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1.0 OVERVIEW OF BACULOTITER™ ASSAY TECHNOLOGY

The BaculoTiter™ Assay is a significant improvement over traditional titering methods. The assay utilizes a stable cell line engineered to express the beta-lactamase reporter gene downstream from a proprietary baculovirus responsive promoter. Cells infected with an increasing amount of baculovirus show a dose dependent increase in beta-lactamase activity, which is then quantified upon loading of the ratiometric live cell substrate and measurement using a bottom-read fluorescence microplate reader. The assay rapidly determines the titer of an unknown baculovirus sample relative to a previously titered baculovirus standard. Due to the fewer handling steps and the ratiometric readout, the BaculoTiter™ Assay method minimizes the risk and instances of experimental variability in titer determinations. An easy to use spreadsheet tool is also available to aid in the analysis of the data (available for download at **www.invitrogen.com/baculotiter**).

The baculovirus expression system is a widely used method for expression of recombinant proteins. While baculovirus cloning methods are relatively simple, titers are generally more cumbersome as they require measurement of cell viability/cytopathic effects (CPE), limiting dilution or plaque assays. Each of these methods can require a week or more and significant handling time. Results obtained can vary widely depending on user experience in the subjective assessment of CPE or counting of plaques. Antisera against the Baculovirus major envelope protein, gp64, have also been used to improve the speed of titer estimation, but these methods still suffer from numerous handling steps and the labor-intensive process of counting numerous plaques or wells and are often highly variable.

Beta-lactamases comprise a family of bacterial enzymes that cleave penicillins and cephalosporins. These enzymes have been extensively studied with respect to their three-dimensional structure, mechanism and substrate specificity. A modified version of the commonly studied isoform TEM-1 beta-lactamase from *E. coli* was identified and determined to be an excellent candidate for reporter gene detection in non-bacterial cells. To detect beta-lactamase activity, the FRET-based substrate LiveBLAzer™-FRET B/G (CCF4-AM) is added to live cells, where endogeneous esterases rapidly convert it to its negatively charged form, which is retained in the cytosol, Major advantages of this reporter gene system include its use in live cell experiments, the ratiometric readout, compatibility with flow cytometry, and amenability high-throughput microplate-based assays.

2.0 MATERIALS SUPPLIED

Catalog no. K1271 (30 titers)				
Component	**Shipping Condition**	**Storage Temperature**	**Amount**	**Part#**
LiveBLAzer™ FRET- B/G Loading Kit	Room Temp	**CCF4-AM:** –20°C, dessicate and protect from light **DMSO for Solution A:** 22–25°C, protect from light **Solution B:** 22–25°C, protect from light **Solution C:** 22–25°C, protect from light	200 µg	K1095
Solution D	Dry Ice	–20°C	2 1 ml	K1156

Catalog no. K1272 (150 titers)				
Component	**Shipping Condition**	**Storage Temperature**	**Amount**	**Part#**
LiveBLAzer™ FRET- B/G Loading Kit	Room Temp	**CCF4-AM:** –20°C, dessicate and protect from light **DMSO for Solution A:** 22–25°C, protect from light **Solution B:** 22–25°C, protect from light **Solution C:** 22–25°C, protect from light	1 mg	K1096
Solution D	Dry Ice	–20°C	1 × 10 ml	K1274

Catalog no. K1273 (750 titers)				
Component	**Shipping Condition**	**Storage Temperature**	**Amount**	**Part#**
LiveBLAzer™ FRET- B/G Loading Kit	Room Temp	**CCF4-AM:** –20°C, dessicate and protect from light **DMSO for Solution A:** 22–25°C, protect from light **Solution B:** 22–25°C, protect from light **Solution C:** 22–25°C, protect from light	5 mg	K1030
Solution D	Dry Ice	–20°C	2 × 25 ml	K1157

3.0 MATERIALS REQUIRED, BUT NOT SUPPLIED

Media/Reagents/Other	Recommended Source	Part #
BaculoTiter™ Sf21 cells	Not a product	Not a product
Recovery™ Cell Culture Freezing Medium	Invitrogen	12648-010
Grace's Insect Media, Supplemented	Invitrogen	11605-094
DMSO	Fluka	41647
Sf900II SFM (1x), liquid	Invitrogen	10902-088
Fetal bovine serum (FBS), Certified (United States), Heat Inactivated (HI)	Invitrogen	10082-147
L-glutamine (200 mM, 100 ml)	Invitrogen	25030-081
Viral Standard Note : A virus with a known titer of 1×10^9 PFU/mL or more is recommended	User Supplied	—

Consumables	Recommended Source	Part #
Black-wall, clear-bottom, 96-well assay plates (with low fluorescence background)	Corning Life Sciences	3603
Compressed air	Various	

Equipment	Recommended Source
Fluorescence plate reader with bottom-read capability	Various
Filters, if required for plate reader (see **Section 5.3**)	Chroma Technology Corp.
27°C Incubator	Various

3.1 Optional Equipment and Materials

- Epifluorescence or fluorescence-equipped microscope with appropriate filters
- Spreadsheet tool for data analysis, available from **www.invitrogen.com/baculotiter**

4.0 CELL CULTURE CONDITIONS

4.1 Media Required

Component	Suspension Growth Medium	Adherent Growth Medium	Assay Medium	Freezing Medium
Sf900II SFM (1x), liquid	100%	—	—	—
Grace's Media, Supplemented	—	90%	90%	60%
FBS, Certified (US), HI	—	10%	10%	30%
L-glutamine	5 mM	—	—	—
DMSO	—	—	—	10%
Recovery™ Cell Culture Freezing Medium (optional)*	—	—	—	*100%

Note: All manipulations with BaculoTiter™ Sf21 cells can be performed under Biosafety Level 1 conditions, and may be grown in adherent or suspension formats.

Note: Unless otherwise stated, have all media and solutions at least at room temperature (we recommend 27°C for optimal performance) before adding them to the cells.

***Note:** Recovery™ Cell Culture Freezing Medium (Invitrogen Part# 12648-010) can be substituted for complete growth medium and DMSO.

4.2 Growth Conditions (See Section 7.0 for detailed cell handling procedures)

1. Thaw cells in Adherent Growth Medium and culture them in the appropriate Growth Medium (Adherent or Suspension). Pass or feed cells at least twice a week and maintain them in a 27°C incubator. Maintain cells between 10% and 90% confluency for adherent and 1.2×10^6 and 2.5×10^6 cells/ml (log phase) for suspension cultures . **Do not allow cells to reach confluence.**

   Note: After thawing cells, we recommend passing cells for three times before using them in the BaculoTiter™ Assay. Thawing a new vial of cells every 4 months is recommended.

2. Freeze cells at 1.1×10^7 cells/ml in Freezing Medium.
3. For detailed cell growth and maintenance procedures, see **Section 7.0**.

   Note: BaculoTiter™ Sf21 cells observed microscopically may not be of uniform size (*e.g.* differences in cell and/or nucleus diameter may be apparent). The ratiometric nature of the titer assay eliminates variables such as cell size and density, and thus these differences are not expected to have a deleterious effect on assay performance or titer determination.

5.0 ASSAY PROCEDURE

The following instructions outline the recommended procedure for determining the titer of baculovirus stocks.

5.1 Quick Reference Guide (for more detailed protocol information, see Section 5.2)

	Standard Virus Wells	Unknown Virus Wells	Cell-free wells
Step 1 **Plate cells**	100 µl cells suspended in Assay Medium (50,000 cells/well)	100 µl cells suspended in Assay Medium (50,000 cells/well)	100 µl Assay Medium (no cells)
Step 2 **Incubate cells**	Incubate at 27°C for 1-4 hours		
Step3 **Dilute Viruses**	Prepare standard and unknown virus dilutions in Assay Medium		
Step 4 **Infection**	10 µl Standard Virus Dilutions	10 µl Unknown Virus Dilutions	10 µl Assay Medium
Step 5 **Incubate cells**	Incubate the plate at 27°C for 18 hours.		
Step 6 **Prepare 6X Substrate Mixture**	12 µl 1 mM LiveBLAzer™ -FRET B/G Substrate (CCF4-AM) + 108 µl Solution B, mix; Add 1880 µl Solution C and 120 µl Solution D, mix		
Step 7 **Load Substrate Mixture**	20 µl per well		
Step 8 **Incubate Substrate + cells**	1 hour at room temperature in the dark		
Step 9 **Detect Activity**	See **Section 5.3**		
Step 10 **Analyze data**	See **Section 6.0**		

5.2 Detailed Assay Protocol

5.2.1 Precautions

1. Work on a dust-free, clean surface. Always handle the 96-well, black-wall, clear-bottom assay plate by the sides; do not touch the clear bottom of the assay plate.

5.2.2 Plate Cells

1. Seed the indicated wells of a 96-well assay plate with 50,000 cells in 100 µl volume Assay Medium (See the next page for diagram).
2. Add 100 µl of Assay Medium to each of the two cell-free control wells (See the next page for diagram).
3. Incubate the plates in a 27°C incubator for 1–4 hours.

5.2.3 Prepare Viral Standard Dilutions (supplied by user)*

***Note: A virus standard with a titer over 1×10^9 PFU/mL is recommended**

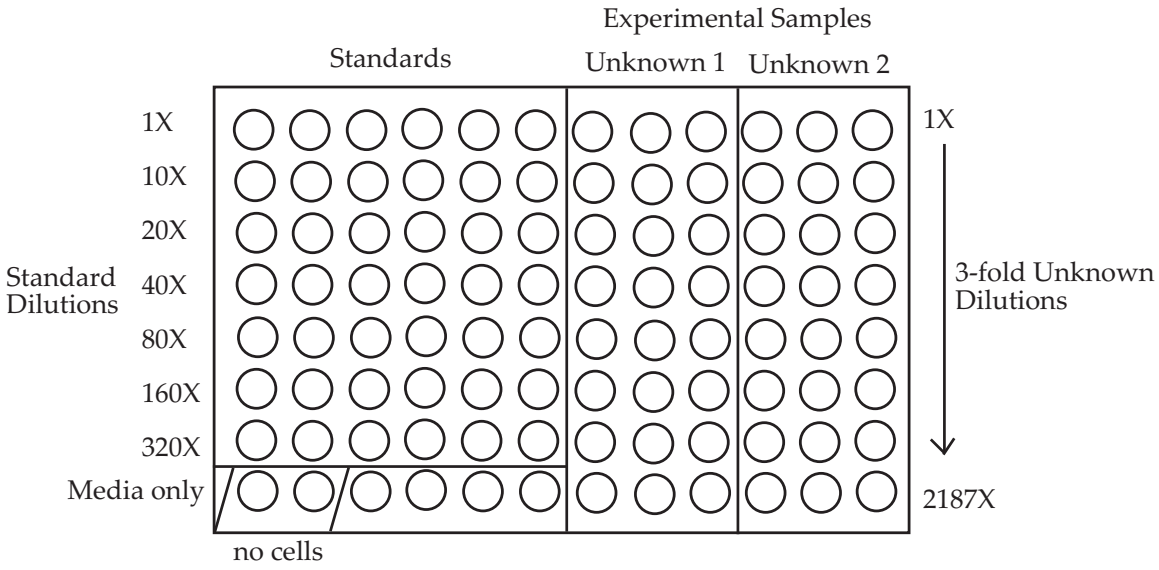
1. Aseptically transfer 25 ml Assay Medium to a 50 ml conical tube.
2. Place eight 1.5 ml polypropylene microfuge tubes in a rack.
3. Aliquot 200 µl of standard virus into first tube.
4. Place 180 µl Assay Medium (from 50 ml conical) into the second tube.
5. Place 100 µl Assay Medium (from 50 ml conical) into the remaining six tubes.
6. Transfer 20 µl of standard virus from the first tube to 180 µl media in the second tube (10X dilution), vortex.
7. Transfer 100 µl of 10x dilution from second tube to third tube (20X dilution), vortex.
8. Repeat for all remaining tubes except the last one, Assay Medium only.

5.2.4 Prepare Viral Unknown Dilutions

1. Place eight 1.5 ml polypropylene microfuge tubes in a rack.
2. Aliquot 240 µl of unknown virus into first tube.
3. Place 160 µl Assay Medium (from 50 ml conical) into the remaining tubes.
4. Transfer 80 µl of unknown virus from the first tube to 160 µl media in the second tube (3X dilution), vortex.
5. Transfer 80 µl of 3x dilution from second tube to third tube (9X dilution), vortex.
6. Repeat this for all remaining tubes.
7. Repeat steps 1-6 using new tubes for other unknown virus samples

5.2.5 Infection and Incubation

1. Add 10 µl of each virus dilution to the wells as shown in the figure below using a repeating pipettor if available. Alternatively, use a manual pipettor. Take care to place the tip into the media of each well to deliver virus evenly. Gently swirl the plate periodically to aid in even distribution.



2. Gently swirl plate one last time, cover plate and place in a 27°C incubator overnight, but for no more than 18 hours.

5.2.6 Substrate Loading and Incubation

Prepare 6X LiveBLAzer™-FRET B/G Substrate (CCF4-AM) Mixture and load cells in the absence of direct strong lighting. Turn off the light in the hood.

1. Prepare Solution A: 1 mM LiveBLAzer™ -FRET B/G Substrate (CCF4-AM, MW = 1096) stock solution in dry DMSO (supplied). Store the aliquots of the stock solution at -20°C until use.
2. Prepare 6X LiveBLAzer™ -FRET B/G (CCF4-AM) Substrate Mixture:

   Add 12 µl of Solution A to 108 µl of Solution B and vortex.

   Add 1880 µl Solution C and 120 µl of Solution D to the combined solutions from above step with vortexing.
3. Remove titer assay plate from the 27°C incubator.
4. Add 20 µl of 6X Substrate Mixture from **Step 2** to each well. Avoid creating bubbles.
5. Cover the plate to protect it from light and evaporation.
6. Incubate at room temperature for 1 hour.

5.3 Detection

All measurements are to be made at room temperature from the bottom of the wells in 96-well, black-wall, clear-bottom assay plates with low fluorescence background. Before reading the plate, remove dust from the bottom with compressed air.

5.3.1 Instrumentation, Filters, and Plates

- Fluorescence plate reader with bottom reading capabilities.
- Recommended filters for fluorescence plate reader:

  Excitation filter: 409/20 nm
  Emission filter: 460/40 nm
  Emission filter: 530/30 nm

5.3.2 Reading an Assay Plate

1. Set the fluorescence plate reader to bottom-read mode, if applicable.
2. Allow the lamp in the fluorescence plate reader to warm up for at least 10 minutes, if applicable, before making measurements.
3. Use the following filter selections:

	Scan 1	**Scan 2**
Purpose:	Measure fluorescence in the blue channel	Measure fluorescence in the green channel
Excitation filter:	409/20 nm	409/20 nm
Emission filter:	460/40 nm	530/30 nm

4. Save/Export the data into the spreadsheet calculator.

6.0 DATA ANALYSIS

6.1 Background Subtraction

We recommend that you subtract the background for both emission channels (460 nm and 530 nm).

1. Use the assay plate layout to identify the location of the Cell-free wells. These control wells are used for background subtraction.
2. Determine the average emission from the Cell-Free wells at both 460 nm (Average Blue Background) and 530 nm (Average Green Background).
3. Subtract the Average Blue Background (data collected at 460 nm) from all of the blue emission data.
4. Subtract the Average Green background (data collected at 530 nm) from all of the green emission data.

6.2 Visual Observation of Intracellular Beta-lactamase Activity Using LiveBLAzer™-FRET B/G Substrate (CCF4-AM)

***Note*:** Microscopic visualization of cells will cause photobleaching. Always read the assay plate in the fluorescence plate reader before performing microscopic visualization.

An inverted microscope equipped for epifluorescence and either a xenon or mercury excitation lamp is typically required to view the LiveBLAzer™-FRET B/G Substrate (CCF4-AM) signal in cells. To visually inspect the cells, you will need a long-pass filter passing blue and green fluorescence light so that your eye can visually identify whether the cells are fluorescing green or blue.

Recommended filter sets for observing beta-lactamase activity are described below and are available from Chroma Technologies (800-824-7662, www.chroma.com).

Chroma Set # 41031

Excitation filter:	HQ405/20x (405 ± 10 nm)
Dichroic mirror:	425 DCXR
Emission filter:	HQ435LP (435 long-pass)

Filter sizes vary for specific microscopes and need to be specified when the filters are ordered. For epifluorescence microscopes, a long-pass dichroic mirror is needed to separate excitation and emission light and should be matched to the excitation filter (to maximally block the excitation light around 405 nm, yet allow good transmission of the emitted light).

6.3 Titer Calculation Using The Spreadsheet Tool

6.3.1 Data Input

1. Once data is obtained from the plate reader and background subtracted, open the spreadsheet tool and go to the **Input** tab/worksheet. Enter or paste the appropriate blue and green emission values obtained from the plate reader into the corresponding blue and green fields as shown below:

	STANDARD						SAMPLE A			SAMPLE B		
BLUE	1	2	3	4	5	6	7	8	9	10	11	12
A	9710	9608	9392	9464	9448	9555	9136	9485	9018	2239	2288	2154
B	8473	8522	8575	8669	8629	8789	8117	8274	7756	2290	2374	2365
C	6824	7543	7980	7731	8021	7996	8400	8449	8354	2212	2449	2582
D	4682	5928	6286	6218	6341	6447	7565	7533	7521	2130	2260	2339
E	3291	4580	4714	4860	4653	4720	4914	4968	4870	2043	2176	2125
F	2228	3224	3570	3503	3540	3648	2755	2704	2775	1908	1922	1825
G	1743	2121	2221	2548	2351	2411	1924	1770	1855	1897	2031	1951
H	0	0	1345	1390	1312	1347	1628	1599	1720	1902	2035	1954
A	2336	2447	2297	2354	2343	2382	2267	2363	2268	4694	4644	4874
B	2574	2305	2345	2275	2332	2327	1973	2009	1898	4381	4444	5264
C	2915	2488	2473	2461	2413	2450	2057	2099	2080	4328	4372	5335
D	3547	3010	2833	2769	2732	2789	2485	2464	2498	4439	4706	5344
E	3770	3205	3151	3082	3142	3175	3007	3008	3014	4516	4711	5107
F	3175	3020	3081	3108	3180	3140	3288	3315	3378	4392	4402	4557
G	3589	3637	3504	3401	3485	3543	3559	3522	3588	4519	4739	4969
H	-3	2	4355	4078	3968	3984	4169	4210	4312	4827	4989	5153
GREEN	1	2	3	4	5	6	7	8	9	10	11	12
	STANDARD						SAMPLE A			SAMPLE B		

2. Scroll down in the **Input** tab/worksheet and enter or paste the appropriate Plaque Forming Unit (PFU) values (using a scientific number format) for your STANDARD Curve dilutions, as represented in the table below:

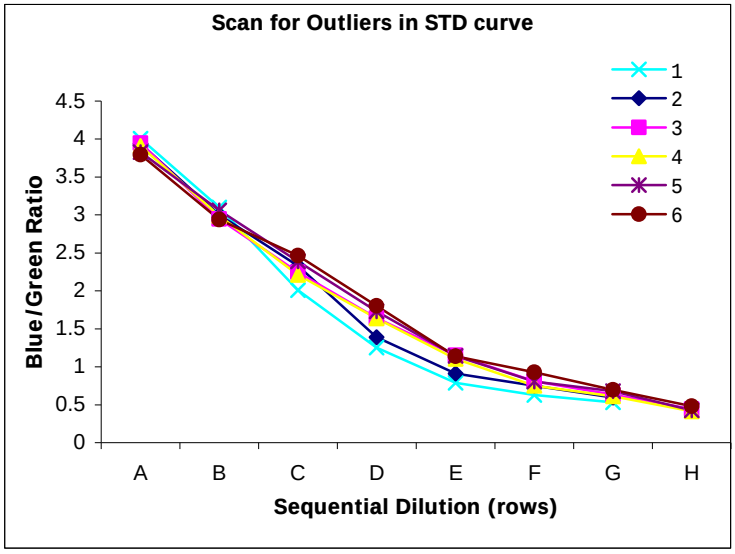
	STANDARD
A	1.00E+09
B	1.00E+08
C	5.00E+07
D	2.50E+07
E	1.25E+07
F	6.25E+06
G	3.13E+06
H	0.00E+00

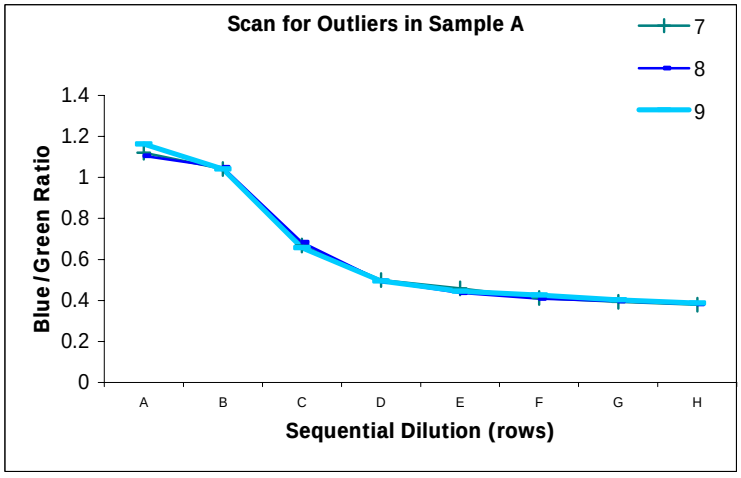
3. Scroll down in the **Input** tab/worksheet and enter or paste the appropriate fold-dilutions of Samples (A & B) from undiluted states, as illustrated in the table below:

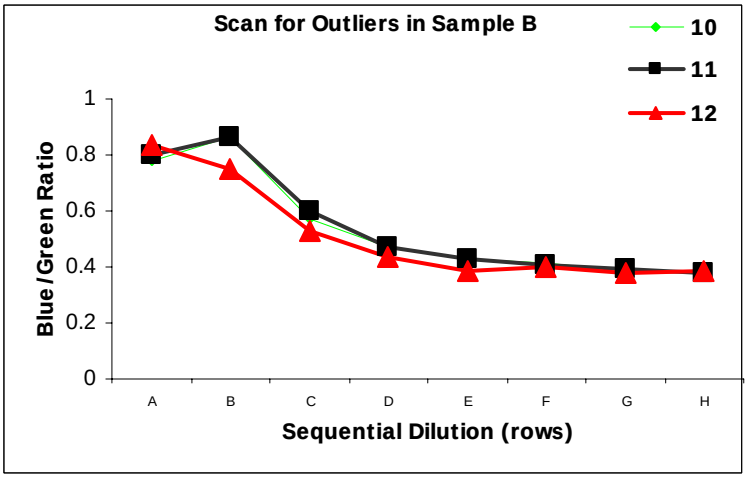
	SAMPLE A
A	1
B	3
C	9
D	27
E	81
F	243
G	729
H	2187

	SAMPLE B
A	1
B	3
C	9
D	27
E	81
F	243
G	729
H	2187

4. Scroll down in the **Input** tab/worksheet and examine the scatter plots (see figure below) for potential outliers in the Standard Curve, Sample A and Sample B data sets. Scatter plots allow quick identification of outliers that can drastically alter your results. Outliers can occur for a variety of reasons, such as dust particles, fingerprints, or pipetting errors. If you find outliers, place the pointer on the data point(s) and the coordinate of the errant point(s) will be displayed. You can eliminate data (described in Step 5) that is clearly aberrant.







5. Scroll down in the **Input** tab/worksheet and locate the average Blue/Green ratio field below the scatter plots (see table below). Highlight the cell(s) containing the errant data point(s) as determined from your examination of the scatter plot in Step 4, and clear the data content from the cell(s). The point will disappear from the scatter plot(s) and will not be included in subsequent titer calculations. Once the data is free of outliers, open the **Output** tab/worksheet.

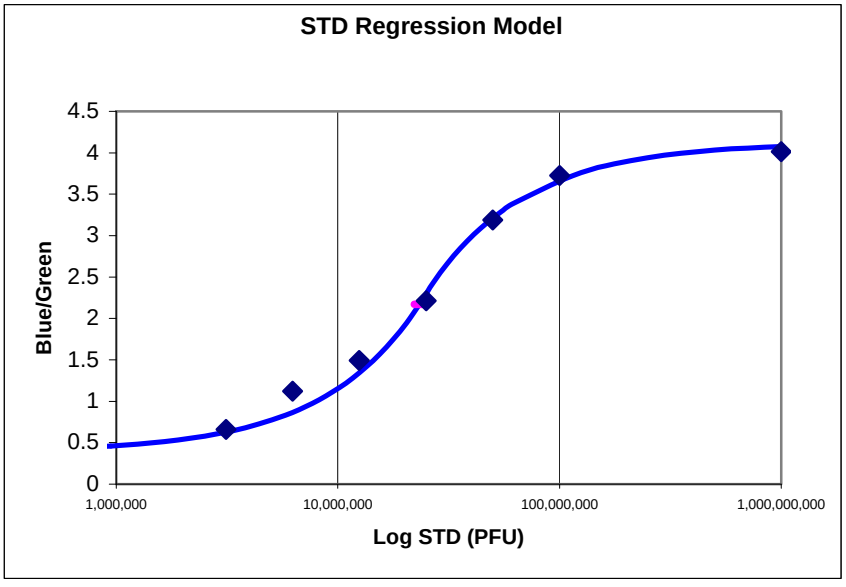
Blue/Green	STANDARD						SAMPLE A			SAMPLE B		
	1	2	3	4	5	6	7	8	9	10	11	12
A	4.01134	3.92644	4.08881	4.02039	4.03244	4.01134	4.03	4.01397	3.97619	0.47699	0.49268	0.44194
B	3.70026	3.69718	3.65672	3.81055	3.70026	3.77697	4.11404	4.11847	4.08641	0.52271	0.5342	0.44928
C	3.14141	3.03175	3.22685	3.14141	3.32408	3.26367	4.08362	4.02525	4.01635	0.51109	0.56016	0.48397
D	2.21885	1.96944	2.21885	2.24558	2.32101	2.31158	3.04427	3.05722	3.01081	0.47984	0.48024	0.43769
E	1.48661	1.42902	1.49603	1.5769	1.4809	1.48661	1.63419	1.6516	1.61579	0.45239	0.4619	0.4161
F	1.11321	1.06755	1.15871	1.12709	1.11321	1.16178	0.8379	0.81569	0.82149	0.43443	0.43662	0.40048
G	0.63385	0.58317	0.63385	0.74919	0.67461	0.6805	0.5406	0.50256	0.517	0.41978	0.42857	0.39263
H			0.30884	0.34085	0.33065	0.3381	0.3905	0.37981	0.39889	0.39403	0.4079	0.3792

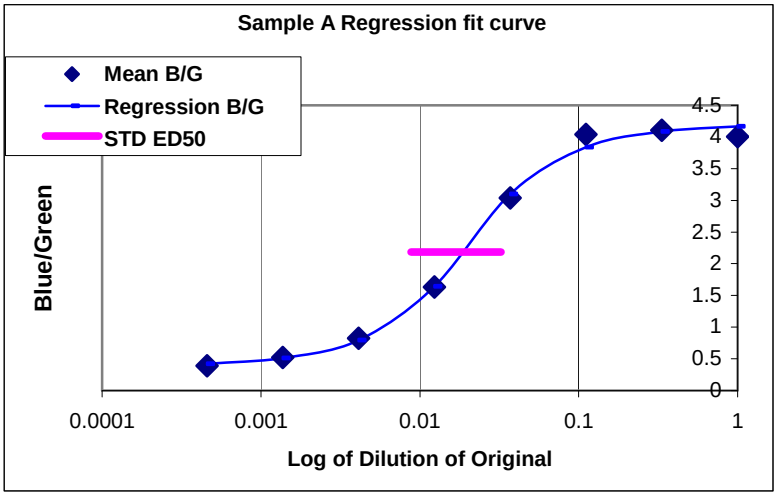
6.3.1 Data Output

1. In the **Output** tab/worksheet, the titers with confidence limits and regression curves for the Standard, Sample A and Sample B (unknowns) are displayed based on the data entered in the **Input** tab/worksheet. The first column in the table for Sample A & B displays the dilution required to obtain the Blue/Green ratio that is ½ maximal (ED_{50}), the 95% Confidence Interval (CI), and Upper and Lower Limits (UL and LL, respectively). The second column for Sample A & B shows the Plaque Forming Units (PFU), the 95% Confidence Interval (CI), and Upper and Lower Limits (UL and LL, respectively). The last row will display whether or not the sample is within the proper range for accurate results, as determined by the ED_{50} value(s) being within the Upper and Lower Limits. Note the **Output** tab below:

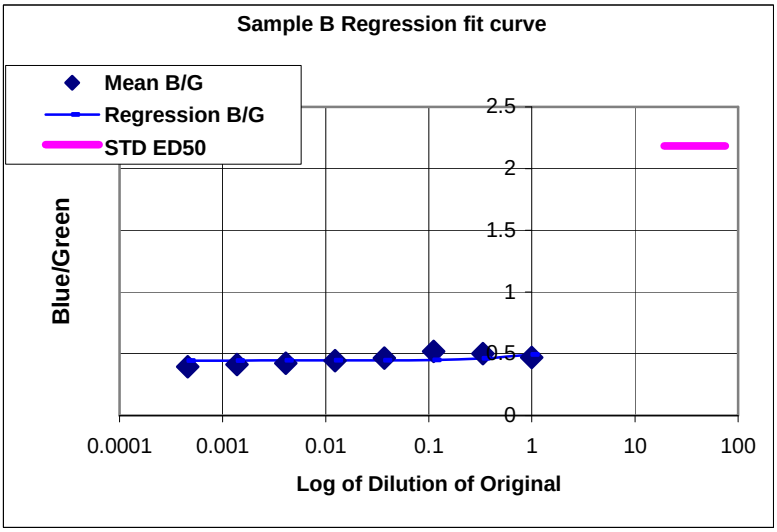
	Dilution/ED_{50}		PFU/Undiluted SAMPLE A		
ED_{50}=	0.020422	dilution	Undiluted SAMPLE A	16185698	PFU
CI=	0.001211	dilution	CI=	6883491.5	PFU
UL=	0.021633	dilution	UL=	23069190	PFU
LL=	0.019211	dilution	LL=	9302206.7	PFU
Range of Sample A was good for accurate ED_{50} measurement.					

	Dilution/ED_{50}		PFU/Undiluted SAMPLE B		
ED_{50}=	5.0424289	dilution	Undiluted SAMPLE B	9134788.3	PFU
CI=	1.5967045	dilution	CI=	3895919.9	PFU
UL=	6.6391335	dilution	UL=	13030708	PFU
LL=	3.4457244	dilution	LL=	5238868.4	PFU
Sample B was NOT within proper range for accurate results.					





Titer of Sample A: 1.195×10^9 pfu/ml



Titer of Sample B: 5.213×10^5 pfu/ml

7.0 DETAILED CELL HANDLING PROCEDURES

7.1 Thawing Method

1. Place 8 ml of pre-warmed Adherent Growth Medium into a T25 flask.
2. Remove the vial of cells to be thawed from liquid nitrogen and thaw rapidly by placing at 27–37°C in a water bath with gentle agitation for 1-2 minutes. Do not submerge vial in water.
3. Decontaminate the vial by wiping with 70% ethanol before opening in a Class II biological safety cabinet.
4. Transfer the vial contents drop-wise into the T25 flask.
5. Allow cells to attach for 1 hour in a 27°C incubator.
6. Remove media and add 8 ml fresh Adherent Growth Medium.
7. Return cells to 27°C incubator.

Note: BaculoTiter™ Sf21 cells observed microscopically may not be of uniform size (*e.g.* differences in cell and/or nucleus diameter may be apparent). The ratiometric nature of the titer assay eliminates variables such as cell size and density, and thus these differences are not expected to have a deleterious effect on assay performance or titer determination.

7.2 Propagation Method

7.2.1 Adherent Cell Culture

1. Passage cells at least twice a week. Cells should be maintained between 10% and 90% confluence.
2. To passage cells, gently slough cells from the T25 flask and add 1 to 2 ml cells to 6 ml Adherent Growth Medium.

7.2.1 Suspension Cell Culture

1. Passage cells at least twice a week. Maintain cells between 1.2–3.0 × 10^6 cells/ml.
2. To passage cells, calculate the cell density of working vessel and seed the new vessel at the desired density in Suspension Growth Medium.

7.3 Freezing Method

1. Calculate the total number of cells, then spin cells down and resuspend in 4°C Recovery™ Cell Culture Freezing Medium or complete medium with DMSO to ~1.0 × 10^7 cells/ml.
2. Dispense 1.0-ml aliquots of cells into cryogenic vials.
3. Place cells in an insulated container for slow cooling and store overnight at –80°C.
4. Transfer cells on the next day to liquid nitrogen for storage.

8.0 PURCHASER NOTIFICATION

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