table 1. Components for QPCR Mastermix reaction

Reagents	***1 QPCR reaction***	***Example*** 1 set of DNA standards + control samples + 5 virus DNA samples + 2 extra reactions*
PCR SuperMix-UDG	12.5 µl	437.5 µl
Water	7.5 µl	262.5 µl
Forward primer (2.5 µM)	1 µl	35 µl
Reverse primer (2.5 µM)	1 µl	35 µl
Probe (2.5 µM)	1 µl	35 µl
Total Volume	23 µl	805 µl

NB: It is advised that the user prepares enough Mastermix to allow for a few extra QPCR reactions, to ensure sufficient final volume as shown by the example in Table 1*.

4. Aliquot 23 µl of Mastermix into the appropriate number of wells in a 96-well plate. Each reaction will be carried out in triplicate i.e. 3× 5 wells for standards, 3

wells for water control and 3 wells per unknown virus.

5. To each set of triplicate wells, add 2 µl of the purified viral DNA from step 1, or 2 µl of the DNA standards (step 2) or control water, to give a final reaction volume of 25 µl in each well.

6. Seal the 96-well plate using an adhesive plate seal and plastic spreader.

7. Centrifuge the 96-well plate briefly at low speed to bring the reactions to the bottom of the wells. Ensure there are no bubbles on the surface of the reactions.

8. Place the 96-well plate within the Sequence Detection System (SDS) and enter the required information into the software, e.g. the position of each reaction, the fluorescent dyes used (6FAM and TAMRA) and the standard DNA dilutions.

9. Perform the DNA amplification following the manufacturer's instructions (as for Quantitative Analysis if using the SDS 7500) using the cycling conditions detailed in Table 2.

tage 1: ***ING*** ***ctivation***	***tage 2:*** **Taq *activation & UNG* *de-activation***	***tage 3:*** ***nnealing/Extension***
ycles: 1	ycles: 1	ycles: 40
0°C	5°C	5°C/60°C
:00	.5:00	:15/1:00

Table 2. SDS 7500 cycling conditions

10. On completion of the QPCR cycle programme, the most exponential part of each of the amplification curves will have been automatically detected by the SDS software and their C_t values calculated from the default threshold (see Figure 2). Occasionally, this may need to be adjusted manually and the threshold may need to be moved higher in the exponential phase of amplification to give improved slope and correlation coefficient values. However, where

possible, baseline setting and threshold levels should remain consistent between assays to improve accuracy and reproducibility. Ideally, the standard curve should have an R^2 value >0.95 and an amplification efficiency of around -3.32 (Applied Biosystems).

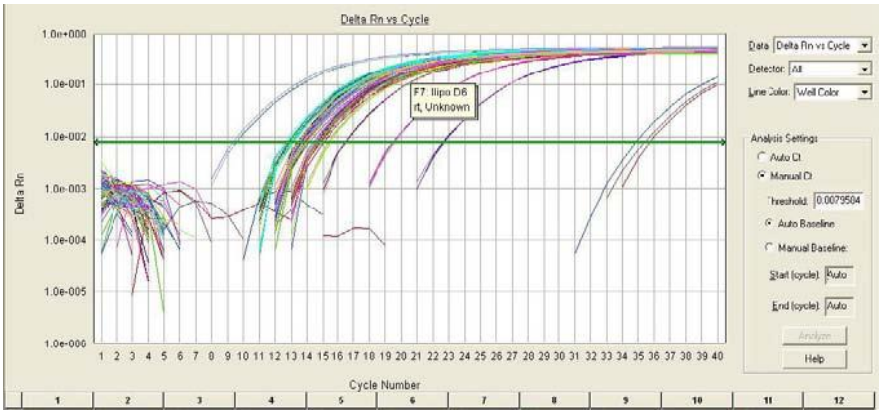


Figure 2. Typical amplification plot from ABI's SDS 7500

11. Export the ct values into a data analysis program (e.g. Microsoft Excel) and calsulate the mean ct for each virus
12. Go to www.expressiontechnologies.com and click the "BaculoQuant" tab, then "Online Service"
13. Enter your username and password
14. Enter your first mean ct value and click "calculate"
15. Your virus titre will be displayed and the number of remaining credits will be shown (maximum 26 per kit)

Threshold

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