Chapter 3

Use of a GFP-PML-Expressing Cell Line as a Biosensor for Human Cytomegalovirus Infection

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Summary

Human cytomegalovirus (HCMV) infection has a marked effect on promyelocytic leukemia (PML) bodies. Here, we describe a novel real-time monitoring system for HCMV-infected cells *in vitro* using a newly established cell line that stably expresses GFP-PML protein. Upon infection, HCMV causes specific dispersion of GFP-PML bodies, thereby allowing the infected cells to be monitored by fluorescence microscopy without immunostaining. Quantitative protocols using either an NPB fluorescence assay or a GFP-PML imaging assay are also described. The NPB fluorescence assay is rapid, sensitive, and sufficiently simple for screening of inhibitory reagents, while the GFP-PML imaging assay is highly sensitive and applicable to drug susceptibility testing of low-titer clinical isolates.

Key words: PML body, GFP-PML, SE/15 cell, HCMV, Clinical isolate, Drug susceptibility testing.

1. Introduction

Promyelocytic leukemia (PML) bodies, also known as ND10 (for "nuclear domain 10"), PODs (for "PML oncogenic domains"), and Kr bodies, are present in the nuclei of most mammalian cells as 10–20 spherical structures ranging in size from 0.3 to 0.5 μm *(1)*. They are composed of multiprotein complexes, including the PML, Sp100, NDP55, and Daxx proteins *(2)*. Infection with DNA viruses, such as herpes simplex virus type 1 (HSV-1), human cytomegalovirus (HCMV), and adenoviruses, has a marked effect on PML bodies. HCMV deposits its genome adjacent to PML bodies and starts transcription of immediate early (IE) gene, thereby causing dispersion of the PML bodies in the

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nucleus through de-SUMOylation of the PML protein *(3)*. We exploited a unique event observed in HCMV-infected cells, that is, the dispersion of PML bodies upon IE1 expression. For this purpose, we established a new cell line (SE/15) that stably expresses green fluorescent protein (GFP)-PML protein *(4)*. SE/15 cells have functional GFP-PML bodies, whose morphology is specifically changed to a diffuse GFP pattern throughout the nucleus upon HCMV infection (**Fig. 1**). Thus, visual assessment by fluorescence microscopy is sufficient for sensitive detection of infected cells without the necessity for any further procedures, such as fixation and staining. Subsequently, we developed a new quantitative system for HCMV titration using this cell line, which is applicable to the screening of antiviral drugs. In addition, we established a simple, sensitive, and quantitative protocol for drug susceptibility testing of low-titer clinical isolates, most of which are cell associated.

2. Materials

2.1. Cultivation of SE/15 Cells (see Note 1*)*

1. SE/15 cells are available from Nippi Inc. (*see* **Notes 2** and **3**).
2. G418 (GIBCO/BRL) stock solution.
3. Zeocin (Invitrogen): 100 mg/mL stock solution, stored at –20 °C, light sensitive.
4. Growth medium for SE/15 cells: Dulbecco's modified Eagle's medium (DMEM, GIBCO/BRL) containing 10% fetal bovine serum (FBS), 100 μg/mL G418, and 100 μg/mL zeocin.
5. Phosphate-buffered saline (PBS).
6. 0.25% trypsin and 0.02% EDTA in PBS.

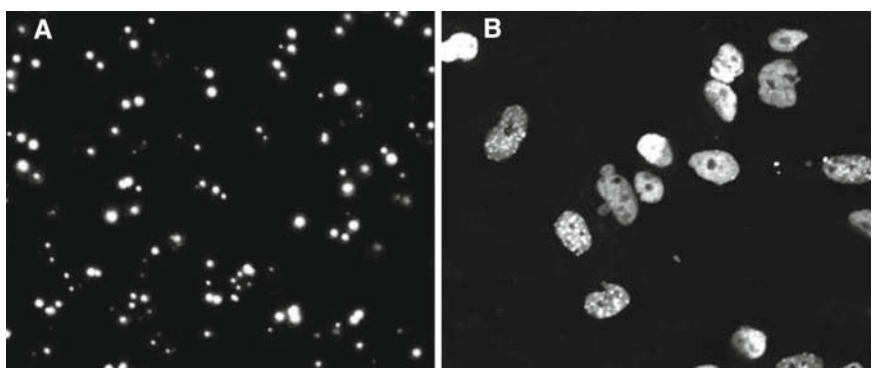

Fig. 1. Fluorescence microscopic images of SE/15 cells showing aberrant localization of GFP-PML upon HCMV infection. SE/15 cells were mock-infected (**A**) or infected with Towne (MOI 3) (**B**). GFP fluorescence micrographs at 18 h p.i. are shown.

2.2. Inoculation of SE/15 Cells

1. Human fibronectin (GIBCO/BRL) solution for coating of dishes. Stock and working concentrations are 1 mg/mL and 10 μg/mL, respectively (*see* **Note 4**).
2. Maintenance medium for HCMV-infected SE/15 cells: F12/DMEM (GIBCO/BRL) containing 5% FBS lacking phenol red. DMEM can be used instead.
3. HCMV solution: HCMV laboratory strains Towne (ATCC, #VR-977) and AD169 (ATCC, #VR-538) are propagated and titrated in HEL cells (or MRC-5 cells, ATCC, #CCL-171) using a standard protocol (*see* **Note 5**).

2.3. Quantification of HCMV-Infected SE/15 Cells by an NPB Fluorescence Assay

1. NPB elution buffer (NPB): 50 mM Tris-HCl, pH 7.4 containing 0.15% NP-40, 150 mM NaCl, 15 mM $MgCl_2$, and 5 mM EDTA.
2. uClear Fluorescence Black P 96-well plates with transparent bottoms (Greiner, #655090).

2.4. Heparin Inhibition Assay

1. Heparin: Stock solution of 1 mg/mL in distilled water. Store at –20 °C.

2.5. Quantification of HCMV-Infected SE/15 Cells by a GFP-PML Imaging Assay

1. Glass Bottom Fluorescence Black Microplates (96-wells, Greiner, #655896).
2. F12/DMEM (GIBCO/BRL) containing 10% FBS.
3. Computer software package for imaging assays: Image J (http://rsb.info.nih.gov/ij).

2.6. Susceptibility Testing of HCMV Clinical Isolates by a GFP-PML Imaging Assay Coupled with Co-Culturing

1. Human diploid fibroblasts (e.g., MRC-5 or HEL fibroblasts).
2. Maintenance medium for HCMV-infected HEL cells: DMEM (GIBCO/BRL) containing 5% FBS, streptomycin, and penicillin.
3. Disposable cell counter: C-chip (DHC-N01, IN CYTO).
4. HCMV clinical isolates propagated in HEL cells.
5. Antiviral stock solutions: 5 mM ganciclovir (GCV) and 100 mM foscarnet (FOS).

3. Methods

Chinese hamster ovary (CHO) cells have been reported to be as sensitive to HCMV as human fibroblasts regarding the initial entry and IE1 expression *(5)*. Since we previously found that HCMV infection frequently causes detachment of CHO cells and that this detachment is efficiently avoided by human p180

overexpression, we generated new cell lines coexpressing GFP-PML and the human host-cell factor p180 *(6)*. Since transfectants containing abnormally large GFP-PML structures appeared to exhibit difficulties in maintaining the structures in a stable manner, we carried out limited dilution to carefully select a suitable clone with respect to the stability and morphogenesis of its GFP-PML bodies, and finally cloned a cell line designated SE/15.

SE/15 cells have functional GFP-PML bodies whose morphology is specifically changed to a diffuse GFP pattern throughout the nucleus upon HCMV infection (**Fig. 1**). The unique appearance of nuclei containing dispersed GFP-PML is never observed in mock-infected cells, begins at postinfection (p.i.) 3–4 h, and continues to increase until postinfection 10–16 h. Thus, visual assessment by fluorescence microscopy is sufficient for sensitive detection of infected cells without the necessity for any further procedures, such as fixation and staining.

3.1. Cultivation of SE/15 Cells

1. For maintenance, culture SE/15 cells in DMEM/10% FBS containing 100 μg/mL G418 and 100 μg/mL zeocin. Cells up to the 25th passage can be used for HCMV quantification assays (*see* **Note 6**).
2. Pass SE/15 cells approaching confluence with trypsin/EDTA to provide new maintenance cultures on 60- or 100-mm dishes using split ratios of 3:1 or 4:1. For assay cultures, prepare fibronectin-coated plates (*see* **Subheading 3.2**).

3.2. Inoculation of SE/15 Cells

1. Add fibronectin solution (10 μg/mL) to the individual wells of tissue culture plates.
2. Incubate the plates overnight at 4 °C. Volumes of 1, 0.5, and 0.1 mL of the solution are sufficient for 12-, 24-, and 96-well culture plates, respectively.
3. Seed SE/15 cells approaching confluence onto fibronectin-coated plates at cell densities of 2×10^5, 1×10^5, and 0.2×10^5 cells/well for 12-, 24-, and 96-well culture plates, respectively (*see* **Note 7**).
4. On the day of the virus inoculation, remove the culture growth medium by aspiration (*see* **Note 8**), and immediately add a small volume (0.05–0.2 mL) of viral stock solution carefully to each well to give an appropriate multiplicity of infection (MOI). Gently rock the plate to achieve even distribution of the virus.
5. To allow adsorption, incubate the cells for 1 h in a CO_2 incubator. The cells may need to be gently tilted every 10 min to avoid cell drying.

6. Remove unabsorbed virus by aspiration and carefully wash three times with prewarmed F12/DMEM/5% FBS (*see* **Note 9**).
7. Add an appropriate volume of F12/DMEM/5% FBS containing no antibiotics to each well and incubate in a CO_2 incubator at 37 °C (*see* **Note 10**).
8. An example of the diffuse GFP-PML in Towne-infected SE/15 cells at 18 h p.i. is shown in **Fig. 1**.

3.3. Quantification of HCMV-Infected SE/15 Cells by an NPB Fluorescence Assay

GFP-PML is present as an insoluble form in normal nuclei *(7)*. In contrast, the diffuse PML in infected nuclei becomes soluble in NP-40-containing buffer (NPB). Therefore, NPB extraction-based fluorescence measurements in SE/15 cells allow quantification of HCMV-infected cells.

1. On the day of the experiment, prepare the following materials: precooled (on ice) NPB buffer, a labeled microcentrifuge tube for each sample, ice container for incubation, PBS.
2. At appropriate times p.i., gently wash HCMV-infected and mock-infected SE/15 cell cultures twice with PBS.
3. Aspirate the residual solution from the cell cultures and place the plates on ice. Add 0.250 mL of ice cold NPB to each well of the 12-well plate (*see* **Note 11**).
4. Incubate the cells for exactly 15 min at 4 °C. Carefully transfer the NPB solutions to 1.5-mL labeled microcentrifuge tubes (*see* **Note 12**), and clarify by centrifugation at 3,000 rpm for 5 min at 4 °C.
5. Transfer the supernatants to a black fluorescence P 96-well plate.
6. Prepare total cell lysates in a parallel experiment. After washing with PBS, scrape the material into appropriately labeled tubes. Add 0.250 mL of prechilled NPB to the 12-well plate samples. Incubate for 15 min on ice and transfer to a black fluorescence P 96-well plate.
7. Measure the NPB fluorescence intensity in a multiwell fluorescence plate reader (e.g., CytoFluor series 4000, Perseptive Biosystems) using an excitation wavelength of 485 nm and an emission wavelength of 510 nm.
8. The data sets of NPB fluorescence intensity required for each sample are NPB extracts of infected cells, mock-infected cells, and total cell lysates.
9. Calculate the net fluorescence intensity of the infected cells as follows: subtract the NPB fluorescence intensity of the mock-infected cells from that of the infected cells.
10. Express the HCMV infectivity as a ratio of the net fluorescence intensity of the NPB extracts to the fluorescence intensity of the total cell lysates.

3.4. Heparin Inhibition Assay Using an NPB Fluorescence Assay

1. Seed SE/15 cells as described in **Subheading 3.2**.
2. On the day of the experiment, prepare the following materials: HCMV (Towne strain) solution which has been thawed and kept on ice, serially diluted solutions of heparin in PBS.
3. Mix HCMV solutions with serial dilutions of heparin to give appropriate concentrations of the virus and heparin.
4. Incubate the mixed virus-heparin solutions at 37 °C for 1 h.
5. Remove the growth medium from the cultures and add the pretreated virus solutions to each well as described in **Subheading 3.2**.
6. Incubate plates in a CO_2 incubator at 37 °C for 18–24 h.
7. Quantify the Towne-infected SE/15 cells by the NPB fluorescence assay as described in **Subheading 3.3**.
8. An example of an inhibition curve for Towne-infected SE/15 cells at 18 h p.i. is shown in **Fig. 2**.

3.5. Quantification of HCMV-Infected SE/15 Cells by a GFP-PML Imaging Assay

Since GFP-PML is dispersed throughout the nuclei of HCMV-infected SE/15 cells and the GFP-PML areas are markedly larger than the GFP-PML dots in uninfected cells (see **Fig. 1**), imaging analysis of fluorescence micrographs enables direct counting of the number of infected nuclei. This protocol allows highly sensitive quantification even in miniscale cultures, and permits assaying low titer clinical isolates (*see* **Note 13**).

As shown in **Fig. 3**, a typical profiling pattern by particle analysis of mock-infected cells shows one peak representing the small GFP-PML foci (**Fig. 3**, left). A second broad peak appears in the profiling pattern of HCMV-infected cells, corresponding to the sizes of nuclei bearing diffuse GFP signals (**Fig. 3,** right). Therefore, HCMV infectivity is expressed as the ratio of the

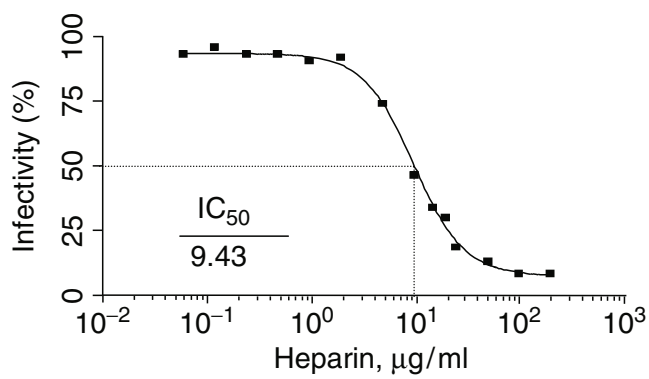

Fig. 2. Heparin inhibition assay using an NPB fluorescence assay. HCMV was preincubated for 1 h at 37 °C with various concentrations of heparin, an entry process inhibitor of the virion into the cells. SE/15 cells were infected (MOI 1) in a 24-well plate for 18 h, followed by the standard NPB fluorescence assay as described in the text. (Reproduced from *(4)* with permission from American Society for Microbiology.).

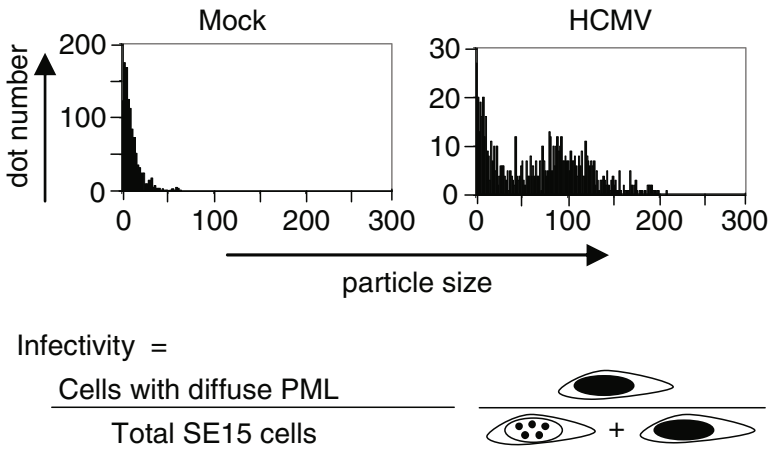

Fig. 3. Profile patterns of the particle sizes of mock-infected and Towne-infected cells. GFP fluorescence images of mock-infected (*left*) and Towne-infected (*right*) cells were captured at 18 h p.i., and analyzed according to the procedure described in the text. (Reproduced from **ref.** *(4)* with permission from American Society for Microbiology).

number of nuclei possessing diffuse GFP signals to the total number of SE/15 cell nuclei.

1. Prepare HCMV-infected and mock-infected SE/15 cells as described above. Growth medium containing no phenol red, G418, or zeocin is highly recommended (*see* **Note 14**).
2. At appropriate times p.i., capture GFP fluorescence images as TIF files using a fluorescence microscope equipped with a cooled charge-coupled device (CCD) camera and a computer (e.g., Olympus model IX71 fluorescence microscope system equipped with an Olympus DP50 CCD camera) (*see* **Note 15**). A 20× objective lens and a fixed data collection time of 0.2 s are used for the conventional assay.
3. When the captured images are not 8-bit gray images, convert them to 8-bit gray images using an image processing software package (e.g., Adobe Photoshop, ImageJ) (*see* **Note 16**).
4. Evaluate the numbers of cells displaying GFP-PML bodies and diffuse GFP-PML using particle counting in ImageJ according to the following procedures.
5. Using the pull-down menu "File" – "Open," open the 8-bit gray images of mock-infected and HCMV-infected cells.
6. Using the pull-down menu "Image" – "Adjust" – "Threshold," set the lower and upper threshold values. When the "apply" button is clicked, the image will be converted into a binary image on the desktop (*see* **Note 17**).
7. Evaluate the total number of mock-infected cells by particle counting as follows.
8. Using the pull-down menu "Analyze" – "Analyze particles," select "outlines" in the show column and 0.00–1.00 in the circularity column.

9. Set the minimum and maximum sizes (pixels) for GFP-PML bodies, where "minimum" refers to the smallest pixels of GFP-PML bodies in mock-infected cells that can segregate electric noise from GFP-PML and "maximum" for GFP-PML bodies is the threshold of diffuse PML, namely the smallest nucleus that can segregate GFP-PML bodies from diffuse PML (*see* **Note 18**).
10. Check "Display results" and "Summarize" and press "OK." The total number of GFP–PML dots is shown in a table of results.
11. Estimate the total number of cells bearing GFP-PML bodies (A) by using the following formula (I): Total number of cells = 0.632 × total number of GFP-PML dots (*see* **Note 19**).
12. Evaluate the cell numbers of infected and noninfected cells using binary images of infected cells as follows.
13. Using the pull-down menu "Analyze" – "Analyze particles," set the "minimum" and "maximum" pixel numbers of diffuse PML using HCMV-infected cells (*see* **Note 20**).
14. "Minimum" is the same value as the maximum pixel number for GFP-PML bodies, while "maximum" is nearly equal to the "upper threshold."
15. Check "Display results" and "Summarize" and press "OK." The number of cells bearing diffuse PML (*B*) is shown.
16. Evaluate the number of cells bearing GFP-PML bodies (C) as described in **Points 7–11**.
17. Evaluate the HCMV infectivity (%) using $B/(B + C) \times 100$ (*see* **Note 21**).

3.6. Susceptibility Testing of HCMV Clinical Isolates by a GFP-PML Imaging Assay Coupled with Co-Culturing

Since SE/15 cells do not support a full replication cycle, co-culturing of SE/15 cells with infected HEL cells is required for susceptibility testing. A schematic representation of the protocol for susceptibility testing is shown in **Fig. 4**.

1. Coat glass-bottom black fluorescence 96-well microplates with fibronectin as described in **Subheading 3.2**
2. Culture HCMV clinical isolates in HEL cells in 24-well plates (1×10^5 cells/well).
3. On the day of the experiment, make serial dilutions of antiviral drugs such as GCV and FOS at tenfold higher concentrations than the final required concentrations.
4. Add antiviral drugs (GCV, FOS, etc.) to the wells, and culture for 48–72 h. Final concentrations of 0.1–100 μM GCV and 1–1,000 μM FOS are recommended.
5. On the day of the co-culturing, harvest the HCMV-infected HEL cells and SE/15 cells with trypsin/EDTA and count the cell numbers.

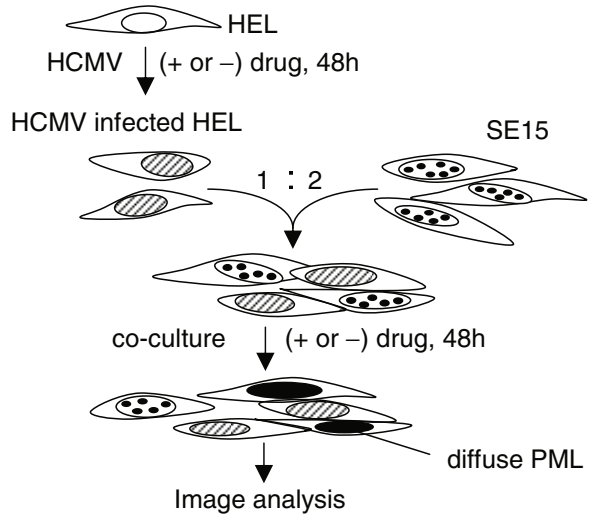

Fig. 4. Schematic representation of the protocol for drug susceptibility testing of clinical isolates using the co-culture system. (Reproduced from **ref.***(4)* with permission from American Society for Microbiology.

6. Prepare serial dilutions of GCV and FOS.
7. Resuspend the cells in DMEM/5% FBS at a density of 2×10^5 cells/mL.
8. Mix HCMV-infected HEL cells (1×10^4 cells/50 µL) and SE/15 cells (2×10^4 cells/100 µL) at a ratio of 1:2. Seed 150 µL of the mixed cell suspension into glass-bottom black fluorescence microplates precoated with fibronectin.
9. Add antiviral drugs (GCV or FOS) to each sample (*see* **Note 22**).
10. Culture the cells for 24–48 h.
11. Analyze each sample by the GFP-PML imaging assay as described in **Subheading 3.5**
12. Estimate the HCMV infectivity at each drug concentration.
13. Calculate the 50% inhibitory concentration (IC_{50}) values using dose–response curves (*see* **Note 23**).

Notes

1. In this chapter, cultivation of cells is performed at 37 °C in a humidified incubator in an atmosphere of 5% CO_2.
2. The following requirements should be considered when selecting stable cell lines expressing GFP-PML for HCMV assays: (1) GFP-PML morphology should be reminiscent of that of

native PML bodies in the nucleus, that is, not too big or too small; (2) cells should possess functional GFP-PML bodies whose morphology is specifically changed upon HCMV infection; (3) cells should maintain their GFP-PML structures in a stable manner after repeated passages.

3. In addition to GFP-PML, SE/15 cells overexpress human p180 protein. We previously found that HCMV infection frequently causes detachment of CHO cells and that this detachment is efficiently avoided by human p180 overexpression.
4. Stock solutions of fibronectin (0.5–1.0 mg/mL) should be stored at –20 °C and not subjected to repeated freezing and thawing. A working solution (10 μg/mL) should be freshly prepared before use. Fibronectin-coated plates wrapped tightly in vinyl can be preserved in a refrigerator for several weeks at least.
5. HCMV replicates slowly in monolayer cultures of diploid fibroblast cells, and exhibits cytopathic effects in 3–6 days for laboratory strains and 12–30 days for clinical isolates. HCMV laboratory strains, including Towne and AD169, are efficiently shed in culture media, allowing the culture supernatants to be collected as high-titer virus solutions on postinoculation days 4–6. On the other hand, since most of the HCMV clinical isolates are cell associated and only small amounts of virus are shed into the culture media, these virus solutions must be prepared via cell lysis by ultrasonication.
6. Cells at young generations (10–15th passage) should be stocked multiply before propagation of SE/15 cells. Whenever subcultivate, surplus SE/15 cells should be also stocked. Beyond the 30th passage, cells lacking or harboring small amounts of GFP-PML may increase, and these are nonapplicable to quantification assays. Therefore, it is important to regularly check the numbers of cells harboring GFP-PML. In bad or severe culture conditions, nonexpressing cells quickly overgrow, thereby necessary to start with a new cell stock of young generation. Healthy SE/15 cells are a prerequisite for quantification.
7. Even distribution of the cells in each well is required for reproducible assays. After seeding, gently rock the plate to achieve an even distribution and carefully transport the plates to the CO_2 incubator to maintain the distribution.
8. All procedures involving HCMV should be performed in a Class II biological safety cabinet under strict aseptic conditions to minimize the risk of contamination.
9. Since HCMV-infected cells are easily detachable from dishes, wash the cells carefully.

10. Under fluorescent microscopy observation, cells containing diffuse GFP-PML are detectable as early as 4 h p.i., increase until 10–12 h p.i. and then almost reach a plateau.
11. For 96- and 24-well plates, add 30 and 150 μL of NPB, respectively.
12. In the presence of detergent, SE/15 cells are easily detachable from dishes. Add NPB gently via the wall surface of the wells.
13. The NPB fluorescence assay provides a simple and rapid protocol, in which there are only two steps: "extraction" and "measurement by a plate reader." For high titer viruses such as experimental strains Towne and AD169, NPB fluorescence assay is useful. As higher sensitivity was required for assaying clinical isolates, we developed the imaging assay which enables assay of low titer viruses of clinical isolates.
14. Conventional culture plates show a high level of background fluorescence. We recommend using culture plates specified for fluorescence measurements (e.g., glass-bottom black fluorescence microplates, Greiner, #655896 or #662896 are one of the most suitable types of plates for imaging assays).
15. Growth medium containing phenol red makes the *S/N* ratio lower. Cells fixed with paraformaldehyde or formaldehyde can be used in the same protocol.
16. The automatic processing functions in Photoshop (Adobe) may be helpful.
17. Some of the PML signals bearing low intensity may disappear after conversion to a binary file. If the outcome is not appropriate, reset the parameters.
18. In **Fig. 3**, a range of 20–299 is used to represent the sizes (pixels).
19. You can verify your protocol by comparison with data from DAPI-stained images.
20. In **Fig. 3**, a range of 300–1,000 is used to represent the sizes (pixels).
21. Data are presented as mean values from 3 to 5 fields taken from one sample.
22. An even distribution of cells in each well is required for reproducible assays. After seeding, gently rock the plate to achieve an even distribution and carefully transport the plates to the CO_2 incubator to maintain the distribution.
23. The statistical software package Prism 4 (Graphpad) may be useful for fitting of logistic curves and calculating the 50% inhibitory concentrations (IC_{50}) statistically.

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