Takara Bio USA

BacPAK™ qPCR Titration Kit User Manual

Cat. No. 631414
(050819)

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Table of Contents

Table of Figures

Table of Tables

I. Introduction

A. Summary

The **BacPAK qPCR Titration Kit** provides a fast and simple method for titrating baculoviral stocks generated from AcMNPV-based baculoviral vectors, including all of Takara Bio's BacPAK vectors. The kit employs a quick DNA purification step and allows determination of viral DNA genome content in just 4 hours, using qPCR and TB Green® detection technologies. Because qPCR titration is so fast, target cells can be infected with accurately titrated virus on the same day the virus is harvested. Delays and freeze-thaw cycles that reduce virus infectivity can be avoided. This method makes it possible to infect cells at a known multiplicity of infection (MOI), allowing you to produce results that are precise, consistent, and interpretable.

B. Protocol Overview

Viral supernatant is collected and then, using a viral DNA purification kit, genomic DNA is extracted from a small aliquot of the supernatant. Serial dilutions of the viral DNA sample are subjected to qPCR to determine threshold cycle (Ct) values for each dilution. ROX™ Reference Dye may be used to normalize fluorescent signal intensity between reactions when using qPCR instruments that are equipped with the option to do so. The DNA genome copy number in a sample dilution is determined by finding the copy number that corresponds to its Ct value on a standard curve generated from serial dilutions of the calibrated BacPAK DNA Control Template.

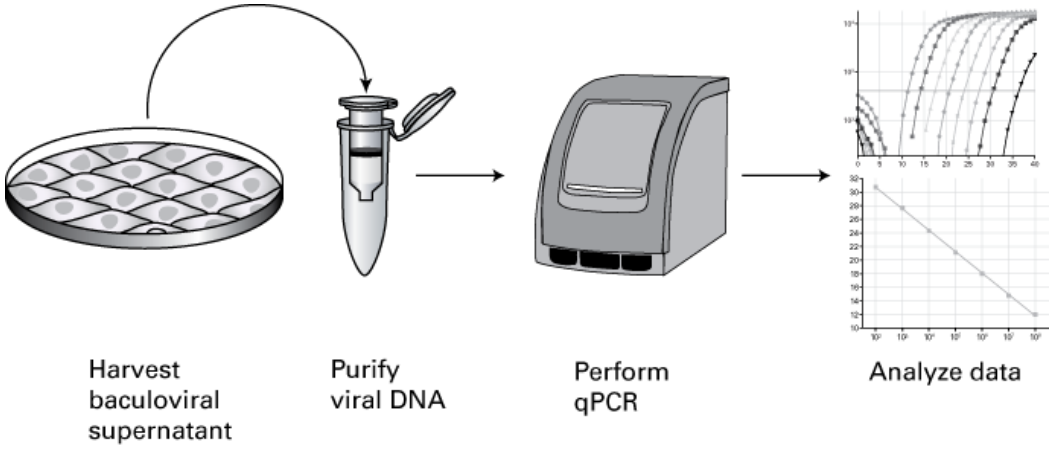

Figure 1. Flowchart of the procedure used for titrating baculovirus supernatants with the BacPAK qPCR Titration Kit.

C. Correlating DNA Titer with Infectivity

The first time you perform the qPCR titration to determine DNA copy number, you may wish to measure viral infectivity (focus forming units (FFU), plaque forming units (PFU), etc.) by independent means (e.g., via rapid titer assay or plaque assay) in order to establish an infectivity coefficient (copy number/FFU; see Table IV, Section IV.E). Determination of the infectivity coefficient for a given prep allows you to normalize the amount of prep used in each experiment, for consistent interassay results. Representative infectivity coefficients (determined with different infectivity titration methods) for a typical BacPAK virus are shown in Table IV. These values should be consistent for similarly prepared viral stocks. However, the calculated ratio may vary due to differences in the amount of virus obtained from individual baculoviral amplifications. Variations in the amount of virus amplified can be caused by differences in cell number, inoculum amount, or time elapsed before the cytopathic effect is observed; therefore, a standardized amplification procedure should be used to help ensure consistent results.

II. List of Components

Store the BacPAK qPCR Titration Kit Components at –20°C.

Store the TB Green Advantage® qPCR Premix and ROX Reference Dyes (Cat. No. 639676) at –70°C in the dark. After thawing, store at 4°C in the dark. Do not refreeze.

Store the NucleoSpin Virus kit (Cat. No. 740983.10) at room temperature.

BacPAK qPCR Titration Kit (200 rxns; Cat. No. 631414)

- **BacPAK qPCR Titration Kit Components** (not sold separately)
  - 40 µl BacPAK DNA Control Template (1.4 x 10^8 copies/µl)
  - 100 µl BacPAK Forward Titer Primer (10 µM)
  - 100 µl BacPAK Reverse Titer Primer (10 µM)
  - 4 tubes EASY Dilution Buffer (1 ml per tube)
- **TB Green Advantage qPCR Premix** (200 rxns; Cat. No. 639676)
  - 4 tubes 2X TB Green Advantage qPCR Premix (0.625 ml per tube)
  - 100 µl 50X ROX Reference Dye LSR
  - 100 µl 50X ROX Reference Dye LMP
- **NucleoSpin Virus** (10 preps; Cat. No. 740983.10)
  - 13 ml Lysis Buffer VL
  - 6 ml Wash Buffer VW1
  - 6 ml Wash Buffer VW2 (Concentrate)
  - 13 ml RNase-free H_2O
  - 300 µg Carrier RNA (lyophilized)
  - 120 µl Liquid Proteinase K
  - 20 ml Collection tubes (1.5 ml) for lysis and elution
  - 10 NucleoSpin Virus Columns (light read rings, plus Collection Tubes)
  - 30 tubes Collection Tubes (2 ml)

III. Additional Materials Required

- Ethanol and PCR-Grade H_2O
- Quantitative real-time PCR thermocycler (any of the following, or equivalent):
  - ABI PRISM 7000/ 7700/ 7900HT, Applied Biosystems 7300/ 7500 Real-Time PCR System, Applied Biosystems 7500 Fast Real-Time PCR System
  - Mx3000P (Stratagene)
  - LightCycler (Roche)
  - Thermal Cycler Dice Real-Time System (TP800, Takara Bio, Japan)
- 96-well PCR plates and 8-well PCR strips that are compatible with your PCR thermocycler
- Repeating pipettor with 23 µl capacity (Section IV.D)
- Multichannel pipettor(s) with 2–25 µl capacity

IV. Baculoviral Titration Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING. Successful titration results depend on performing the following steps in sequence.

A. General Recommendations

Due to the tremendous amplification power and sensitivity of qPCR, even trace amounts of contaminating DNA will be amplified and will affect Ct and final copy number values. Before you begin, prepare work areas free of potentially contaminating DNA and DNases. If possible, dilute your samples and controls in one work area with a dedicated set of pipettors, and assemble your qPCR reactions in a separate area or noncirculating containment hood, using a different set of dedicated pipettors. Wear gloves at all times and use PCR pipettor tips with hydrophobic filters, and dedicated solutions. We also recommend setting up negative template control (NTC) reactions lacking any template. Finally, perform all post-PCR analyses in a separate area, preferably in a separate room, with different pipettors.

B. Preparation of NucleoSpin Virus Kit Buffers

IMPORTANT: Lysis Buffer VL and Wash Buffer VW1 contain guanidine salts! Wear gloves and goggles!

- **RAV3 Buffer:**

  Add 24 ml of ethanol (96–100%; nondenatured ethanol is recommended) to the bottle of Wash Buffer VW2 (Concentrate). Mark the label of the bottle to indicate that the ethanol has been added. Store the Wash Buffer VW2 at room temperature (20–25°C).

C. Protocol: Purifying Baculoviral DNA

1. Centrifuge the medium from baculovirus-infected cells for 5 min at 2,000 rpm to remove cells and debris. Save the supernatant.
2. Purify DNA from 200 µl of the baculoviral supernatant. Smaller volumes (50–100 µl) can be used if necessary, but you must bring the sample volume up to 200 µl with medium or PBS. For your convenience, the NucleoSpin purification protocol is outlined in Steps 3–7, below.

   NOTE: The lysis and Proteinase K treatment step included in the NucleoSpin user manual (Section 5.2, Step 1) is unnecessary for the BacPAK qPCR Titration protocol.

3. Add 400 µl ethanol (96–100%) to 200 µl of baculoviral supernatant and mix by vortexing (10–15 sec). Incubate for 5 min at room temperature (18 -25°C). Briefly centrifuge the collection Tube (~ 1 sec at ~ 2,000*g*) to remove drops from the lid (short spin only). Do not centrifuge at a higher g-force in this step!
4. For each sample, place one NucleoSpin Virus column in a 2 ml collection tube and load 600 µl of the sample. Centrifuge for 3 min at 4,000*g*.

   NOTE: If the lysate is not completely drawn through the membrane, repeat the centrifugation at higher g-forces (15,000–20,800*g* for 1 min). In case the lysate still does not pass the membrane completely, discard the sample and repeat the isolation with new sample material.

5. Place the NucleoSpin Virus Column into a new Collection Tube (2 ml, provided) and discard the Collection Tube with flow-through from the previous step.

6. Wash and dry the silica membrane as follows:
    a. Add 400 µl Wash Buffer VW1 to the NucleoSpin Virus Column. Centrifuge for 30 sec at 11,000*g*. Place the NucleoSpin Virus Column into a new Collection Tube (2 ml, provided) and discard the Collection Tube with flow-through from the previous step.
    b. Add 400 µl Wash Buffer VW2 to the NucleoSpin Virus Column. Centrifuge for 30 sec at 11,000*g*. Place the NucleoSpin Virus Column into a new collection tube (2 ml, provided) and discard the Collection Tube with flow-through from the previous step.

    NOTE: Make sure that residual buffer from the previous step is washed away with Buffer VW2, especially if the lysate has been in contact with the inner rim of the column during loading of the lysate onto the column. For efficient washing of the inner rim, flush it with Buffer VW2.

    c. Add 200 µl Wash Buffer VW2 to the NucleoSpin Virus Column. Centrifuge for 5 min at 20,000*g* (or full speed). Place the NucleoSpin Virus Column in a clean Elution Tube (1.5 ml, provided) and discard the Collection Tube with flow-through from the previous step. Incubate the assembly for 5 min at 56 °C with open column lid.
7. To elute the DNA, add 30 µl RNase-free H2O (pre-heated to 70°C) onto the column. Incubate for 3 min at room temperature. Centrifuge 3 min at 20,000*g* to elute nucleic acid from the column.
8. Keep eluted DNA on ice or freeze for storage. Perform qPCR (Section D).

D. Protocol: qPCR Amplification of Baculoviral DNA

1. In your reaction assembly work area, make a Master Reaction Mix (MRM) on ice consisting of the following reagents (Table I). Make sufficient MRM for the required number of wells. Each control, no-template control (NTC), and sample reaction should be performed in duplicate:

Table I. Example Master Reaction Mixes for Different qPCR Instruments

	qPCR Instruments			
	Stratagene Mx3000P	**Takara Bio Thermal Cycler Dice Real Time System**	**Applied Biosystems Instruments**	**Roche LightCycler**
Reagents	**Volumes of each reagent per well (µl) for each instrument**			
PCR-Grade H_2O	9.0	9.5	6.8	7.2
BacPAK Forward Primer (10 µM)	0.5	0.5	0.4	0.4
BacPAK Reverse Primer (10 µM)	0.5	0.5	0.4	0.4
ROX Reference Dye LSR or LMP (50X)*	0.5	—	0.4	—
TB Green Advantage qPCR Premix (2X)	12.5	12.5	10.0	10.0
Total volume per well	**23.0**	**23.0**	**18.0**	**18.0**

*The kit is supplied with two different ROX formulations that allow you to normalize fluorescence signals on instruments that are equipped with this option. ROX Reference Dye LSR is for instruments whose excitation source is a 488 nm laser, while ROX Reference Dye LMP is for instruments whose excitation source is either a lamp or an LED. **Be certain to use the formulation that is appropriate for your real-time instrument!**

NOTE: To ensure sufficient volume, prepare approximately 10% more Master Reaction Mix than is necessary for all reactions (see example, below).

EXAMPLE CALCULATION:

Calculating Total Master Reaction Mix (MRM) Volume:

Total MRM Volume = 1.10 x Total wells x Total volume per well

1. **Controls:** 5 dilutions in duplicate; 1.10 x 10 wells x 23 µl = 253 µl
2. **NTCs:** 3 each in duplicate; 1.10 x 6 wells x 23 µl = 152 µl
3. **Samples:** 4 dilutions in duplicate; 1.10 x 8 wells x 23 µl = 202 µl

Table II. Control and Sample Dilutions for qPCR

Well	Strips 1 & 2 controls*	Strips 3, etc. samples
1	1.4×10^8	Sample 1 (1X)
2	1.4×10^7	0.1X
3	1.4×10^6	0.01X
4	1.4×10^5	0.001X
5	1.4×10^4	Sample 2 (1X)
6	NTC	0.1X
7	NTC	0.01X

*copies/µl

2. **In your sample dilution work area,** and using PCR grade 8-well strips, dilute the BacPAK DNA Control Template and purified sample(s) with EASY Dilution Buffer as shown in Table II.
    a. Dilute the BacPAK DNA Control Template in an 8-well strip (Table II, 'Strip 1') as follows:
        i. Pipet 20 µl of undiluted BacPAK DNA (1.4×10^8 copies/µl) into the first well.
        ii. Subsequent dilutions (Wells 2–5) can be made by serially transferring 3 µl of the preceding dilution into 27 µl of buffer in the next well.
    b. Make a duplicate series of dilutions in a second 8-well strip (Table II, 'Strip 2').
    c. Pipet only EASY Dilution Buffer into the last 3 wells of both strips for NTCs.
    d. Dilute your DNA sample(s) in another set of 8-well strips; each strip can be used to dilute either 2 duplicate samples or 2 different samples (Table II, Strip 3). **We recommend making duplicate dilutions of all samples.**
        i. The first well in each series (Wells 1 & 5) should contain 20 µl of undiluted sample (1X).
        ii. Subsequent 10-fold sample dilutions (Wells 2–4 & 6–8) can be made by serially transferring 3 µl of the preceding dilution into 27 µl of buffer in the next well.
    e. Centrifuge the strips at 2,000 rpm (4°C) for 1 min to remove any bubbles.
3. **In your qPCR reaction assembly area,** place a 96-well PCR plate on ice (or a blueblock; 4°C), and dispense the appropriate total volume of MRM/well for your PCR thermocycler (e.g., 23 µl/well for Stratagene's Mx3000P, see Table I) into the appropriate wells (in duplicate) using a repeating pipettor.
4. Using a multichannel pipettor, transfer 2 µl/well of the control dilutions, NTCs, and sample dilutions (in duplicate) from the 8-well PCR strips to the PCR plate containing the MRM.

5. We recommend that you program your real-time qPCR instrument for the following qPCR reaction cycles (see Table III). Include a final dissociation curve cycle.

Table III. Recommended Thermal Cycling Conditions for Different Real-Time PCR Instruments

	qPCR instruments									
	Stratagene Mx3000P		Takara Bio Thermal Cycler Dice™ Real Time System		ABI7500 Fast		ABI7000		Roche LightCycler	
qPCR Reaction Cycles	**Cycling conditions for each instrument**									
Initial Denaturation (1 Cycle):	95°C	10 sec	95°C	30 sec	95°C	30 sec	95°C	30 sec	95°C	20 sec
qPCR (40 Cycles):	95°C 60°C	5 sec 20 sec	95°C 60°C	5 sec 30 sec	95°C 60°C	3 sec 25 sec	95°C 60°C	3 sec 31 sec	95°C 60°C	5 sec 20 sec
Dissociation Curve (1 Cycle):	95°C 55°C 95°C	1 min 30 sec 30 sec	95°C 60°C 95°C	15 sec 30 sec 15 sec	95°C 60°C 95°C	15 sec 1 min 15 sec	95°C 60°C 95°C	15 sec 1 min 15 sec	95°C 60°C 95°C	0 sec[a] 15 sec 0 sec[b]

[a]20°C/sec
[b]0.1°C/sec

NOTE: Although Table III shows the recommended cycling conditions for a selection of commonly used instruments, the BacPAK qPCR Titration Kit can be used with a variety of real-time instruments and is not limited to those listed in the table. For instruments not listed, please refer to the TB Green qPCR Premix User Manual and/or your instrument's user manual to determine cycling conditions for your particular thermal cycler.

E. Data Analysis

1. Determine average Ct values from the control dilution duplicates and plot vs. copy number (log scale) to generate a standard curve (Figure 2).
2. Determine average Ct values for each duplicate sample dilution and read the corresponding copy number value from the standard curve. Use all Ct values that are below that of the NTC.
3. For each dilution, back-calculate a starting copy number value for the original sample using the example given below. Generate a mean value to determine the DNA genome content of the sample.

EXAMPLE CALCULATIONS:

Calculating DNA Copy Numbers and Focus Forming Units:

1. **Copy numbers:** 150 µl of a sample was purified and eluted in 50 µl. The undiluted sample corresponded to a raw copy number of 2.4 x 10^7 copies on the qPCR Standard Curve.

   - Copies/ml = $\frac{\text{[raw copy number (copies)] x [1,000 µl/ml] x [elution volume (µl)]}}{\text{[sample size (µl)] x [volume (µl) per well]}}$
   - Copies/ml = $\frac{(2.4 \times 10^7 \text{ copies}) \times (1{,}000 \text{ µl/ml}) \times (50 \text{ µl elution*})}{(150 \text{ µl sample*}) \times (2 \text{ µl per well})}$
   - Copies/ml = 4.0 x 10^9 copies/ml

   *These values are user defined.

2. **Focus forming units:** If you have also determined viral infectivity (e.g., via the BacPAK Baculovirus Rapid Titer Assay), calculate a qPCR:Rapid Titer ratio (copies/FFU), or focus forming units, for your virus by dividing the qPCR copies/ml by the FFU/ml value from your Rapid Titer Assay (see Table IV, below). This coefficient can then be used to calculate the FFU/ml for subsequent qPCR titration results.

   - Using the copy number (copies/ml) from the above example (4.0 x 10^9) and the qPCR:Rapid Titer ratio from Table IV (105), FFU/ml = (4.0 x 10^9 copies/ml) / (105 copies/FFU)
   - FFU/ml = 3.8 x 10^7

Table IV. BacPAK qPCR Titer Correlation

Baculovirus	Titration Method			BacPAK qPCR Titration Ratio	
	BacPAK Rapid Titer Assay[a] (FFU/ml)	Plaque Assay (PFU/ml)	BacPAK qPCR[b] (copies/ml)	qPCR/Rapid Titer (copies/FFU)	qPCR/Plaque Assay (copies/PFU)
BacPAK6	3.8 x 10^7	7.6 x 10^7	4 x 10^9	105	53

[a]Focus forming units were determined using Takara Bio's BacPAK Baculovirus Rapid Titer Kit (Cat. No. 631406)
[b]Baculoviral copy numbers were determined using the BacPAK qPCR Titration Kit (Cat. No. 631414).

NOTE: The data shown in Table IV are intended for illustrative purposes only. Users should determine infectivity coefficients that are specific for their viral preparation.

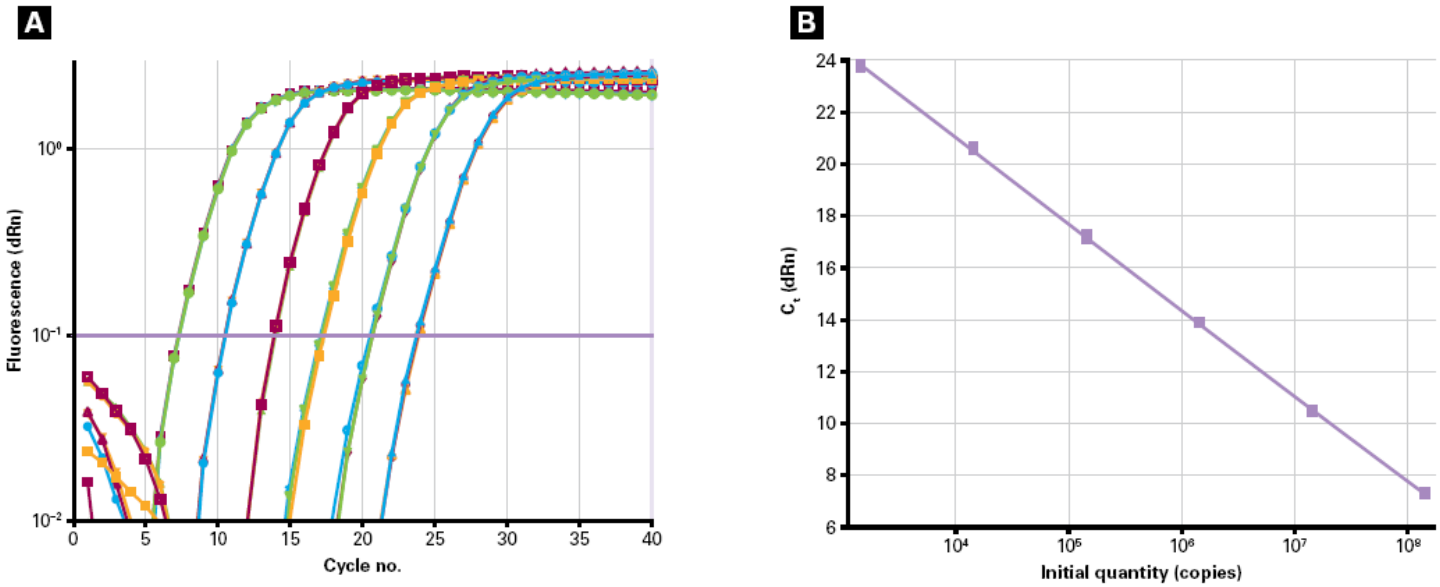

Figure 2. Using the BacPAK DNA Control Template to generate a standard curve. **Panel A.** Amplification plots of qPCR reactions using serial dilutions of the BacPAK DNA Control Template (2.8×10^8–2.8×10^3 copies) and the BacPAK qPCR Titration Kit. The assay shows a dynamic range of at least six orders of magnitude (each dilution is represented by a different colored plot) with no NTC background. **Panel B.** A standard curve created from the plots shown in Panel A demonstrates a strong linear correlation between the Ct values and the DNA copy number (log scale), with R2 = 1.000 and a PCR efficiency of 100.2%.

V. Troubleshooting

Table V. Troubleshooting Guide for BacPAK qPCR Titration

Problem	Possible Explanation	Solution
High signal in NTC reactions	Contamination of buffer, pipettors, or work area from improper handling of samples or control template	Diagnosis: Run control and NTC qPCR sample on 3% agarose gel to visually compare size of product bands (baculoviral-specific vs. nonspecific) and compare dissociation curves. Specific amplimer used for titration is ~114 bp. Dissociation curves should reflect the presence of a single product of this size, which should also be visible in the agarose gel.
		Prepare work area properly and use clean, dedicated pipettors for each phase of the protocol: dilution, reaction set-up, and analysis.
Poor efficiency or R^2	Poor technique or pipetting inconsistent	Review qPCR techniques; use repeating pipettors and multichannel pipettors for improved accuracy; calibrate pipettors.
Viral signal is higher than expected	High virus yield or residual plasmid DNA contamination.	Treat samples with DNase I prior to purification. Ensure samples are free of contaminating plasmid DNA. Perform a control reaction.
Viral signal absent or lower than expected	Low titer sample	Reamplify virus or concentrate viral stock and retitrate.
	Forgot to purify viral DNA via NucleoSpin column. DNase 1 treatment after NucleoSpin column purification.	Purify virus through the NucleoSpin column. Treat samples with DNase I prior to purification.

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