Takara Bio USA

BacPAK™ p35 ELISA Kit (Single Wash) User Manual

Cat. No. 631477
(040324)

Takara Bio USA, Inc.
2560 Orchard Parkway, San Jose, CA 95131, USA
U.S. Technical Support: technical_support@takarabio.com

United States/Canada	Asia Pacific	Europe	Japan
800.662.2566	+1.650.919.7300	+33.(0)1.3904.6880	+81.(0)77.565.6999

Table of Contents

Table of Figures

Table of Tables

I. Introduction

The **BacPAK p35 ELISA Kit (Single Wash)** (Cat. No. 631477) enables you to determine baculovirus titers based on the expression of p35, an early-expressed viral protein that prevents cellular apoptosis and is essential for infectivity. p35 is initially detected in the host cell cytosol 4–12 hours after infection, before morphological changes or virus budding are observed (Gershburg et al. 1997; Hershberger et al. 1994). The kit accurately measures the ability of the virus to infect cells and express virally encoded proteins, reliably quantifying titers greater than 10^4 PFU/ml (IFU/ml).

The protocol enables rapid Baculovirus titer determination through a two-day process. On Day 1, insect cells are seeded in a 96-well plate, infected with serially diluted virus samples, then incubated overnight at 27°C. On Day 2, infected cells are lysed and p35 levels are measured by ELISA alongside a p35 standard dilution series. p35 in the samples binds to plate-bound capture antibody then are labeled by HRP-anti-p35 detection antibody. After washing and addition of TMB Substrate and Stop Solution, the p35 concentration of the sample dilutions is quantified against a standard curve.

To determine the correlation between titer and p35 values, we recommend using a reference sample of known titer (not supplied). In the absence of a reference virus, we recommend determining the correlation between p35 and your downstream application, for instance, protein expression, virus sample amplification, et al.

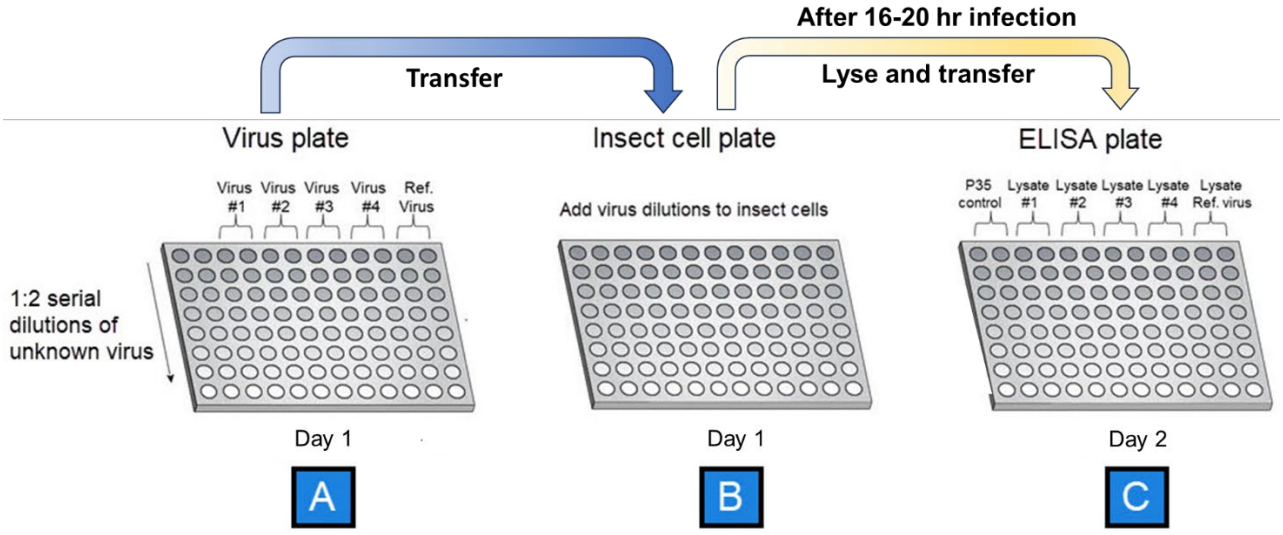

Figure 1. The BacPAK p35 ELISA Kit (Single Wash) workflow. Initial sample dilutions are made before loading into the 96-well plate (not shown). **Panel A.** Serial dilutions are made of samples of an unknown titer. **Panel B.** Insect cells are infected by the prepared dilutions. After incubation (16-20 hr), the cells are lysed. **Panel C.** The lysate is transferred to the ELISA plate included in the kit. ELISA is performed, and the absorbance measured at 450 nm. The standard curve generated from the p35 control (provided) is used to determine the titers of the sample baculoviruses.

II. List of Components

BacPAK p35 ELISA Kit (Single Wash) (Cat. No. 631477) contains sufficient reagents to perform 96 reactions.

NOTE: Do not use reagents beyond the expiration date. Do not combine reagents from different kit lots.

Table 1. BacPAK p35 ELISA Kit (Single Wash) components.

BacPAK p35 ELISA Kit (Single Wash) (Store at 4°C)	631477
Anti-p35 Coated Plate (12 x 8-well microtiter strips)*	1 plate
p35 Control	2 x 500 ng
p35 Buffer	8 ml
Lysis Buffer	8 ml
Anti-p35 (HRP Conjugate)	12 ml
Washing Buffer (20X)†	50 ml
TMB Substrate	12 ml
Stop Solution	12 ml

*Once opened, microtiter strips may be stored at 2–8°C until the expiration date on the label. Microtiter strips must be stored under desiccated conditions. Return unused microtiter strips to their original foil pouch along with the desiccant sachet, and securely reseal the pouch by folding over the open end and securing it with adhesive tape.

†The 20X Wash Buffer can develop a crystalline precipitate during initial storage at 4°C. This will dissolve upon warming to 37°C. After first use, store 20X Wash Buffer at room temperature.

III. Additional Materials Required (Not Provided)

The following materials, or their equivalents, are required to perform the BacPAK p35 ELISA Kit (Single Wash) assay:

- A reference baculovirus with known titer (recommended)
- Two 96-well tissue culture plates
- Microtiter plate reader with 450 nm filter
- Sf21, Sf9, or HighFive insect cells

  NOTE: This kit has been validated using Sf21, Sf9, and HighFive cell lines. For use with other insect cell lines not listed here, optimization and validation studies must be performed by the user.
- Insect cell complete medium
- Hemacytometer and Trypan Blue, or equivalent cell counting instrument
- Cell culture incubator (27°C; humidified by water)
- Micropipettes for delivering volumes of 2 µl, 20 µl, 100 µl, 200 µl, and 1,000 µl. A multichannel pipette is preferred for dispensing reagents into microtiter plates.
- 15 ml and 100 ml beakers
- 1 L graduated cylinder
- Filtered 1 ml, 5 ml, 10 ml, and 25 ml pipettes
- Distilled or deionized water
- 25 ml reagent reservoirs
- Microtube rack
- 2 ml centrifuge tubes
- Absorbent paper towels

- Automatic microtiter plate washer or a vacuum line fitted with a vacuum pump trap to collect liquid
- Latex gloves, safety glasses, and other appropriate protective garments
- Biohazard infectious waste containers
- Timer

IV. General Considerations and Safety Guidelines

A. General Requirements

- The BacPAK p35 ELISA Kit (Single Wash) contains reagent systems which are optimized and balanced for each kit lot. Do not interchange reagents from kits with different lot numbers. Do not interchange vial caps or stoppers either within or between kits.
- Allow foil bags to warm to room temperature before opening to avoid condensation on the inner surface of the bag, which may contribute to a deterioration of the microtiter strips intended for future use.
- Reagents should be dispensed with the tip of the micropipettes touching the side of the well at a point about midsection. Follow manufacturer's recommendations for automatic processors.
- Always keep the upper surface of the microtiter strips free from excess fluid droplets. Reagent and buffer overspill should be blotted dry on completion of the manipulation.
- Do not allow the wells to completely dry during an assay.

B. Sample Recommendations and Requirements

- Thoroughly mix thawed samples before testing.
- The assay cannot be used to quantitate samples having p35 values greater than the highest value on the p35 standard curve, unless the samples are diluted sufficiently. See Appendix A for more information.
- The kit includes sufficient reagents for titrating 5 samples if using two 8-well strips per sample.
- Baculovirus samples with titers of $>10^4$ PFU/ml or IFU/ml can be titered using this kit.

V. Protocol

IMPORTANT: Please read the entire protocol before starting.

BacPAK p35 ELISA Kit (Single Wash) follows a two-day protocol which includes diluting viral samples, seeding and infecting insect cells, and performing the ELISA. On the first day, you will dilute viral samples, seed insect cells (see Note below) in a 96-well tissue culture plate and infect them with the different dilutions of your virus samples of unknown titer, then incubate overnight at 27°C. On the second day, you will lyse the infected cells, measure p35 levels by ELISA, and use the results to calculate the p35 concentrations and subsequently the infectious titer of your baculovirus samples.

This kit was validated using Sf21, Sf9, and HighFive insect cell lines. When adapting this kit to new cell types, it is recommended to optimize parameters like cell density, baculovirus infection time, and culture conditions to achieve robust p35 expression for accurate titer determination. Other insect cell lines can differ in protein

expression profiles and growth characteristics compared to Sf21, Sf9, and HighFive cells. Both cell number and viral titer impact the amount of p35 antigen produced.

IMPORTANT: We strongly recommend using a reference sample of known titer to determine the correlation between titer and p35 values. The kit reliably quantifies titers greater than 10^4 PFU/ml (or IFU/ml). To achieve optimal performance, we recommend the following dilution procedure:

- For early passaged or low titer samples, begin with either an as-is or 1:10 dilution, and then proceed with a 1:2 serial dilution.
- For late passaged or high titer samples, initiate with a 1:100 or 1:1,000 dilution, followed by a 1:2 serial dilution.

In either case, ensure a minimum of seven dilutions for each unknown baculovirus sample before infecting insect cells.

A. Protocol: (Day 1) Infect Insect Cells (~1 hr)

1. Seed Insect Cells

NOTE: The quantity of p35 antigen detected in each well depends on both the viral titer and the number of cells per well. While viral titer has a greater influence, having consistent cell numbers across wells is still important to normalize the background and enable accurate titer determination.

1. Collect cultured insect cells with ≥90% viability from culture (spinner flask or plate). Count viable cells and dilute in insect medium to a final concentration of 1 x 10^6 cells/ml.
2. Seed 100 µl of insect cell suspension (10^5 cells/well) into each well of the required number of columns of a sterile 96-well culture plate. Use twice as many columns as the total number of samples being tested.

   Example:
   If testing 5 samples total, seed 10 columns of a 96-well plate.
   (5 samples x 2 columns per sample = 10 columns needed)
3. Let the cells attach for at least 15 min at room temperature. They should be ~90% confluent.

2. Prepare Serial Dilutions of Virus Samples

NOTES:
– The kit reliably quantifies titers greater than 10^4 PFU/ml or IFU/ml.
– Include a negative control well containing media without virus.
– We strongly recommend using a reference sample of known titer to determine the correlation between titer and p35 values.

To achieve optimal performance, we recommend the following dilution procedure each virus sample. This protocol will create a total of eight dilutions (initial + 7 serial) per sample, with each sample assayed in duplicate (Appendix A, Table 2).

- Initial Dilution:
  - Early Passage/Low Titer: Use the virus sample either as-is or dilute 1:10 in a total volume of 250 µl per single replicate.
  - Late Passage/High Titer: Dilute the virus sample 1:100 or 1:1,000 in a total volume of 250 µl per single replicate.

- Serial Dilutions:
  - Following the initial dilution, perform seven 1:2 serial dilutions using 125 µl of media for each dilution for each replicate.

An example preparation of serial dilutions for an early-passage sample stock with an infectious titer range of 10^5–10^7 pfu/ml is provided in the Appendix A.

3. Infect Insect Cells

1. Gently transfer 100 µl of the diluted sample stocks from Step 2 to the corresponding wells containing the insect cells (seeded in Step 1).
2. Incubate in a cell culture incubator (27°C; humidified by water) for 16–20 hr.

B. Protocol: (Day 2) Perform ELISA (~2 hr)

1. Prepare p35 Standard Dilutions

To determine accurate sample titers, prepare a p35 standard curve (0–200 ng/ml).

1. Allow all reagents, except lysis buffer, to reach room temperature (18–25°C).
2. Prepare a p35 stock solution by adding 1,250 µl of p35 Buffer to the p35 Control vial (500 ng lyophilized powder) to make the 400 ng/ml stock solution. Vortex for 5 sec.

NOTE: The reconstituted p35 stock (400 ng/ml) is stable for 4 weeks when stored at 4˚C.

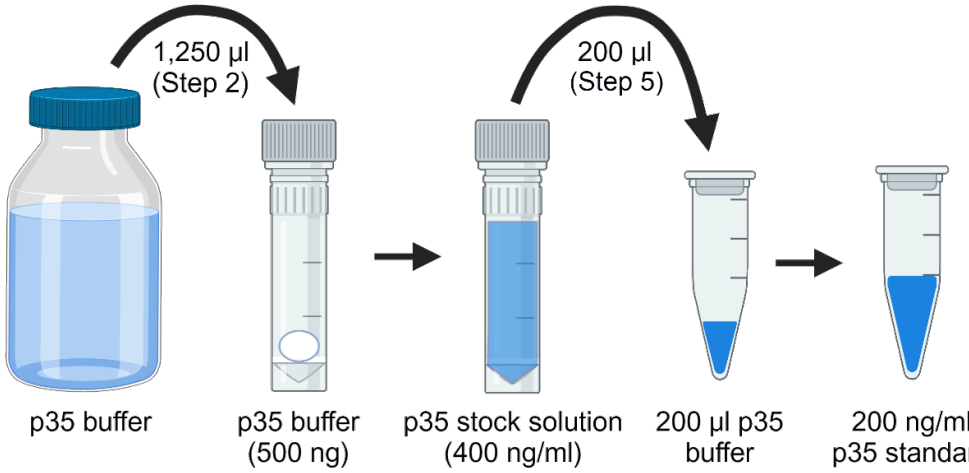

Figure 2. p35 stock solution preparation steps.

3. Prepare eight centrifuge tubes with appropriate labeling for the following dilutions for the standard curve:

200 ng/ml	100 ng/ml	50 ng/ml	25 ng/ml
12.5 ng/ml	6.25 ng/ml	3.125 ng/ml	0 ng/ml*

*Negative control (NTC), p35 Buffer only

4. Add 200 µl of p35 Buffer to each of the eight prepared tubes.
5. Add 200 µl of p35 stock solution (from Step 2) to the tube labeled for the 200 ng/ml dilution and vortex for 5 sec to mix. Use Figure 3 (next page) as a guideline to continue creating each subsequent dilution for the standard curve series, vortexing each new dilution for 5 sec to mix before proceeding to the next one.

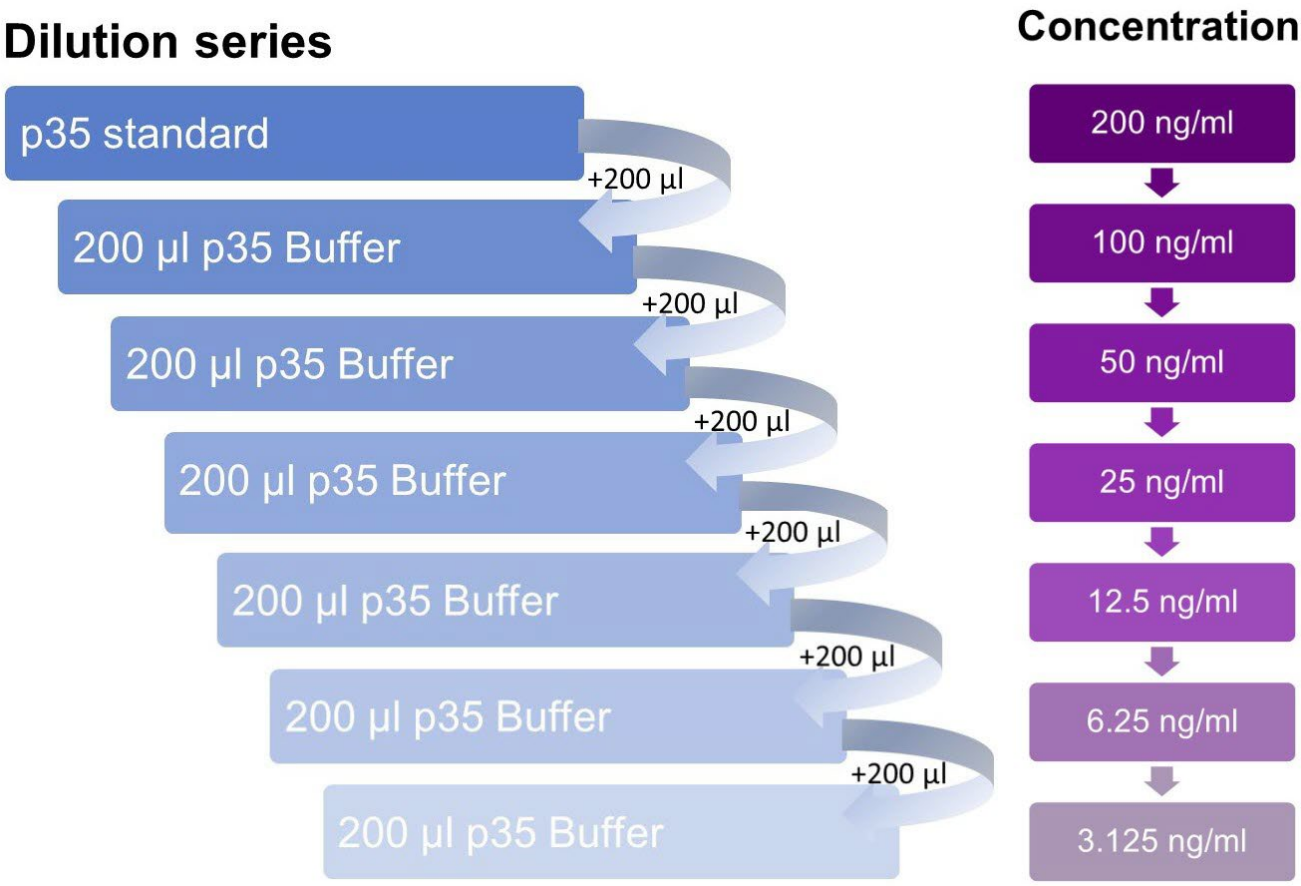

Figure 3. Preparing the p35 curve serial dilutions. 200 µl of the solution from the previous step in the series is added to 200 µl of p35 Buffer in a fresh tube to make each subsequent solution (3.125–100 ng/ml).

2. Lyse Infected Cells

1. Take the infected insect cells from Day 1 and aspirate the medium from the plate.
2. Add 60 µl of ice-cold lysis buffer to each well containing cells.

   NOTE: Buffer can either be added directly from storage at 4°C or prechilled at the bench on ice to ensure it is ice-cold.
3. Incubate the plate at room temperature for 20 min to lyse the cells.

3. Perform ELISA

1. Place the Anti-p35 Coated Plate microtiter strips into the holding frame to accommodate samples, negative controls, reference baculovirus (if used), and all p35 standards (tubes 1–8 of the p35 standard dilution series from Step 1 of Day 2).
2. Transfer 50 µl of p35 standard dilutions to the corresponding p35 standard wells of the ELISA plate.
3. Transfer 50 µl of the insect cell lysates to the corresponding sample wells of the ELISA plate.
4. Add 100 µl of Anti-p35 (HRP conjugate) to each well. Gently agitate the plate.
5. Incubate the plate at room temperature (18–25°C) for 60 min ± 5 min.

6. Aspirate the contents of the wells and wash the microtiter plate either via automatic or manual plate washing, following the appropriate protocol below.

NOTES:

– Using an automatic plate washer is recommended to enhance speed, efficiency, and well-to-well consistency. Manual plate washing can yield equivalent results if performed carefully.

– A single six-rinse/wash cycle is required.

Automatic Plate Washing

a. The rinse cycle must consist of six consecutive washes.

b. After final rinse cycle, flip the plate side down and tap firmly.

c. Check for any residual wash buffer in the wells and blot the upper surface with a dry paper towel.

Manual Plate Washing

For manual plate washing, perform the following steps for the rinse cycle:

a. Use a vacuum line fitted with a vacuum pump trap to aspirate liquid from all wells.

b. With a multichannel pipette, fill wells with ~300 µl of Washing Buffer (to the brim).

c. Aspirate the wells with the vacuum line.

d. Repeat steps b. and c. five more times (Total: 6 rinse/wash cycles).

e. Flip the plate well-side down and tap firmly on a paper towel to remove any excess liquid. Repeat this twice to ensure most of the liquid is removed.

7. After washing, immediately, dispense 100 µl of TMB Substrate into each well.
8. Incubate the plate in the dark at room temperature (18–25°C) for 10 ± 2 min.
9. Add 100 µl of Stop Solution to each well to stop the reaction and mix gently. The blue solution should change to a homogeneous yellow color in each well.
10. Ensure that the undersides of the wells are dry and that there are no air bubbles in the wells.
11. Immediately read the absorbance values at 450 nm using a microtiter plate reader.
12. Calculate the standard curve based on the standard dilution series, then use the curve to determine the p35 concentration values of both your known and unknown samples.

VI. References

Gershburg, E., Rivkin, H. & Chejanovsky, N. Expression of the Autographa californica nuclear polyhedrosis virus apoptotic suppressor gene p35 in nonpermissive Spodoptera littoralis cells. *J. Virol.* **71,** 7593–7599 (1997).

Hershberger, P. A., LaCount, D. J. & Friesen, P. D. The apoptotic suppressor P35 is required early during baculovirus replication and is targeted to the cytosol of infected cells. *J. Virol.* **68,** 3467–3477 (1994).

Appendix A. Dilution Series Example

Provided below is a dilution series example for an early-passage virus stock with an expected titer range of 10^5–10^7 pfu/ml.

1. Add 225 µl of insect cell medium to Row A and 125 µl of insect cell medium to the Row B–H.
2. Add 25 µl of the first baculovirus sample to the first well of the Column A. Mix well by gently pipetting up and down several times.
3. Using a multichannel pipette, transfer 125 µl from Row A of the column to Row B. Mix thoroughly by pipetting.
4. Transfer 125 µl of the suspension to the next row of wells (Row B to Row C). Mix thoroughly by pipetting.
5. Repeat Step 4 five times, for each subsequent row, until the serial dilution is completed (Row G to Row H).

Table 2. Example series dilution panel.

	1	2	3	4	5	6	7	8	9	10	11	12
A			Sample 1 1:10	Sample 1 1:10	Sample 2 1:10	Sample 2 1:10	Sample 3 1:10	Sample 3 1:10	Sample 4 1:10	Sample 4 1:10	Reference* 1:10	Reference* 1:10
B			Sample 1 1:20	Sample 1 1:20	Sample 2 1:20	Sample 2 1:20	Sample 3 1:20	Sample 3 1:20	Sample 4 1:20	Sample 4 1:20	Reference* 1:20	Reference* 1:20
C			Sample 1 1:40	Sample 1 1:40	Sample 2 1:40	Sample 2 1:40	Sample 3 1:40	Sample 3 1:40	Sample 4 1:40	Sample 4 1:40	Reference* 1:40	Reference* 1:40
D			Sample 1 1:80	Sample 1 1:80	Sample 2 1:80	Sample 2 1:80	Sample 3 1:80	Sample 3 1:80	Sample 4 1:80	Sample 4 1:80	Reference* 1:80	Reference* 1:80
E			Sample 1 1:160	Sample 1 1:160	Sample 2 1:160	Sample 2 1:160	Sample 3 1:160	Sample 3 1:160	Sample 4 1:160	Sample 4 1:160	Reference* 1:160	Reference* 1:160
F			Sample 1 1:320	Sample 1 1:320	Sample 2 1:320	Sample 2 1:320	Sample 3 1:320	Sample 3 1:320	Sample 4 1:320	Sample 4 1:320	Reference* 1:320	Reference* 1:320
G			Sample 1 1:640	Sample 1 1:640	Sample 2 1:640	Sample 2 1:640	Sample 3 1:640	Sample 3 1:640	Sample 4 1:640	Sample 4 1:640	Reference* 1:640	Reference* 1:640
H			Sample 1 1:1,280	Sample 1 1:1,280	Sample 2 1:1,280	Sample 2 1:1,280	Sample 3 1:1,280	Sample 3 1:1,280	Sample 4 1:1,280	Sample 4 1:1,280	Cell media (NTC)†	Cell media (NTC)†

*Reference is the baculovirus with known titer.
†Insect cell complete medium is the negative control.

6. To calculate the actual IFU/ml for an unknown stock, a reference virus (a virus stock for which the IFU/ml is known) must first be tested to obtain both an infectious unit value as well as a p35 value.
7. Calculate the IFU/p35 value ratio for the reference virus.
8. Perform calculations to determine your IFU/ml using the following formula:

$$\textbf{Infectious titer (unknown)} = \text{p35 (sample)} \times \frac{\text{Infectious titer (reference)}}{\text{p35 (reference)}}$$

Example:

Table 3. Example data. The values represent the p35 determined by the results of the protocol against the calculated standard curve.

Viral prep	Infectious titer (IFU/ml)	p35 (ng/ml)
Reference	6.0×10^6	**9.18×10^5**
Unknown_1	?	**5.93×10^6**
Unknown_2	?	**3.50×10^4**

Putting the values of Table 3 into the formula, the infectious titers of samples Unknown_1 and Unknown_2 can be calculated as 3.8 x 10^7 IFU/ml and 2.3 x 10^5 IFU/ml, respectively.

Infectious titer (Unkown_1) = $5.93 \times 10^6 \times \frac{6.0 \times 10^6}{9.18 \times 10^5}$ = **3.8×10^7 IFU/ml**

Infectious titer (Unkown_2) = $3.50 \times 10^4 \times \frac{(6.0 \times 10^6)}{9.18 \times 10^5}$ = **2.3×10^5 IFU/ml**

Appendix B. Troubleshooting Guide

Problem	Possible Explanation	Solution
Reagents become visibly cloudy or contain a precipitate.	The 20X Wash Buffer can develop a crystalline precipitate during storage at 2–8°C.	Precipitate will dissolve upon warming the 20X Wash Buffer to 37°C.
	For other reagents, this may be caused by a kit with previously opened reagents or past its expiration date.	Use a new kit with fresh reagents. If this is a newly opened kit not past the expiration date, contact technical support.
The TMB Substrate solution becomes dark blue in color.	This is likely caused by chemical contamination of the substrate solution.	Use a new kit with fresh reagents. If this is a newly opened kit not past the expiration date, contact technical support.
Signals obtained from samples are too high	The samples are not diluted sufficiently	Perform serial dilution of the sample until the dilution falls into the p35 standard curve.
Signals obtained from samples are too low	The samples are too diluted	Use the sample as-is to infect insect cells.
The kit fails to meet the required criteria for a valid test	Check possible issues in the table above.	If no issues match your experimental situation, contact technical support.

Contact Us	
Customer Service/Ordering	**Technical Support**
tel: 800.662.2566 (toll-free)	tel: 800.662.2566 (toll-free)
fax: 800.424.1350 (toll-free)	fax: 800.424.1350 (toll-free)
web: takarabio.com/service	web: takarabio.com/support
e-mail: ordersUS@takarabio.com	e-mail: technical_support@takarabio.com

Notice to Purchaser

Our products are to be used for **Research Use Only**. They may not be used for any other purpose, including, but not limited to, use in humans, therapeutic or diagnostic use, or commercial use of any kind. Our products may not be transferred to third parties, resold, modified for resale, or used to manufacture commercial products or to provide a service to third parties without our prior written approval.

Your use of this product is also subject to compliance with any applicable licensing requirements described on the product's web page at takarabio.com. It is your responsibility to review, understand and adhere to any restrictions imposed by such statements.

This document has been reviewed and approved by the Quality Department.