

# Rapid diagnosis and quantification of herpes simplex virus with a green fluorescent protein reporter system

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Received 10 May 2000; received in revised form 12 July 2000; accepted 12 July 2000

## Abstract

A genetically modified cell line (Vero–ICP10–EGFP) was constructed for detection of herpes simplex virus (HSV) by a simple, rapid and direct method. The cell line was developed by stable transfection of Vero cell with a plasmid encoding the green fluorescent protein (GFP) driven by the promoter of the HSV-2 ICP10 gene. As early as 6 h after infection with HSV, fluorescence-emitting cells can be observed under a fluorescence microscope. A single infected cell emitting fluorescence can be observed with soft agar overlay by inverted fluorescence microscopy. No induction of detectable fluorescence was seen following infections with human cytomegalovirus (HCMV), varicella zoster virus (VZV), coxsackievirus A16 and enterovirus 71. Analysis by flow cytometry also demonstrated that intensity of the triggered fluorescence is proportional to the titer of HSV inoculated. Taken together, this novel GFP reporter system could become a useful means for rapid detection and quantification of HSV in clinical specimens. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Herpes simplex virus; Green fluorescent protein; ICP10 promoter

## 1. Introduction

Herpes simplex virus types 1 and 2 (collectively, HSV) infections are the significant causes of morbidity and mortality among immunocompromised persons, such as newborns, leukemia patients,

transplant recipients and patients with acquired immunodeficiency syndrome (AIDS) (Whitley, 1996). Laboratory diagnosis for HSV has thus become one of the most frequent tests conducted in clinical virology laboratories. Development of chemotherapeutic agents for HSV underlines further the need for rapid, definitive and sensitive detection assays (Hammer and Inouye, 1997). Currently, diagnostic tests for HSV detection can be categorized into assays for viral antigen, viral

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DNA and infectious virus. Antigen-detection assays are rapid and specific although sensitivity is wanting. Most serological assays are often of little clinical value. Virus DNA amplification using the polymerase chain reaction (PCR) offers sensitivity, but rigorous quality control is critical for routine use. Despite the rapid progress in protein- and nucleic-based assays, virus culture remains the only method for the detection of infectious virus although it is time-consuming, labor-intensive and relatively insensitive (McIntosh, 1996). Taken together, there is room for improvement of current diagnostic methods.

With advances in the understanding of HSV replication, it is possible to take advantage of virus-specific events, such as transcription from a viral promoter, to identify virus-infected cells. Lines of evidence indicated that the promoter that controls the large subunit of ribonucleotide reductase, the ICP6 from HSV-1 or the ICP10 from HSV-2, may be suitable for this goal. Both promoters have been studied by linkage to various reporter genes, including the chloramphenicol acetyltransferase (CAT, Wymer et al., 1989, 1992; Hanson et al., 1994; Zhu and Aurelian, 1997), the *lacZ* (Goldstein and Weller, 1988; Stabell and Olivo, 1992; Stabell et al., 1993; Patel et al., 1999) and the luciferase (Olivo, 1994). Accumulated data indicated that the background expressions driven by these promoters are extremely low, and that the expressions are induced specifically by HSV-1 or HSV-2 infection within several hours post-infection. In addition, a number of stable lines with the *lacZ* gene behind the ICP6 promoter have been used for clinical virology assays (Stabell et al., 1993; Patel et al., 1999), including development of an assay for antiviral susceptibility testing (Tebas et al., 1995, 1998). However, these assays require fixation of cells and the addition of exogenous substrates (*lacZ*), and/or preparation of cell lysates (CAT and luciferase). With these experimental procedures, cells of interest are killed and not suitable for further study. Thus, we sought to establish a simple and direct system for continuous monitoring of HSV infections in living cells.

The green fluorescent protein (GFP), identified originally from jellyfish, *Aequorea victoria* has sev-

eral promising features that make it ideal for the purpose. The GFP emits bright green light after exposure to ultraviolet or blue light without extrinsic labeling or substrates. With the wild-type (wt) GFP gene being cloned, it has been used as a reporter gene in a real-time and noninvasive fashion (Chalfie et al., 1994). Recently, variants of GFP have also been designed in that they are adapted better to mammalian expression and signal detection. The enhanced GFP (EGFP), for example, contains double-amino-acid substitutions, i.e. Phe-64 to Leu and Ser-65 to Thr (Cormack et al., 1996; Yang et al., 1996). This derivative, thus, has a single, strong, red-shifted excitation peak at 488 nm, which is well suited for detection by fluorescence microscopy and flow cytometry. Moreover, the EGFP fluoresces 35-fold more intensively than what wt GFP does as measured by standard FITC filtering procedures (Cormack et al., 1996; Yang et al., 1996).

We report the establishment of a stable cell line harboring a plasmid encoding the EGFP reporter gene driven by the HSV-2 ICP10 promoter. Within 6–10 h after infection with either HSV-1 or HSV-2, fluorescence-emitting cells can be observed under a fluorescence microscope. The high sensitivity of the system was demonstrated in that a single infected cell with fluorescence can be observed on soft agar overlay by inverted fluorescence microscopy. Infection with clinical isolates of human cytomegalovirus (HCMV), varicella zoster virus (VZV), coxsackievirus A16 and enterovirus 71 did not trigger the system, suggesting its high specificity. Analysis with flow cytometry showed also that the intensity of fluorescence from the infected cells is proportional to the titer of HSV inoculated, making it possible to quantify HSV infectious particles.

## 2. Materials and methods

### 2.1. Plasmids

The plasmid pEGFP-1 (Clontech, Palo Alto, CA) encodes a human codon-optimized GFP downstream of a multiple cloning site, and a neomycin resistance gene (*NeoR*) for antibiotic

neomycin (G418) selection in mammalian cells. A DNA fragment, containing the ICP10 promoter (–535 to +113 relative to the mRNA cap site; Wymer et al., 1989) of HSV-2 (strain 186) was obtained by PCR using the following set of primers. Forward, 5'-CGAGATCTGTGTCGACAG-GCTGTACCGCTGG-3'; reverse, 5'-CCAAGCT-TGTCGACAGGACAGCACCGGCC-3' with the following parameters, 98°C for 10 min, then 30 cycles with denaturation step at 94°C for 1 min, hybridization 56°C for 2 min, and elongation at 72°C for 3 min with a final elongation at 72°C for 7 min. The primer pair was designed in such a way that the *Bgl*III and *Hind*III restriction sites were added at the 5'- and 3'- ends, respectively. The PCR fragment was subsequently restricted and directionally cloned into the corresponding restriction sites in the pEGFP-1 plasmid. The insert was then confirmed by sequencing. The resultant plasmid was designated as pICP10-EGFP.

## 2.2. Cells and viruses

Vero (African green monkey kidney) cells were propagated and maintained in Dulbecco's modified eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. Laboratory strains of HSV-1 (KOS) and HSV-2 (186) were used, and their titers determined by a standard plaque assay on Vero cells. Clinical isolates of HSV-1 and 2 were identified by an immunofluorescence assay according to manufacturer's instructions (DAKO Diagnostics Ltd., Cambridgeshire, UK). Clinical isolates of HCMV, VZV, enterovirus 71 and coxsackievirus A16 were collected from routine isolations in Division of Clinical Virology, Veteran General Hospital-Taipei.

## 2.3. Establishment of stably transfected cell lines

Vero cells were split 1:6 from a fully confluent 6-well plate 24 h before transfection. Approximately, 2- $\mu$ g pICP10-EGFP plasmid was transfected into the Vero cell using Lipofectamine

(Gibco-BRL, Gaithersburg, MD) as per the manufacturer's instructions. Forty-eight hours after the transfection, the transfected cells were grown in DMEM, containing G418 at 0.8 mg/ml, until the mock-transfected cells were all dead (about 2 weeks). To obtain stable clones, G418-resistant cells were isolated further by plating at limiting dilution onto 96-well tissue culture dishes. Each clone was grown and maintained in DMEM containing 0.4 mg/ml G418.

## 2.4. Fluorescence microscopy

For direct visualization under an inverted fluorescence microscope (Leica DMIRB), Vero-ICP10-EGFP cells were seeded on a bottom glass microwell dish (MatTek Corp., Ashland, MA) a day before infection. Cells were infected with HSV in DMEM without phenol red. Cells were overlaid with DMEM (without phenol red) in the presence of 2% FBS and 0.8% agar noble (Difco Laboratory, Detroit, MI) to observe plaque morphology.

To screen clinical specimens for HSV, Vero-ICP10-EGFP cells were plated onto 18  $\times$  18 mm coverslips placed in six-well trays (Corning Inc., Corning, NY) a day before infection. Following infection for 8 h, tissue culture media were removed and cells were washed three times with 1XPBS. The coverslips were mounted onto glass microscopic slides and subjected to observation under an upright fluorescence microscope (Nikon).

## 2.5. Flow cytometric analysis

Flow cytometric analysis was conducted using a FACSORT (Becton Dickinson) with a 488-nm argon ion laser. The FL-1 emission channel normally used to detect fluorescence isothiocyanate (FITC) was used to monitor the EGFP expression. Fifteen hours after infection, the cells were trypsinized and washed once with 1XPBS. The cells were then filtered and counted; approximately 10<sup>6</sup> cells were used for each run of flow cytometric analysis.

### 3. Results

#### 3.1. Preparation of the Vero-ICP10-EGFP cell line

The pICP10-EGFP plasmid was constructed by insertion of the HSV-2 ICP10 promoter to the upstream of the EGFP reporter gene in plasmid pEGFP-1. The pEGFP-1 plasmid contains the neomycin (G418) resistant gene that confers neomycin resistance to the transfectants. A functional feature map of the plasmid pICP10-EGFP is shown in Fig. 1. The pICP10-EGFP plasmid was linearized with the enzyme *Bgl*II and subsequently transfected into Vero cells. Stable transfectants were isolated by selection for G418 resistant colonies followed by limiting dilution, as described in Section 2. The clones that displayed undetectable level of the EGFP under a fluorescence microscope were subjected to infection with HSV-2 at three multiplicity of infection (MOI), and those with approximately 100% expression of the EGFP following the infection were chosen for further study. The same process was repeated for two more rounds to obtain stable clones. The

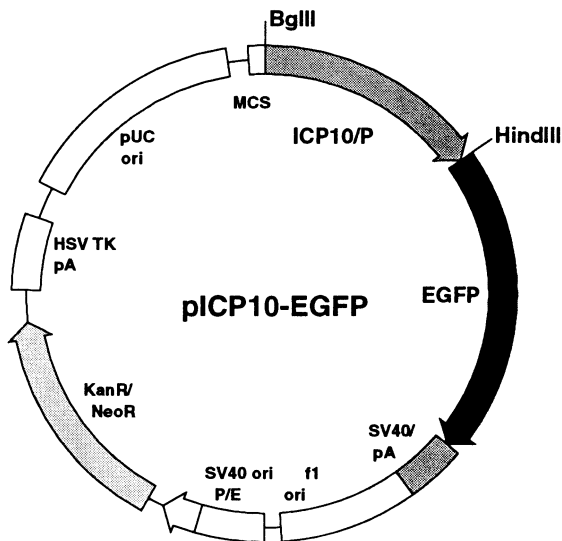


Fig. 1. Schematic representation of plasmid pICP10-EGFP. The PCR product containing the HSV-2 ICP10 promoter (ICP10/P) was cloned upstream to the EGFP reporter gene in the plasmid pEGFP-1 (Clontech). The plasmid was linearized with *Bgl*II prior to transfection into Vero cells. The presence of the *neo* selectable marker (*NeoR*) is also shown.

most appropriate clone from the procedure above was designated as Vero-ICP10-EGFP.

#### 3.2. Analysis of the EGFP expressions by fluorescence microscopy

The EGFP expression induced by infection of HSV was monitored by three methods. First, to learn the minimal time point for the appearance of fluorescence, cells infected with HSV-1 at an MOI of 0.5 were analyzed at 6, 8 and 10 h post-infections with an inverted fluorescence microscope (Fig. 2). The fluorescence was not visible (Fig. 2a) until 6 h post-infection (Fig. 2b), with pronounced increase in the intensities at later hours (Fig. 2c and d). Notably, the cells displayed no apparent cytopathic effect (CPE) 10 h post-infection (Fig. 2e), while bright green signals were readily detected. As a comparison, extensive CPE was discernible 24 h post-infection under a phase-contrast microscope (Fig. 2f).

As a second method, the infected cells were overlaid with soft agar for observation of plaque formation. At 24 h post-infection, inverted fluorescence microscopy allows direct observation of fluorescence-emitting cells (Fig. 3c), while phase-contrast microscopy detected no significant plaque formation (Fig. 3d). At 48 h post-infection, characteristic plaques with infected cells that become large, round and refractile can be seen under a phase-contrast microscope (Fig. 3f). At this time point, cells within the center of the viral plaque emitted bright green fluorescence, while newly infected cells in the periphery of the viral plaque appeared to fluorescence less intensely under a fluorescence microscope (Fig. 3e). No fluorescent cells or plaques were seen with mock-infected cells at this time point (Fig. 3a and b). In addition, the number of fluorescent cells at 24 h post-infection is identical to that of the plaques inspected 48 h post-infection, indicating that the viral titer estimated by a conventional plaque assay is reproducible with the EGFP system at much earlier time. In line with the finding, the sensitivity of the EGFP system probably approximates to that of the plaque assay since each bright green cell later developed into a corresponding plaque.

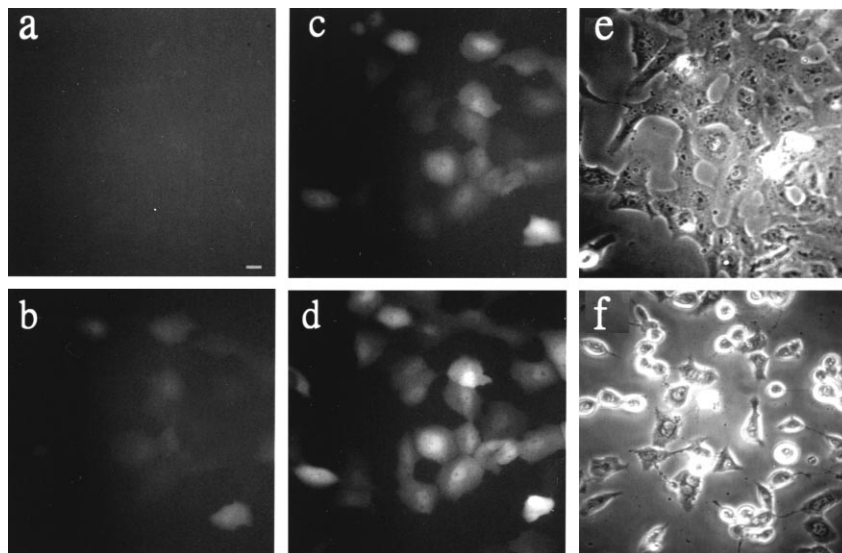


Fig. 2. The EGFP expression kinetics of the infected Vero-ICP10-EGFP cell line. The Vero-ICP10-EGFP cell line was mock-infected (a) or infected with KOS strain of HSV-1 at an MOI of 0.5, and directly observed under an inverted fluorescence microscope at 6 (b), 8 (c) and 10 h (d) post-infections. The infected cells were also visualized at 10 (e) and 24 h (f) with a phase-contrast microscope. Bar, 5  $\mu$ M.

Considering that an inverted fluorescence microscope is not available generally in a clinical virology laboratory, the third method that allows cells seeded on a coverslid and observed with a regular upright fluorescence microscope was applied. The results were identical to those obtained by the first method in that the fluorescent cells were visualized at 6 h after infection with significant increase in the brightness at later time of infections. Eight hours post-infection was chosen for larger scale screening of HSV in clinical specimens since the fluorescence is well discernible at this time point. Using this protocol, 15 HSV-1 and 10 HSV-2 clinical isolates, identified previously by the immunofluorescence assay, were examined and all of them resulted in generation of readily detectable fluorescent signal (data not shown). The stable line was then infected with clinical isolates of two other herpes viruses, HCMV and VZV, as well as RNA viruses including enterovirus 71 and coxsackievirus A16. None of the viral infections led to detectable expressions of the EGFP reporter gene at 8 h post-infection (data not shown), suggesting that the EGFP system is highly specific for HSV infections.

### 3.3. Flow cytometry

EGFP has a single red-shifted excitation peak at 488 nm which is ideal for automated analysis by FACS instruments using argon ion lasers and standard FITC filter sets. To assess the ability of infected cells to be detected and quantified by FACS analysis, fluorescence intensities from HSV-1-infected cells were measured 15 h post-infection as a function of the amount of virus inocula; approximately  $10^6$  cells were subjected to infections with HSV-1 starting at 10 PFU in a 10-fold increment up to  $10^5$  PFU (Fig. 4). The infected cells exhibited a marked increase in the numbers of EGFP-positive cells with increasing amount of virus added. The system is approximately sensitive to HSV-1 infections with a titer as low as 10 PFU (equivalent to  $10^{-5}$  MOI) within the time-frame of a single virus life cycle (20–24 h). Nevertheless, virus-induced expression of the EGFP, following infection with 10 PFU, is well above background (6–9 fold) by 48 h (data not shown). Similar results were found for both HSV-1 and HSV-2 in triplicate (data not shown). This study stresses the usefulness of flow cytome-

try for quantitating the number of HSV infectious particles.

#### 4. Discussion

This report addressed the development and characterization of an EGFP-based reporter system for detection of HSV-1 and 2 infections. The Vero-ICP10-EGFP cell line allows monitoring of the EGFP expression by several complementary methods. Inverted fluorescence microscopy enables direct visualization of fluorescent cells on a set field over time (Fig. 2). When overlaid with soft agar, the system demonstrated its reproducibility and sensitivity when compared with the plaque assay (Fig. 3). To adapt this reporter cell line to routine uses in clinical virology laboratories, the protocol with the cells seeded on coverslips followed by inspection using an upright fluorescence microscope was developed. It permitted rapid diagnosis of all the HSV specimens tested but not those selected viruses other than HSV, consistent with the results documented (Stabell and Olivo, 1992). Analysis with flow cytometry revealed the parallel increase in fluorescence

and input inocula (Fig. 4), suggesting its potential application for rapid quantification of viral infectivity titers.

Recently, Gervaix et al. (1997) reported the isolation of a genetically modified cell line containing the EGFP reporter gene controlled by the long terminal repeat (LTR) of human immunodeficiency virus type 1 (HIV-1). The reporter cell line was used to determine the values of 50% inhibitory concentrations (IC<sub>50</sub>) of certain drugs against HIV. Given that several anti-HSV agents have been available and drug-resistant HSV strains emerged, the Vero-ICP10-EGFP cell line may be developed into novel systems for drug susceptibility testing as well as antiviral drug screening.

Since the observation of the EGFP requires no further invasive steps, it would be possible to sort the EGFP-positive cells, which remain alive, followed by outgrowth of individual EGFP-positive cell. This proposed method could shorten the time for isolation of viral specimens, which normally takes several days by a plaque purification assay.

In summary, the establishment of a stable cell line expressing the HSV-inducible EGFP reporter gene has provided a fast, easy, economical and

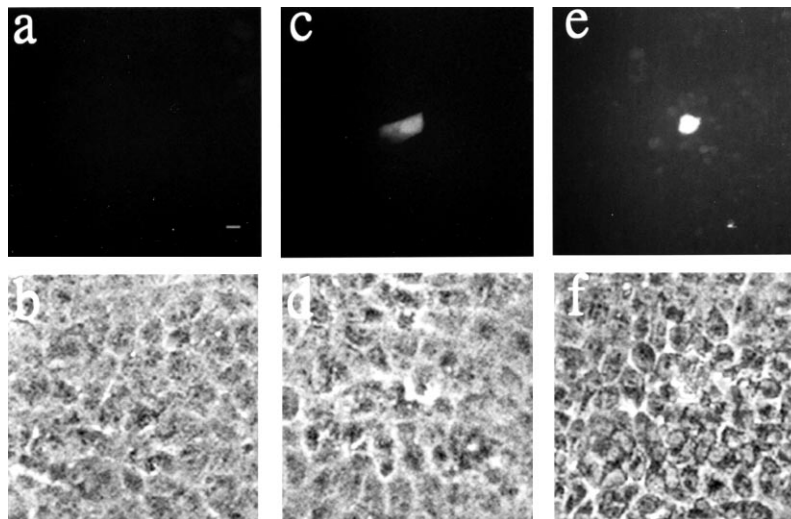


Fig. 3. The EGFP expression and plaque formation of the infected Vero-ICP10-EGFP cell line. The Vero-ICP10-EGFP cell line was mock-infected (a and b) or infected with KOS strain of HSV-1 at an MOI of 0.001 and then overlaid with agar noble. The cells were detected 24 (c and d) and 48 h (e and f) post-infections by inverted fluorescence microscopy (a, c and e) or phase-contrast microscopy (b, d and f). Bar, 5  $\mu$ M.

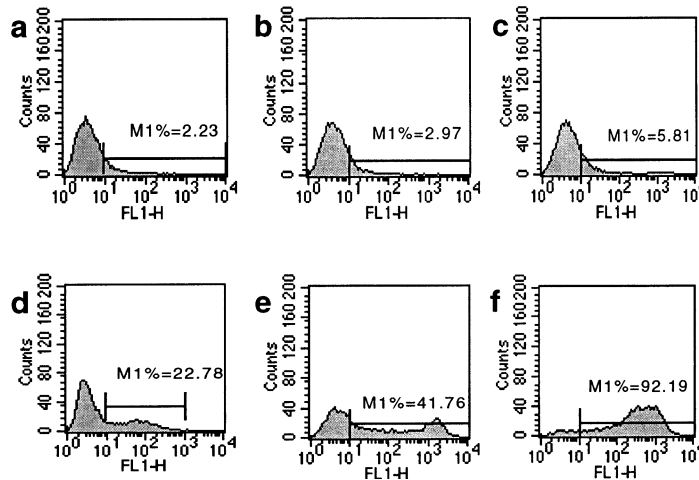


Fig. 4. Analysis of infected Vero-ICP10-EGFP cell line by flow cytometry. Approximately,  $10^6$  Vero-ICP10-EGFP cells were infected with HSV-1 with MOIs ranging from  $10^{-5}$  (b),  $10^{-4}$  (c),  $10^{-3}$  (d),  $10^{-2}$  (e) and  $10^{-1}$  (f) and fluorescence measured at 15 h post-infection. The sorting gate M1 was determined arbitrarily to include 2.23% of the mock-infected (a) cells. Data from a single experiment of three replicate experiments are shown.

accurate tool for monitoring and investigating HSV infections. The unique feature of direct visualization and counting, and the possibility of sorting fluorescent cells may lead to future applications for antiviral drug susceptibility testing and virus isolation. Evaluation of its use for clinical virology with large number of samples is currently in progress.

### Acknowledgements

We wish to thank the financial support from National Science Council, ROC (NSC88-2312-B-010-0005).

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