Takara Bio USA

BacPAK™ Baculovirus Rapid Titer Kit User Manual

Cat. No. 631406
(042222)

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

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

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I. Introduction

The BacPAK Baculovirus Rapid Titer Kit provides a quick method for determining titers of baculovirus stocks, typically the most time-consuming part of baculovirus expression protocols. In conjunction with the BacPAK Baculovirus Expression System (Cat. No. 631402) or any AcMNPV-based baculovirus system, this kit can reduce the total time needed to express proteins by as much as six days. The Rapid Titer immunoassay utilizes a standard immunoassay for a viral envelope glycoprotein to accurately identify virally infected cells in only two days—well before plaques can be detected in standard plaque assays (Figure 1).

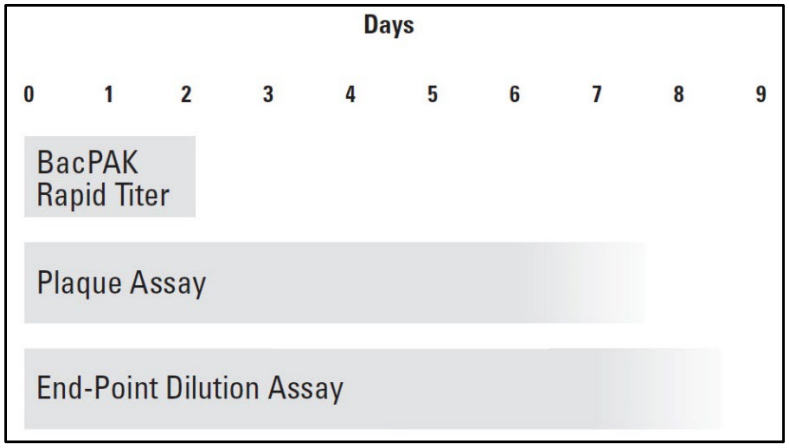

Figure 1. The Rapid Titer assay is significantly faster than other baculovirus titering methods.

A. Benefits of the BacPAK Rapid Titer Kit

Viral infection at the correct multiplicity of infection is critical to achieving optimal protein yields with baculovirus gene expression. There are some drawbacks to common titering methods, including plaque and endpoint dilution assays: these assays generally take 5–8 days and require extensive baculovirus experience to perform. In contrast, the Rapid Titer Kit is a standard immunological assay (Figure 2) which takes only two days. Infected cells express viral antigens long before plaques are formed, so titers are determined by antibody detection of those antigens after a much shorter incubation period (Volkman & Goldsmith, 1982).

Although the results of these assays are often found to be similar (Figure 3), the plaque assay and endpoint dilution assay are dependent on the ability of the virus to replicate in infected cells (Table I). In contrast, the immunostaining method is not dependent on the ability of the virus to replicate, only on its ability to infect and express a virally-encoded protein. In this sense, the results of immunostaining (expressed as infectious units per ml, or IFU/ml), are more relevant to the situation when the virus is used to express a protein of interest than are the results of the plaque or endpoint dilution assay (expressed as PFU/ml).

Table 1. Comparison of titration methods

Procedure or measurement	What is required	Virus replication required?
Infection of cells for protein expression	Virus must infect cells and express virally-encoded proteins, including protein-of-interest	No
Detection of virus by immunostaining (Rapid Titer Kit)	Virus must infect cells and express viral protein (Gp64)	No
Plaque assay or endpoint dilution assay (PFU/ml)	Virus must infect cells, replicate, and then infect surrounding cells	Yes

In this titering method (Figure 2, below), a primary monoclonal antibody raised to an AcMNPV envelope glycoprotein (gp64) labels infected cells in replicate samples. A secondary HRP-conjugated antibody stains the infected cells so the number of infected foci can be counted under light microscopy. Then the titer can be determined since the number of infected foci corresponds to the multiplicity of infection. Assaying multiple wells at the same viral concentration is essential since there may be a slight well-to-well variation. The Rapid Titer Kit provides sufficient reagents for performing five assays (one assay is 12 wells in a 96 well plate), and is suitable for titering any virus stock with a titer of more than 2 x 10^4 PFU (plaque-forming units)/ml or IFU (infectious units)/ml. The gp64 antibody is only available as part of the Rapid Titer Kit.

B. The BakPak system

The BacPAK Baculovirus Expression System (Takara Bio, Cat. No. 631402) routinely delivers protein expression of 10–100 mg/L and features BacPAK6, a specially engineered viral DNA (Kitts & Possee, 1993) that facilitates the construction and selection of recombinant expression vectors at frequencies >90%. We offer a complete line of BacPAK baculovirus products that are compatible with most other baculovirus expression systems.

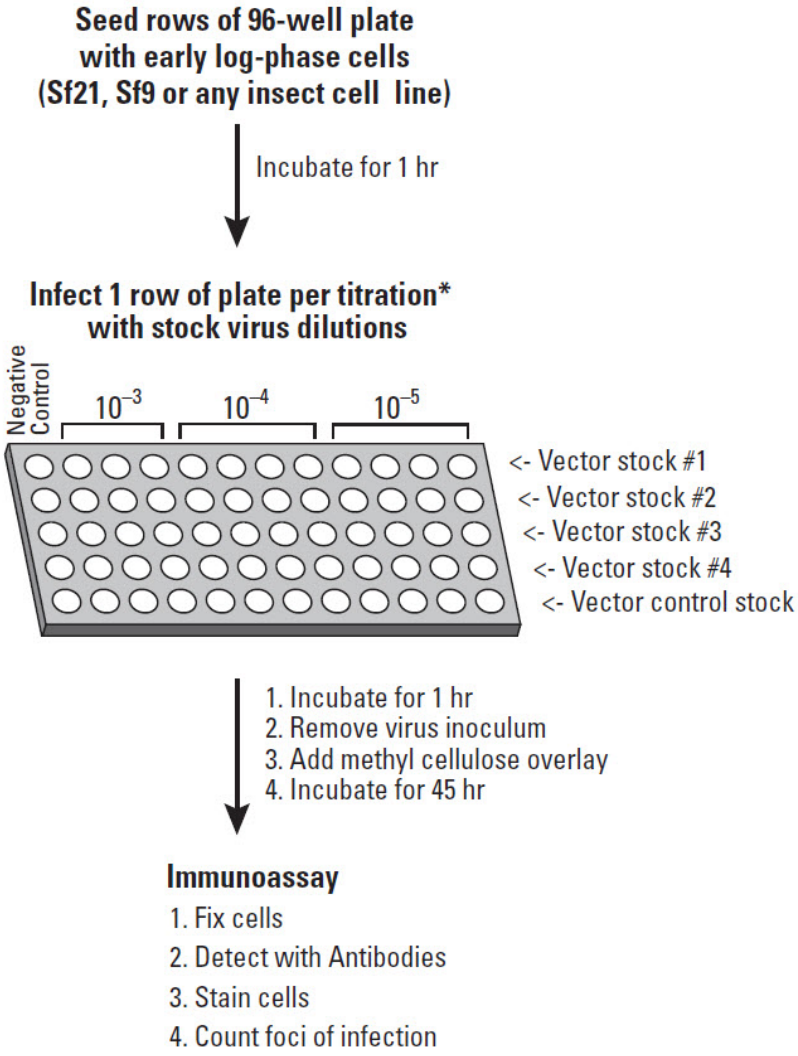

Figure 2. Flowchart of the BacPak Rapid Titer assay procedure. *Sufficient reagents are supplied for 5 titrations (60 wells).

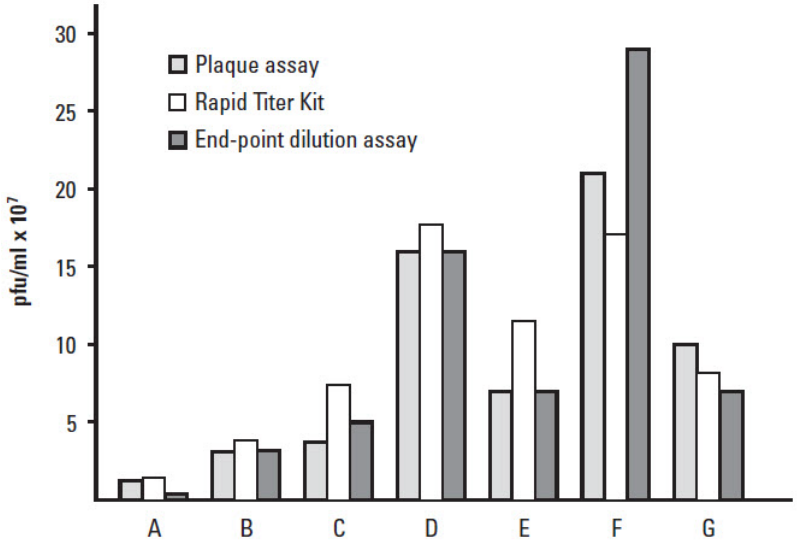

Figure 3. Comparison of viral titering methods. Viral titers obtained with BacPAK Rapid Titer method are similar to titers obtained with other assay methods. Seven virus stocks with titers between 1 x 10^7 and 3 x 10^8 plaque forming units (PFU)/ml were harvested at different times and assayed in parallel with the Baculovirus Rapid Titer Kit, plaque assay, and endpoint dilution assay (Luckow, 1993; Miller, 1993; O'Reilly et al. 1992). Stock type: A: AcMNPV; B: BacPAK6 Virus; C: AcMNPV; D: BacPAK6 Virus; E: BacPAK6 Virus; F: BacPAK6 Virus; G: BacPAK6 Virus.

II. List of Components

Table 2. Components of the BacPAK Baculovirus Rapid Titer Kit (Cat. No. 631406)

BacPAK Baculovirus Rapid Titer Kit	631406
Box 1 (Store at –20°C)	
Goat Anti-mouse Antibody/HRP Conjugate	30 µl
Normal Goat Serum	0.5 ml
Box 2 (Store at 4°C)	
Mouse gp64 Antibody	13 µl
Blue Peroxidase Substrate	4 ml
Methyl Cellulose Overlay	4 ml
Resealable Plastic Bags	2

III. Additional Materials Required, Not Provided

- Phosphate buffered saline (PBS) with $CaCl_2$ and $MgCl_2$ (Sigma, Cat. No. D8662)
- Formyl buffered acetone (ice-cold)*—prepare in a glass or acetone-resistant plastic container.

  To prepare 4 ml, add the following in the order indicated and place at –20°C for at least 3 hours prior to starting immunoassay.
  - 1.2 ml PBS
  - 1.0 ml 37% formaldehyde solution
  - 1.8 ml acetone

***NOTE:** Alternative to formyl buffered acetone—we have found that paraformaldehyde consistently produces much better signal to noise than formyl buffered acetone. Please see Appendix A for details of this recommended alternative. The materials required for this alternative:

- Paraformaldehyde solution
- 0.2 M sodium phosphate buffer (pH 7.4)

- Tween 20 (Sigma, Cat. No. P9416)
- Microtiter plates (tissue culture grade; clear plastic)
- Pipette tips (standard and gel loading tips), pipettors and multichannel pipettor

IV. Rapid Titer Assay Protocol

NOTE: PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING.

A. General Considerations

- Use standard cell culture microtiter plates.
- Always perform replicates of each viral dilution to minimize the variation of individual samples.
- After the cells are fixed to the plate, we recommend you shake out reagents over the sink instead of aspirating them. This technique gives better results because it limits the amount of cell scraping.

B. Assay Setup and Infection

1. Seed one row (12 wells) of a 96-well microtiter plate with early log phase Sf21 or Sf9 cells (6.5×10^4 cells/well). Label wells as shown in Figure 2. The density of early log phase cells used for the viral infection in Step 3 should be 3–4 x 10^5 cells/ml.
2. Incubate the plate in a sealed plastic bag containing a moist towel at 27°C for 1 hr.
3. Make serial dilutions of the virus sample(s) by adding 100 µl of virus stock to 900 µl of Complete Medium (or TNM-FH + 10% FBS) to give final dilutions of 10^{-3}, 10^{-4}, and 10^{-5}. Mix thoroughly between dilutions.

   These dilutions are appropriate for virus stocks with titers of 5×10^5 to 1×10^8 IFU/ml. Adjust the dilutions if the expected titer is not in this range.
4. Carefully aspirate the medium from microtiter plate using a multichannel pipettor with gel loading tips. Take care not to scrape the bottom of the wells.
5. Add 25 µl aliquots of the viral dilutions to the appropriate wells (3 wells for 10^{-3}; 4 wells for 10^{-4} and 10^{-5}). Add 25 µl of medium to the negative control well. Gently rock the plate to evenly distribute the virus.
6. Incubate for 1 hr at room temperature in a sealed plastic bag containing a moist towel.
7. Aspirate inoculum as in Step 4 above. Be careful not to scrape the cells from the bottom of the wells.
8. Add 50 µl Methyl Cellulose Overlay, wrap microtiter plate in a moist paper towel, and incubate in a sealed plastic bag at 27°C for 43–47 hr.

C. Virus Detection

1. Before beginning the assay, prepare the following reagents. All volumes are sufficient for one assay (12 wells); for multiple assays, increase volumes accordingly.
   - PBS + 0.05% Tween 20:
     - 25 ml PBS
     - 12.5 µl Tween 20

- Diluted Normal Goat Serum:
  - 80 µl concentrated serum
  - 2.3 ml PBS + 0.05% Tween 20
- Mouse gp64 Antibody:
  - 2.5 µl gp64 antibody
  - 497.5 µl Diluted Normal Goat Serum
- Goat Anti-mouse Antibody/HRP Conjugate (mix thoroughly):
  - 4 µl anti-mouse conjugate
  - 996 µl Diluted Normal Goat Serum
- 4 ml of ice-cold formyl buffered acetone (see Section III, Additional Materials Required)

  -OR-

  Paraformaldehyde solution and sodium phosphate buffer (see Appendix A for details of using paraformaldehyde instead of formyl buffered acetone; also see note below)

2. Carefully add 150 µl of freshly prepared ice-cold formyl buffered acetone* to each well containing the methyl cellulose overlay and incubate for 10 min at room temperature.

   NOTE: We have found that paraformaldehyde consistently produces much better signal to noise than formyl buffered acetone. Please see Appendix A for details of this recommended alternative.

3. Shake reagent out in the sink. Tap plate lightly on paper towel and wash 3X with 200 µl PBS + 0.05% Tween 20 (5 min per wash).
4. Add 50 µl diluted Normal Goat Serum. Incubate on shaker for 5 min at room temperature.
5. Shake out reagents in sink, tap plate lightly on paper towel. **Do not wash.**
6. Add 25 µl diluted Mouse gp64 Antibody and incubate at 37°C for 25 min.
7. Shake out the plate in sink. Tap plate lightly on a paper towel, and wash 2X with 200 µl PBS + 0.05% Tween 20 (5 min per wash with shaking).
8. Add 50 µl diluted Goat Anti-mouse Antibody/HRP Conjugate and incubate for 25 min at 37°C.
9. Shake out the plate in sink. Tap plate lightly on paper towel, and wash 3X with 200 µl PBS + 0.05% Tween 20 (5 min per wash with shaking).
10. Add 50 µl Blue Peroxidase Substrate and incubate for 3 hr at room temperature. You can obtain a preliminary but less accurate estimate of viral titer as early as 10 min after adding the substrate.

D. Determining Virus Titer

1. Use light microscopy to count stained foci of infection in the highest dilution wells containing a reasonable number of foci (~5–25). Count each discrete cluster of stained cells as one focus.
2. Determine the average number of foci per well for all the wells at that dilution. You will obtain the best results when the average number of foci per well is 5–25.

3. Multiply the average number of foci by the corresponding dilution factor and an inoculum volume normalization factor of 40 to determine the virus titer in infectious units per ml (IFU/ml).

   Virus titer (IFU/ml) =

   average no. of foci per well * ***dilution factor*** * 40

   Sample calculation:

   15 foci per well * $(10^{-5})^{-1}$ * 40 = **6 x 10^7 IFU/ml**

4. There may be an occasional well with no foci. This is to be expected given the variability of this assay and does not mean that you have performed the experiment incorrectly. As long as foci are counted in multiple wells at each dilution, you will get a reliable and reproducible measure of titer.

V. References

Kitts, P. A. & Possee, R. D. (1993) A method for producing recombinant baculovirus expression vectors at high frequency. *BioTechniques* **14,** 810–817.

Luckow, V. A. Baculovirus systems for the expression of human gene products. *Curr. Opin. Biotechnol.* **4,** 564–572 (1993).

Miller, L. K. Baculoviruses: high-level expression in insect cells. *Curr. Opin. Genet. Devel.* **3,** 97–101 (1993).

O'Reilly, D. R., Miller, L. K. & Luckow, V. A. Baculovirus Expression Vectors: A Laboratory Manual (W. H. Freeman & Co., NY) (1992).

Volkman, L. E. & Goldsmith, P. A. *Appl. Envir. Microbiol.* **44,** 227–233 (1982).

Appendix A. Paraformaldehyde Staining Protocol

We have found that paraformaldehyde consistently produces much better signal to noise than formyl-buffered acetone. We suggest switching to paraformaldehyde, which can be made following the recipe below, and frozen in aliquots for at least six months at –20°C. Once thawed, the aliquots can be stored at 4°C for up to one week.

The fixation step should be increased to 30 minutes from the original 10 minutes.

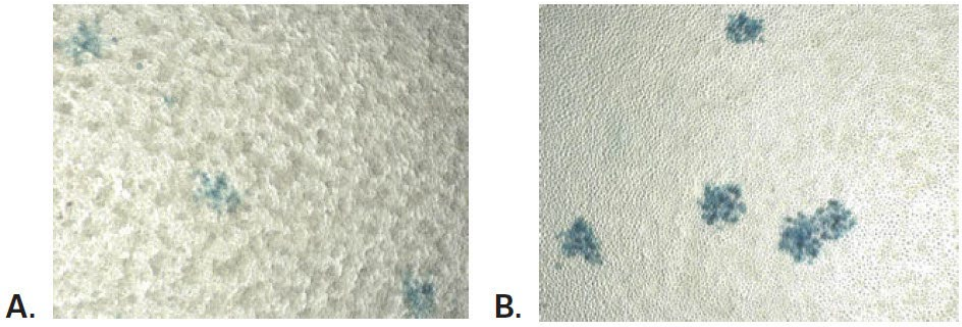

Figure 4. Paraformaldehyde versus formyl-buffered acetone. **Panel A** shows Rapid Titer results using formyl-buffered acetone (according to User Manual protocol). **Panel B** shows Rapid Titer results when 4% paraformaldehyde is substituted for formyl-buffered acetone.

Prepare 100 ml of 4% paraformaldehyde as follows:

WARNING: Paraformaldehyde is toxic. Avoid inhaling. Use a fume hood when making this solution, and wear protective clothing (gloves, lab coat and safety glasses). Consult MSDS for more details (e.g., MSDS for Sigma P6148, available online).

1. Weigh out 4 g of paraformaldehyde (e.g., Sigma P6148) and add to 50 ml distilled water in a glass beaker.
2. Heat to 60°C in the hood on a hot plate (do not exceed 65°C) using a magnetic stir bar. While stirring, add 1 drop of 10N NaOH. This should neutralize the solution.

   NOTE: THE pH IS EXTREMELY IMPORTANT. It is recommended to use a long Pasteur pipet and add one drop, and to check the resulting pH using pH paper. The pH should be neutral (~pH 6-8).
3. Allow the solution to cool.
4. Filter the solution using Whatman paper or a syringe filter (optional).
5. Dilute the solution with an equal volume of 0.2M sodium phosphate, pH 7.4. The final pH should be pH 7–8, adjust if necessary.
6. Aliquot and store at –20°C. Remove from –20°C and bring to room temperature before use. Once thawed, store at 4°C for up to one week.

   NOTE: Commercially available solutions of paraformaldehyde usually contain methanol, which can negatively affect fixation of membrane proteins such as gp64.

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