Rapid Titration of Viruses by Flow Cytometry

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ABSTRACT

Traditionally, the most common methods used to titrate virus stocks are the plaque assay and the hemagglutination assay. The protocol presented here is based on the detection of viral-expressed proteins in infected cells by flow cytometry. It is simpler and more rapid than the traditional plaque-forming assay and it enables high-throughput analyses. *Curr. Protoc. Cell Biol.* 51:26.11.1-26.11.7. © 2011 by John Wiley & Sons, Inc.

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BASIC PROTOCOL

There are a myriad of methods to determine virus titer. The most common traditional methods are perhaps the plaque assay and hemagglutination assay. The protocol presented here is based on detection of viral expressed proteins in the infected cells by flow cytometry. It is simpler and more rapid than the traditional plaque-forming assay and it enables high-throughput analyses. Titration of different viruses by flow cytometry has been reported to correlate with their respective traditional methods of titration (Gueret et al., 2002; Lonsdale et al., 2003; Drayman et al., 2010; Li et al., 2010). Monitoring expression of an early protein, following infection at several dilutions of the virus stock, measures the titer of infectious units with high confidence.

This protocol describes the preparation and infection of cells with the SV40 virus for analysis by flow cytometry. Some of the steps (such as the method of infection) are left to the researcher's preferences. While the protocol presented here was calibrated for SV40, it can be easily adopted for titration of other viruses.

Materials

Cells (e.g., CV1 cells for SV40 titration)
Cell culture medium
Viral stock (e.g., SV40)
Serum-free medium (SFM; cell culture medium without serum)
Phosphate-buffered saline (PBS; Biological Industries, cat. no. 02-023-1)
0.05% EDTA in PBS
Trypsin solution, to detach cells from the plate (Biological Industries, cat. no. 03-050-1)
100% Methanol (keep at −20°C)
Primary antibody in blocking solution (for SV40 titration: anti-T antigen; Pab 101, Santa Cruz)
Blocking solution: 5% bovine serum albumin (BSA) in PBS
Fluoropohore-conjugated secondary antibody

6-well plates
37°C incubator
Hemacytometer
1.5-ml microcentrifuge tubes
Vortex
15-ml conical centrifuge tubes

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Centrifuge
53-μm filter
FACS tubes

Plate cells for infection

1. Plate the cells in 6-well plates in 2 ml final volume, at a concentration that will yield confluent monolayers the following day. One plate will serve for a control (uninfected) and the others for viral infection, at two fold serial dilutions. Mark the plates accordingly. Incubate the plates overnight at 37°C.

   For SV40 titration, seed ~6 × 10^5 CV1 cells a day before.

Count the cells at time of infection

2. Count the number of cells harvested from three wells from the control plate using a hemacytometer. The average of the three counts is the number of cells at the time of infection. Note this number as it will serve later to determine the viral titer.

Infect the cells with the virus

3. Make serial 2-fold dilutions of the viral stock in serum-free medium (SFM) in 1.5-ml microcentrifuge tubes. Start by adding 500 μl SFM to each tube. Mix the tubes well (vortex) at each dilution step. Record the volume of the original virus stock used for infection at each dilution, as seen in Figure 26.11.1. This volume will be used later to calculate the titer of the virus stock.

   The starting dilution will depend on your experience and the presumed titer of the viral stock. See the example in Figure 26.11.1. For accurate titer determination, it is recommended to perform the serial dilutions and infection in duplicates. After gaining experience, you may find that 3-fold serial dilutions are sufficient for accurate titer determination.

4. Wash the cells in the infection plate twice with ~2 ml PBS.

5. Infect the cells according to your standard protocols.

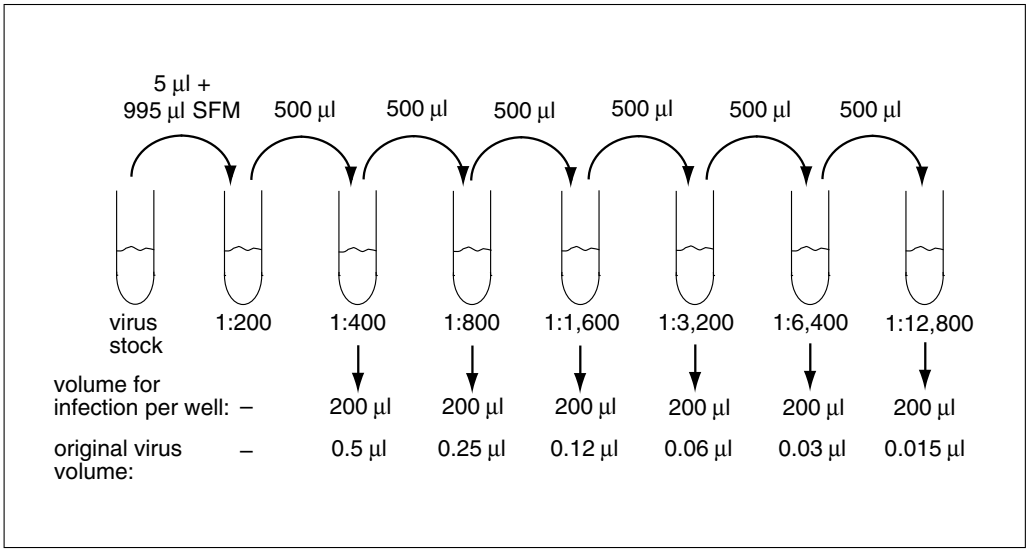

Figure 26.11.1 Dilution curves for titration. The example depicts 2-fold serial dilutions recommended for high-titer (10^8–10^{10} infectious units/ml) virus stocks. At the start, each microcentrifuge tube contains 500 μl of SFM. Serial dilutions are performed by transferring 500 μl each time. The bottom of the figure shows the final dilution in each tube and the volume of original virus stock used for infection.

6. Incubate the plates at 37°C until ready for harvest (see below).

   For SV40, the optimal time point is 24 to 48 hr post infection.

Harvest the cells and fix for FACS

This protocol is calibrated for a final cell density of 1–2 × 10^6 cells per well. Harvesting more cells may lead to inefficient staining and subsequent inaccurate titer determination. It is therefore recommended to count the cells at the time of harvest and take 1–2 × 10^6 cells for subsequent steps.

7. In advance, prepare 15-ml conical centrifuge tubes with 5 ml cold PBS and place on ice. Each tube will be used for harvesting cells from one well. Mark the tubes accordingly.

   The PBS and centrifuge should be precooled to 4°C and 100% methanol should be precooled to −20°C for the following procedures. Prepare the solutions and cool the apparatus in advance.

8. Transfer the medium from each well into its corresponding tube. Start from the three remaining uninfected wells from the control plate and proceed with the cells infected at serial dilutions.

   Start by harvesting and fixing cells from the control plate to avoid contamination with infected cells.

9. Harvest the cells according to your preference. The following steps (10 and 11) may be omitted if your cells detach easily.

   The example here is for CV1 cells infected with SV40.

10. Add 2 ml 0.05% EDTA in PBS to each well. Incubate at room temperature as necessary and add the EDTA to the corresponding tube. Keep the tubes on ice.

11. Add 0.5 ml trypsin solution to each well, incubate at room temperature with gentle mixing until detached. Suspend cells thoroughly by pipetting up and down several times before collecting into the corresponding tube. Keep the tubes on ice.

12. Mix the tubes by inverting them several times or by vortexing (depending on your cells).

    A quick burst of 2 to 3 sec of vortexing should suffice.

13. Pellet the cells by centrifuging for 5 min at 1500 × *g*, 4°C. Discard the supernatant.

14. At this point, you should have a cell pellet in a residual solution volume of ~50 to 100 µl. Resuspend the cells in the residual solution by gently tapping at the bottom of the tube.

15. Carefully pipet 5 ml of 100% methanol solution (precooled to −20°C). To minimize cell clumping add the first 1 ml of methanol dropwise while vortexing. Add the remaining 4 ml slowly while vortexing.

16. Incubate the cells at −20°C for at least 1 hr.

    This step will fix and permeabilize the cells.

    At this stage, the cells may be kept at −20°C for up to several weeks before the FACS analysis.

Perform marker staining for flow cytometry

17. Remove the cells from −20°C. Perform all subsequent steps at room temperature.

18. Wash the cells once as follows:

a. Add 5 ml PBS to each tube, mix, and pellet by centrifuging 5 min at 1,500 × *g*, 4°C. Carefully decant the supernatant.

b. Resuspend the cells in the residual PBS by gently tapping at the bottom of the tube.

19. Add 5 ml PBS to each tube and allow cells to rehydrate by incubating at room temperature for 30 min.

20. Centrifuge and decant the supernatant as described in step 18a. From this point on, do not vortex, as the cells may stick to the side of the tube and they will not be stained.

21. Add 100 µl primary antibody solution.

    For SV40, use 100 µl of PBS containing 5% BSA and 2 µg anti-T antigen monoclonal antibody (Pab 101, Santa Cruz or equivalent).

22. Incubate the cells for 1 hr at room temperature. It is important to resuspend the cells every 15 min by gently tapping at the bottom of the tube, to ensure maximal exposure of the cells to the staining solution. Otherwise, the cells may precipitate and will not stain evenly.

23. Wash the cells with PBS as described above (step 18).

24. Add 100 µl fluorophore-conjugated secondary antibody solution to the cell pellet.

25. Incubate the cells in the dark for 1 hr at room temperature. Resuspend the cells by gently tapping every 15 min.

26. Wash the cells with PBS as described above (step 18).

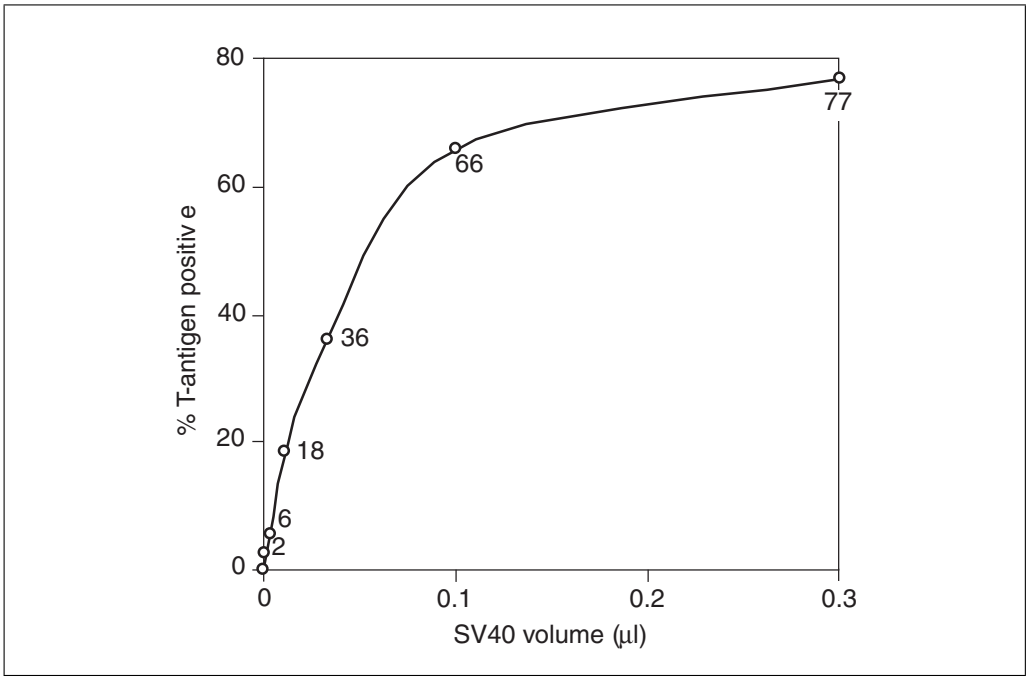

Figure 26.11.2 Titration curve. SV40 stock (diluted in 3-fold serial dilution) was tittered on CV1 cells. The *x* axis shows the volume of original SV40 stock used to infect each well and the *y* axis denotes the percentage of infected cells (measured as T-antigen positive cells). Note that the graph is linear at the three lower volumes (2%, 6%, and 18% infected cells, as expected for 3-fold dilution steps) but starts leveling off at higher volumes. Titer calculation should be made only from the linear part of the titration curve.

27. Add 500 µl PBS to the cell pellet.

28. Thoroughly resuspend the cells by pipetting up and down several times and filter through a 53-µm filter into FACS tubes.

Perform flow-cytometry analysis

29. Count at least 10,000 cells from each sample. Use the three control samples (uninfected) to determine background staining level and for gating by standard flow-cytometry procedures.

30. Determine and note the percentage of antigen-positive cells in each serial dilution of the viral stock.

    Here, the general term "antigen" denotes the viral early protein being monitored.

Determine the viral titer

31. Plot a graph as in Figure 26.11.2, with the *x* axis representing the volume of original viral stock used for each infection and the *y* axis the percentage of antigen-positive cells.

32. Determine which data points are in the linear range of the graph. Data points below 30% infectivity are expected to be within the linear range.

33. Calculate the fraction of infected cells for each serial dilution by dividing the percentage of antigen positive cells by 100.

34. Determine the virus titer as infecting units per ml based on: (a) the number of cells at the time of infection (step 2), (b) the volume of the original viral stock used for infection (step 3), and (c) the fraction of antigen-positive cells (step 33), according to the following formula:

$$\frac{[\text{number of cells at time of infection}] \times [\text{fraction of infected cells}]}{[\text{ml infecting virus}]}$$

Data points in the linear range should give similar titers. The average of these titers, preferably based on at least three data points, is the titer of your virus stock.

COMMENTARY

Background Information

Accurate titration of viral stocks is paramount for reliable research in all fields of virology. Titers of many viruses are traditionally determined by the hemagglutination assay (HA) or the plaque assay (pfu). The hemagglutination assay measures the concentration of particles, including defective (non-infectious) particles, which may constitute a significant part of the virus stock. Since virus stocks contain defective particles, it is important that titration will measure infective particles.

The plaque assay measures infective particles. However, plaques appear after several rounds of infection, taking between a few days and a few weeks, depending on the virus and cell line used for titration. Furthermore, plaques are counted manually, thereby limiting the throughput of this assay.

Rapid titration methods based on measurements of viral total proteins or genome levels have been reported (see for example, Thomas et al., 2007; Nair et al., 2008). However, these suffer from the same problems as the hemagglutination assay. The protocol presented here offers a rapid and high-throughput titration of infective particles.

Critical Parameters

This protocol was calibrated for titration of SV40 on adherent cells using the viral T antigen as a marker for infection, 24 to 48 hr post infection. Since viruses have different life cycles, the following parameters will need to be calibrated.

An appropriate marker for infection: An appropriate marker is (1) a viral protein that is expressed early and is stable, (2) can be detected by flow cytometry, meaning suitable antibodies are available, and (3) is absent from the mature virion—allowing distinction between productive and non-productive entry of

Table 26.11.1 Troubleshooting Guide for Rapid Titration of Viruses by Flow Cytometry

Problem	Possible causes	Possible solutions
No staining of infected cells	1. Inefficient infection.	1. Verify infection by another method and repeat titration.
	2. Virus stock was diluted too much.	2. Repeat titration at lower dilutions.
	3. Antibody does not work in flow cytometry.	3. Replace the antibody.
	4. Methanol fixation is not compatible with the protein being stained.	4. For staining of small proteins, consider using a different fixation, such as 4% formaldehyde, following permeabilization with 0.5% Triton X-100. Remember to add Triton to all antibodies buffers!
High background staining of uninfected cells	1. The concentration of fluorophore-conjugated secondary antibody is too high.	1. Calibrate secondary antibody concentration. Alternatively, you can use a fluorophore-conjugated primary antibody, omitting the secondary antibody from the procedure.
	2. Primary antibody reacts nonspecifically with cellular proteins.	2. This is often the case with polyclonal antibodies. Consider switching to a monoclonal antibody compatible with flow cytometry.
Different viral stock dilutions give the same level of infected cells	Inefficient staining caused by too many cells.	Count the cells at the time of harvest and calibrate the number of cells that give a linear response in staining for the FACS analysis.
Not enough data points are in the linear range of infection.	Dilution points are too far apart.	Add additional dilution points in the linear range.
Titers calculated from different points in the linear range differ greatly.	1. The number of cells that was seeded in each well was not the same (step 1).	1. Repeat the titration experiments, ensuring that equal number of cells are seeded in all wells.
	2. Improper dilution of the virus stock (step 3).	2. Repeat the titration experiment. Mix well at every dilution step.
Titers calculated from duplicate serial dilutions differ greatly	Improper dilution of the virus stock (step 3).	Repeat titration experiment. Mix well at every dilution step.

viruses. Note that viral vectors expressing a fluorescent marker, such as GFP, can be readily tittered by this protocol.

Time point for measuring infection: Determine the optimal time point for detection of the selected marker by following its expression. This will vary between viruses and cell lines.

Calibration of conditions for FACS analysis: The number of cells per FACS assay, as well as concentrations of the primary and secondary antibodies, need to be calibrated. The optimal staining conditions are those that separate infected and non-infected cell unambiguously. Usually, staining protocols for FACS start with 1–2 $\times$ 10^6 cells. We recommend using this number of cells by performing the titration in 6-well plates, as they are easy to work with. If however you wish, or need to, you can calibrate the protocol for 12- or 24-well plates, and a lower number of cells. To calibrate the concentration of antibodies, we recommend comparing mock and highly infected (MOI=10 or greater) cells with varying concentration of antibodies. After calibration, the conditions should be rigorously adhered to. Changing these conditions could lead to high background staining of uninfected cells or inefficient staining of infected ones.

Troubleshooting

Please see Table 26.11.1 for problems that may be encountered using this procedure.

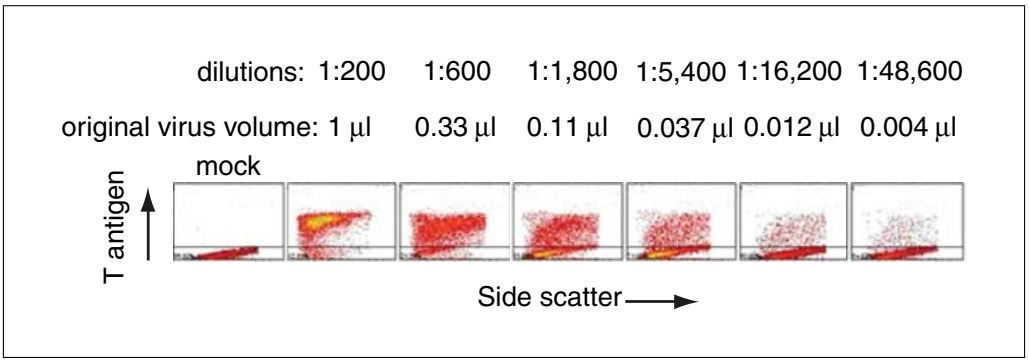

Figure 26.11.3 Typical results from SV40 titration experiments. SV40 stock was diluted 1:200 and then by 3-fold serial dilution steps. CV1 cells, grown to confluence, were infected with the serial dilutions of the viral stock and incubated for 48 hr at 37°C. Cells were harvested, fixed, and stained for SV40 T antigen according to the protocol presented here, and the percentage of infected cells was quantified by flow cytometry, with gating based on mock-infected cells. The number at the upper-right corner of each box denotes the percentage of T-antigen positive cells. Note that only the highest dilutions (1:5,400, 1:16,200, 1:48,600) are in the linear range of the infection. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cb2611*.

Anticipated Results

The outcome of this procedure is the titer of the viral stock. It is important that the titer be calculated from data in the linear range of infection. Therefore, at least three data points in the titration curve should be within the linear range. A typical result from titration of SV40 is shown in Figure 26.11.3.

Naturally, the titers calculated based on points in the linear range should be roughly the same. A big variability between them suggests a problem in either cell seeding or virus serial dilutions (see Table 26.11.1). Note that titer cannot be calculated if the percentage of infected cells is very low (<1% to 2%). The titer in this range is not reliable because of statistical errors.

Although we have shown that titration by flow cytometry yields the same results as the conventional methods of titration (Drayman et al., 2010), we encourage first time users to compare the results obtained using this protocol to the results of another protocol of their choice.

Time Considerations

This procedure can be accomplished in 2 to 3 days, not including culturing cells for infection overnight. Following infection, cells are incubated for 1 to 3 days before harvesting and fixation in methanol. Cells can be kept at this point for several weeks. FACS staining and analysis can be finished in a few hours.

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