



FastPlax™ Titer Kit

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Cell plating

1. Prepare an exponentially growing culture of Sf9 or TriEx™ Sf9 Cells (see User Protocols TB329 or TB314, respectively).
2. Seed each well of the 6-well plate with 2 ml of cells and medium, comprising a total of 1×10^6 cells per well. Label three pairs of wells “-5,” “-6,” and “-7”, respectively. After pipetting cells, rock plates gently (side-to-side and back-and-forth) to ensure an even monolayer. Do not swirl the plates. Doing so will cause cells to cluster in the center of the well. Allow cells to attach (about 30 min at 28°C).

Virus dilution

While the cells are attaching in the wells, prepare the virus dilutions. For optimal accuracy, avoid pipetting volumes of less than 10 µl. Use sterile 1.5 ml microcentrifuge tubes, preferably with screw caps.

1. For each virus stock to be titered, place 5 sterile 1.5 ml microcentrifuge tubes in a rack. Label them “-2”, “-4”, “-5”, “-6”, and “-7”.
2. Add 990 µl sterile medium to the “-2” and “-4” tubes. Add 900 µl sterile medium to the “-5”, “-6”, and “-7” tubes. Begin the dilution series by adding 10 µl virus stock to the tube labeled “-2”; this is the 10^{-2} dilution. Accurate measurement is essential. Be mindful of droplets of virus stock adhering to the outside of the pipet tip. Rinse the inside of the tip several times by pipetting the dilution up and down.
3. Close the cap of the “-2” tube. Invert or vortex gently to mix.
4. Add 10 µl of the “-2” dilution to the tube labeled “-4”. Cap and mix as above.
5. Add 100 µl of the “-4” dilution to the “-5” tube; cap and mix.
6. Add 100 µl of the “-5” dilution to the “-6” tube; cap and mix.
7. Add 100 µl of the “-6” dilution to the “-7” tube; cap and mix.

Infection

1. Aspirate medium from the wells. Slowly tip plate at a $\sim 10^\circ$ angle, and carefully aspirate from edge of each well. Avoid disturbing the monolayer.
2. Slowly add 200 µl of each virus dilution to each of 2 duplicate wells. Add dilution drop by drop to center of each well. Start with the “-7” dilution, then “-6”, then “-5”. The same pipet tip may be used for all dilutions if this order is followed.
3. Incubate plate at room temperature (22°C) for 60 min. During this incubation, rock plate every 10 min to keep cell monolayer wet. Tip plate to one side and allow medium to pool at the lower corner. Then, quickly rock plate in the opposite direction to allow medium to flow across the cell monolayer. Do not allow monolayer to dry out. If necessary, use a humid chamber.
4. At the end of the incubation, carefully add 2 ml medium to each well. Incubate plate at 28°C for 24–30 h in a sealed humid chamber. Include moist paper towels in the container to prevent plate from drying out.

Detection

Subsequent steps may be performed at room temperature on an open bench. A tilting platform or slow orbital shaker may be used during blocking, antibody incubations, and washes.

1. Remove medium 20–30 h post-infection. Dispose of medium as biological waste, since it contains infectious baculovirus.
2. Wash cells twice with 2 ml/well PBS.
3. Fix cells with 2 ml/well 3.7% formaldehyde solution (37% formaldehyde solution diluted 1:10 in PBS). Incubate for 10–15 min.
4. Remove formaldehyde solution. Wash twice with 2 ml/well PBS.
5. Block by adding 2 ml/well 1% Gelatin in TBST (10% Gelatin diluted 1:10 in 1X TBST; melt 10% Gelatin by placing bottle in a beaker filled with hot tap water for a few minutes). Incubate for 30 min with gentle rocking.

Perform all cell and virus manipulations in a laminar flow hood. Employ aseptic technique.

6. Remove Gelatin solution. Wash cells twice with 2 ml/well PBS. Add 1 ml/well of FastPlax™ Antibody diluted 1:10,000 in 1X TBST. Incubate for 60 min with gentle rocking.
7. Wash 3 times with 2 ml/well 1X TBST. Incubate each wash for 10 min.
8. Add 1 ml/well Goat Anti-Mouse β -gal Conjugate diluted 1:100 in 1X TBST. Incubate for 60 min with gentle rocking or agitation.
9. Wash 3 times with 2 ml/well 1X TBST. Incubate each wash for 10 min.
10. During the last wash, mix developing solution by adding 60 μ l X-Gal and 60 μ l NBT to 15 ml PBS plus 5 mM MgCl₂.
11. Remove the last wash. Add 2 ml/well developing solution. Incubate at 37°C for 15–60 min. Check plate every 10 min under a microscope to avoid overdeveloping. Infected cells will appear medium blue to dark purple (color completely filling the dimensions of the cell), or as dark purple foci of staining.
12. Wash twice with 2 ml/well 1X TBST to stop color development.

Note: *The color may continue to develop and darken with time, even after washing. Therefore, stained cells must be counted immediately after washing. Uninfected cells can appear lightly stained if over-developed.*

13. Count the infected cells and foci immediately, using a dissecting microscope (invert the plate to allow foci to be marked) or a low power objective on an inverted microscope. Single cells that are strongly stained should be counted, as well as small clusters of stained cells. The most accurate counts will come from wells with 10–100 infected cells and/or foci. If infected cells are too dense, the overlapping stained foci will be difficult to count.

Calculating the titer

Calculate the titer by the following formula: pfu/ml = 5 \times (# infected cells and foci) \times (dilution factor). Use the average of 6 wells to determine the final titer.

In the following example, a 6-well dish may have the following counts:

Dilution:		10⁻⁵	10⁻⁶	10⁻⁷		
Infected cells/foci per well:		175	18	2		
		190	21	1		
5	\times	175	\times	10 ⁵	=	8.75 \times 10 ⁷
5	\times	190	\times	10 ⁵	=	9.50 \times 10 ⁷
5	\times	18	\times	10 ⁶	=	9.00 \times 10 ⁷
5	\times	21	\times	10 ⁶	=	1.05 \times 10 ⁸
5	\times	2	\times	10 ⁷	=	1.00 \times 10 ⁸
5	\times	1	\times	10 ⁷	=	5.00 \times 10 ⁷
Final titer (average)				=	8.63 \times 10 ⁷ pfu/ml	

Troubleshooting Guide

Problem	Possible Cause	Solutions
No infected cells detected	Cell failure	Check cell viability before plating. Viability should be > 85%. Plate excess cells in a T-25 flask with 5 ml medium and monitor growth.
	Infection failure	Virus stock titer may be very low. Repeat the assay, infecting one well with the 10 ⁻² dilution and one well with the 10 ⁻⁴ dilution. Confirm that the virus dilution has spread evenly across monolayer.
	Antibody binding failure	FastPlax™ antibody will not bind to native gp64. Cells must be fixed. Try using fresh 37% formaldehyde solution.
	Staining failure	No staining will occur if X-Gal is omitted from the staining solution. Failure to include MgCl ₂ in the staining solution will greatly decrease activity of β-galactosidase, resulting in poor staining. Omitting NBT will result in weak, diffuse staining.
Monolayer is damaged in center of well	Monolayer has dried out during infection	Tip plate frequently during the infection (e.g., every 10 min). Keep plate in a humid chamber. Ensure that virus dilution is spreading across center of well. If necessary, hold plate at an angle for a longer period to allow virus dilution to pool in the lower corner of the wells.
Low titer	Infected cells were lost from monolayer	Aspirate medium and solutions gently by tipping plate and aspirating from lower corner of the well. Add solutions slowly into lower corner of the well.
	Recombinant virus has altered amplification rate	A recombinant virus may exhibit altered amplification kinetics, delaying expression of detectable quantities of gp64. Incubate the infected cells 6–28 h longer before fixing (i.e., fix at 32–48 h post-infection).
	Single infected cells were not counted	It is important to count all strongly stained cells, even if no staining is detectable in neighboring cells. The FastPlax method relies on the early detection of infection, before virus has spread.
	Error in dilution series	Repeat the assay, taking extra care while setting up virus dilution series. Be sure each dilution is well-mixed before removing an aliquot for the next dilution.
High staining background in monolayer	Incomplete blocking	Rinse wells twice with TBST after fixation to remove excess formaldehyde before adding blocking solution. Extend blocking incubation to 1 h.
	Incomplete washing	Wells must be washed thoroughly after each antibody incubation. Washes can be extended to 15–20 min each.
	Excessive length of staining incubation	Monitor staining reaction more frequently. Observe plate every 5 min using a dissecting microscope.

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