



# BACULOVIRUS TITERING ASSAY

## Summary:

Accurate titering of recombinant baculovirus is an important step in protein production optimization. An equally important step is screening the various insect cell lines available to determine which is the most beneficial for optimal production. In order to perform the most rigorous determination of cell line and media formulation choice, it is necessary to titer the stock virus in each test condition to assure a direct comparison between conditions. The current “gold standard” plaque assay method of titer determination is prohibitively time and labor intensive; therefore this important step is often omitted. Here we present a high throughput, flow cytometric assay to quantitate infectious baculovirus particles produced in insect cell culture, allowing for accurate virus titer determination for multiple conditions in less than 24 hours. The assay described detects the cell surface expression of gp64 after a short incubation period allowing for a single round of infection. Briefly, insect cells are plated in a 96-well format and 10-fold dilutions of the sample virus stock are used to infect the cells for a period of 18 hours. The gp64 protein is synthesized within 6 hours of infection with accumulation at the membrane surface occurring some hours later. Limiting the incubation to 18 hours maximum prevents an overestimation of viral titer as a consequence of viral spread. Cultures are stained with an antibody specific to baculovirus gp64 followed by a secondary immunostain with a PE-labeled antibody directed against the anti-gp64 antibody. Virus infected cells are then assessed by flow cytometric analysis for gp64. Measurements that fall within the linear portion of the curve generated by the percentage of cells expressing gp64 are used to determine with statistical confidence the titer of the virus stock. The flow cytometric assay was compared to more traditional titering assays such as real-time PCR, end point dilution assay, and the “gold standard” plaque assay and found to be of equal or greater accuracy. The power of this assay lies not only in the user friendly time to titer data and the determination of infectious particles versus viral transcript, but the high throughput format allows for direct comparisons of virus titer specific to target cell as well as media composition.

Note: The protocol below refers to Sf9 cells throughout as the target for baculovirus titering. This protocol is applicable to any insect cell type that is susceptible to infection with AcMNPV.

**Kit Contents:**

PE conjugated anti-gp64  
Control Virus for Standard Curve

**Required items, not included:**

Costar Low Attachment Plate (Cat# 3474)  
Dilution tubes  
Flow cytometer (Guava PCA or Easycyte, BD FACSarray, etc)

**Method:**

1. Plate cells in Costar Low Attachment 96 well plate. Plate 100 ul per well using Sf9 cells at a concentration of  $2 \times 10^6$  cells per ml. The Sf9 cells should be in log phase. For each virus being titered, plate 14 wells as diagrammed below:

	1:10	Stock (1:2)	1:10	1:100	1:1000	1:10000	No Virus	
A								
A								
B								
B								
C								
C								
D								
D								
E								
E								
F								
F								

2. Prepare virus dilutions as follows:

1:10 200 ul stock plus 800 ul media  
1:100 100 ul above plus 900 ul media  
1:1000 100 ul above plus 900 ul media  
1:1000 100 ul above plus 900 ul media

Add 100 ul of the appropriate dilution to each well as diagrammed above. Add 100 ul media to wells containing no virus



3. Incubate overnight at 27° C in orbital shaker incubator with shaking at approximately 170 rpm. Adjust incubator for mixing without splashing.

#### 4. Staining Method

Use gp64-PE antibody at a dilution of 1:200. Dilute antibody in PBS-BSA and use 50 ul per well to stain cells. Prepare irrelevant antibody control at the same dilution.

Spin down plate 3 minutes 1800 rpm. Aspirate off media. Add 50 ul Ab to appropriate wells. Add isotype to yellow wells, add primary antibody to orange wells.

Incubate samples at 4° C for 20 minutes.

Add 150 ul cold PBS to each well. Spin down plate 3 minutes 1800 rpm. Aspirate off media. Add 200 ul cold PBS to each well to wash. Spin down plate and aspirate.

Add 200 ul PBS-BSA to each well. Acquire data with a flow cytometer or Guava cytometer. Refer to instrument applications for setup of acquisition parameters. For data analysis, set irrelevant antibody control as the negative (% positive for PE should be less than 2) and record percent positive for PE-positive cells for each virus dilution.

#### References:

Mulvania T, Hayes B, Hedin D. A Flow Cytometric Assay for Rapid, Accurate Determination of Baculovirus Titers. *BioProcessing Journal* 2004: May/June; 47-53.

Jarvis DL, Garcia A Jr. Biosynthesis and processing of the *Autographa californica* nuclear polyhedrosis virus gp64 protein. *Virology* 1994: 205; 300-13.

Dee KU, Shuler ML. Optimization of an assay for baculovirus titer and design of regimens for the synchronous infection of insect cells. *Biotechnol Prog* 1997: 13: 14-24.

Deparis V, Jestin A, Marc A, Goergen JL. Use of flow cytometry to monitor infection and recombinant human  $\alpha$ -1,3/4 fucosyltransferase production in baculovirus infected Sf9 cell cultures. *Biotechnol Prog* 2003: 19; 624-630.



## Technical Notes:

The culturing conditions of the stock cells used for the assay are very important. The cells have to be in a very healthy log phase of growth in order for the virus to readily infect the cells. Two typical insect cell maintenance schedules are listed below, with the common theme being: don't split them back too far and don't let them overgrow. One maintenance schedule is splitting back to  $5 \times 10^5$  per ml twice a week on a Mon/Thurs or Tues/Fri schedule. The cells never go beyond  $8 \times 10^6$  cells per ml although they certainly are capable of higher densities if you let them go. Never use a culture that is greater than  $6 \times 10^6$ /ml or less than  $2 \times 10^6$ /ml. Typically Expression Systems' seeds a flask at  $10^6$ /ml on Monday for use Wed for titring. Another schedule for passing cells is every two days by splitting back to  $5 \times 10^5$  and not letting them go much higher than  $2 \times 10^6$ . In either case, the cells should not overgrow and should not be spun down and resuspended in a smaller volume to achieve the correct density.

Another point for consideration occurs after the infection incubation and during the staining and washing procedures. Vigorous pipetting of the infected cells in the wells, either to suspend the pellet in the antibody or to suspend the cells in PBS for data acquisition, tends to preferentially lyse the infected, and therefore stained, cells and leads to a drop off in percent positive for the more concentrated virus dilutions. Usually, if the staining is done in the flat bottom plates, the addition of the antibody is sufficient to resuspend the pellet. However a gentle pipetting once or use of a plate mixer at a low speed can help resuspend the pellet if necessary.

### Hints for Guava Users (based on PCA usage):

#### Setting parameters for acquisition

Sf9 cells are larger than mammalian cells so set the FSC gain at X2 or X4. Cells grown in ESF 921 will tend to be a bit larger so try FSC Gain of X4 if using another media. Expression Systems tends to keep the cell population between the  $10^2$  and  $10^3$  area of the FSC axis. The polyploid nature of Sf9 cells can create a double population along the FSC axis. Do not gate to eliminate one population. For the dot plot, FSC is plotted vs. PM1 (PE channel). For the PCA, the voltage ranges between 430 and 490 to get the infected yet unstained (or irrelevant antibody) population so that it is centered in the first decade ( $10^0$  and  $10^1$ ). If this is set too high, it is possible to miss brightly stained events later during analysis. Drag the gate line (the dotted line) to the left to make sure you aren't missing a significant portion of your population but be sure to gate out debris. I also like to increase the voltage of PM2 to equivalent levels, centering the population in the first decade (lower left quadrant) of the PM1 vs PM2 dot plot. Below are some screen shots demonstrating a negative and a positive on the EasyCyte.



# Guava ExpressPlus

## Analysis

Go to Acquisition

Main Menu



Unit Control

Sample Information

Analysis Sample List

- 0001:A01
- 0002:B01
- 0003:C01
- 0004:D01
- 0005:A02**
- 0006:B02
- 0007:C02
- 0008:D02
- 0009:A03
- 0010:B03
- 0011:C03
- 0012:D03
- 0013:A04
- 0014:B04

**Plot 1** Show Histogram  Enable Regions

**Plot 2** Show Histogram  Enable Regions

**Plot 3** Show DotPlot  Allow Overlapping Markers

**Analysis Results Plot 2 - DotPlot: FSC vs PM3**

	Count	Cells/mL	% of Total	x-Mean	y-Mean
Total	5000	1.27e05	100.00%	281.37	4.53
Not Gated	-	-	-	-	-

**Analysis Results Plot 3 - Histogram: PM1**

	Count	Cells/mL	% of Total	x-Mean	% CV
Marker 1	34	8.62e02	0.68%	57.26	135.61%
Marker 2	0	0.00e00	0.00%	0.00	0.00%
Marker 3	0	0.00e00	0.00%	0.00	0.00%
Not Gated	-	-	-	-	-

**Analysis Operations**

Open Data Set

Log Comment

Export to FCS 2.0

View Event Log

Export to Spreadsheet

Apply Current Settings to Selected Samples

Print Preview

Print

Ready to Start

00:00 of 00:00

# Guava ExpressPlus

## Analysis

Go to Acquisition

Main Menu



Unit Control

Sample Information

Analysis Sample List

- 0001:A01
- 0002:B01
- 0003:C01
- 0004:D01
- 0005:A02
- 0006:B02**
- 0007:C02
- 0008:D02
- 0009:A03
- 0010:B03
- 0011:C03
- 0012:D03
- 0013:A04
- 0014:B04

**Plot 1** Show Histogram  Enable Regions

**Plot 2** Show Histogram  Enable Regions

**Plot 3** Show DotPlot  Allow Overlapping Markers

**Analysis Results Plot 2 - DotPlot: FSC vs PM3**

	Count	Cells/mL	% of Total	x-Mean	y-Mean
Total	5000	1.10e05	100.00%	224.48	4.37
Not Gated	-	-	-	-	-

**Analysis Results Plot 3 - Histogram: PM1**

	Count	Cells/mL	% of Total	x-Mean	% CV
Marker 1	756	1.66e04	15.12%	92.29	183.19%
Marker 2	0	0.00e00	0.00%	0.00	0.00%
Marker 3	0	0.00e00	0.00%	0.00	0.00%
Not Gated	-	-	-	-	-

**Analysis Operations**

Open Data Set

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Print Preview

Print

Ready to Start

00:00 of 00:00



## DATA ANALYSIS

This protocol refers to the Excel file available on the Expression Systems website ([www.expressionsystems.com](http://www.expressionsystems.com)). Alternatively, any graphing program can be used to generate an equation using the standard curve.

### Data Analysis Standard Curve Method

1. Insert percent positive for dilution scheme in Columns C and D of worksheet. The worksheet will normalize the data and give you a titer by that method. In the example in the worksheet, we used a positive control known to be  $2 \times 10^9$ . The normalization method determined it to be  $1.16 \times 10^9$ . See Blue box (J15)
2. Column M gives the average percent positive for the data entered in C and D. Enter into column N the expected titer based upon 10-fold dilutions of positive control. Use this data to generate a scatter graph. Plot all dilutions except the stock.
3. To determine standard curve, switch Y axis to log scale (change minimum to 1). Go to chart and add exponential trendline. For options check show equation and show  $R^2$  value. It is desirable to see an  $R^2$  value greater than 90 and preferable to see a value greater than 95.
4. Replace the old equation in the equation boxes with the new equation. Replace the equation in cells G19-G22. The titer highlighted in yellow is the titer of that sample as determined by the standard curve. This is a double check of the data and it should be reasonably close to the expected value.
5. If the two titers determined are close to expected, replace all the equations to the right (column T) to determine your unknowns. The yellow boxes display the titers determined by the standard curve.
6. Refinement of data analysis: Click on the yellow titer box and look at which numbers are included in the average that was used to determine the titer. The highest dilution is usually not used because it overestimates the titer. The exceptions are low titer virus where the stock dilution is within the standard curve parameters. Don't use percent positive data that is less than 2. That is generally near background and will overestimate titers.

### Data Analysis Normalization Method

1. This method will not work for low titer viruses. It depends upon the virus saturating the infectable cells in the culture
2. The blue boxes will give data based on this method. Again, eliminate data that is less than 2% positive.