

Major Article

Evaluating the use of fluorescence-based flow cytometry assay for dengue diagnosis using peripheral blood mononuclear cells

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Abstract

Introduction: Dengue virus (DENV) is the most important arthropod-borne viral disease worldwide with an estimated 50 million infections occurring each year. **Methods:** In this study, we present a flow cytometry assay (FACS) for diagnosing DENV, and compare its results with those of the non-structural protein 1 (NS1) immunochromatographic assay and *reverse transcriptase* polymerase chain reaction (RT-PCR). **Results:** All three assays identified 29.1% (39/134) of the patients as dengue-positive. The FACS approach and real-time RT-PCR detected the DENV in 39 and 44 samples, respectively. On the other hand, the immunochromatographic assay detected the NS1 protein in 40.1% (56/134) of the patients. The Cohen's kappa coefficient analysis revealed a substantial agreement among the three methods. **Conclusions:** The FACS approach may be a useful alternative for dengue diagnosis and can be implemented in public and private laboratories.

Keywords: Dengue virus. Arbovirus. *Flavivirus*. Viral diagnostic. Flow cytometry.

INTRODUCTION

Dengue viruses (DENVs) are classified under the *Flavivirus* genus and the *Flaviviridae* family¹. These viruses are enveloped positive-sense single-stranded RNA viruses that encode three structural proteins and seven non-structural proteins². Based on the neutralization assay, DENVs are classified into four serotypes (DENV-1 to 4), and are transmitted to humans through the bite of infected *Aedes* mosquitoes^{1,3}. Currently, DENV is the most important arthropod-borne viral disease worldwide, with an estimated 50 million infections occurring each year, which may be asymptomatic as in the majority of cases or may result in severe hemorrhagic fevers⁴. In Brazil, all four DENV serotypes have been reported in highly populated areas in the last three decades, such as DENV-1 in 1985, DENV-2 in 1990, DENV-3 in 2000, and DENV-4 in 2008^{5,6}. These viruses have spread and have been reintroduced probably several times, subsequently producing large outbreaks that affect at least ten million people, causing severe disease in tens of thousands of people, eventually leading to thousands of deaths⁵.

Dengue infection usually begins as a mild acute febrile illness, but in some cases, it can progress to severe shock syndrome in a few hours⁴. Therefore, the diagnosis of dengue in the acute viremic phase of the disease is essential for suitable management of patients, especially at severe stages, and for consequent prognostic improvement¹. Patients with dengue infection usually do not present specific antibodies during the acute phase of the disease. The diagnosis is based on isolation of the virus, detection of viral proteins (e.g., NS1 protein) in blood samples by immunochromatographic assay, and detection of the viral genome by RT-PCR⁷. Hence, to provide alternative methods of detection of DENV during the acute phase and for the subsequent effective management of patients, the development of quicker and easier diagnostic protocols for dengue is important. In this study, we have reported an assay for the diagnosis of DENVs in peripheral blood mononuclear cells (PBMCs) using a flow cytometry system with fluorescence-activated cell sorting (FACS).

METHODS

Study design and population

During June to October 2015, 152 patients with acute febrile illness at Basic Health Units from the Ribeirão Preto City and the Jardimópolis municipality, São Paulo, Brazil, were recruited for the study. Samples were collected from the patients from

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the first to fifth day after the onset of symptoms. All participants had agreed and signed the consent form for this study.

Ethical considerations

This study was approved by the Research Ethics Committee of the Hospital of the School of Medicine of Ribeirão Preto, University of São Paulo (Process No. UPC 7641).

Sample collection and preparation of PBMCs

Blood samples were collected by venipuncture in heparin tubes BD Vacutainer® CPT™ containing Ficoll-Hypaque solution (Becton Dickinson, USA). PBMCs were separated from whole blood based on Ficoll-Hypaque density gradient according to the manufacturer's instructions. PBMCs were carefully collected from the thin interface layer between red blood cells and plasma. The cells were then washed to remove platelets, and the PBMCs were suspended in 1ml of phosphate-buffered saline (PBS) solution and counted using the ABX Pentra 60 equipment (Horiba, Japan).

FACS detection of DENVs from PBMCs

To standardize the FACS approach for the detection of DENVs, human PBMCs from a dengue seronegative adult donor were suspended in 1ml of Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, USA) transferred into a 6-well tissue culture plate, and 3ml of RPMI 1640 medium (Sigma-Aldrich, USA) supplemented with 3% fetal bovine serum and antibiotics (penicillin 100U/ml and streptomycin 100mcg/ml) was added per well. Subsequently, a total of 10^6 PBMCs were infected with 3×10^4 PFU/ml of the DENV-4 strain BeH402276 and incubated at 37°C in an atmosphere of 5% CO₂ for six days. The DENV-4 strain BeH402276 was isolated from a patient with dengue fever on the 1st of June, 1982 at Belém City by the Evandro Chagas Institute, Brazil and was kindly provided by Dr. Robert E. Shope from the University of Texas Medical Branch at Galveston. Subsequently, 100µl of PBMCs from healthy individuals was infected *in vitro* with DENVs, and the PBMCs from patients were incubated with 7µl of a mixture of murine ascitic fluids from animals hyper immunized with all four serotypes of DENV⁸. Then, PBMCs infected *in vitro* with DENVs and the PBMCs from patients were permeabilized and fixed with a FIX & PERM Cell Permeabilization Kit (Invitrogen, USA) according to the manufacturer's instructions. Subsequently, the PBMCs were incubated for 20 minutes with 7µl of anti-mouse immunoglobulin G (IgG) antibodies conjugated with fluorescein isothiocyanate (FITC) (Sigma, USA) and submitted to the BD FACSCalibur™ platform (Becton Dickinson, USA).

The subpopulations of PBMCs infected *in vitro* with DENVs and the PBMCs from patients were fractionated by FACS using the following conjugated monoclonal antibodies capable of specifically recognizing different populations of white blood cells (WBCs): allophycocyanin-conjugated anti-CD3 antibodies bind to T cells, phycoerythrin-conjugated anti-CD4 antibodies to T helper cells, protein chlorophyllated piperidine-conjugated anti-CD8 antibodies to cytotoxic T cells, allophycocyanin-conjugated anti-CD14 antibodies to monocytes, protein

chlorophyllated piperidine-conjugated anti-CD19 antibodies to B lymphocytes, and phycoerythrin-conjugated anti-CD56 antibodies to natural killer cells (NKs)

The purity of the fractionated cells was evaluated by immunostaining for cell surface markers using a BD FACSCalibur™ platform (BD Biosciences, USA). The PBMCs infected *in vitro* with DENVs and the PBMCs from patients were analyzed after 8 hours of immunostaining in the BD FACSCalibur™ platform (BD Biosciences, USA) with a 488-nm argon laser and Cell Quest™ Software (BD Biosciences, USA). Monocytes and lymphocytes were identified by their forward and side scatter characteristics, and enclosed in an electronic gate for fluorescence intensity analysis. Nonspecific fluorescence was reduced by using an antibody of the same isotype that was not specific to the antigenic sites of the samples in the analysis, and the background was also quantified in the negative control blood cells to avoid interference in the results. The results were calculated as the average percent of 10,000 single cell events in the analysis gate.

Real-time RT-PCR, nested RT-PCR, and immunochromatographic assay

The viral ribonucleic acid (RNA) in the blood samples was extracted with a QIAamp viral RNA extraction kit (Qiagen, Germany) and converted into double-stranded complementary deoxyribonucleic acid (cDNA) using Superscript III reverse transcriptase (Invitrogen, USA) according to the manufacturer's instructions. We then performed a real-time nested reverse transcription polymerase chain reaction (RT-PCR) generic to flaviviruses⁹, and a nested PCR to identify dengue serotypes¹⁰. In addition, all samples were tested for the presence of non-structural protein 1 (NS1) using the immunochromatographic strip test (BioClin™, Brazil) for NS1 antigen capture according to the manufacturer's instructions. The inter-rater agreement for the three methods employed was measured by the Cohen's kappa coefficient^{11,12}.

RESULTS

The FACS approach detected a total of 4.7% DENV-positive cells from PBMCs infected *in vitro* with the DENV-4 strain BeH402276, and 0.7% DENV-positive cells in uninfected cells (negative control), which was considered as the background in this assay (**Figure 1A** and **Figure 1B**). These results were supported by real-time RT-PCR, which confirmed by amplification, the presence of the DENV viral genome in the PBMCs infected *in vitro* with DENV-4, but not in those of the uninfected sample.

The blood samples of 152 patients were tested by FACS and the NS1 immunochromatographic assay, including the 134 samples that were also examined by real-time RT-PCR. In the 134 samples tested by all assays, we observed a total of 29.1% (39/134) DENV-positive patients (**Figure 2A**). The immunochromatographic assay showed the highest number of DENV-positive samples and detected the NS1 protein in 40.1% (56/134) of the patient samples (**Figure 2A**). The FACS approach and real-time RT-PCR presented positive confirmation for DENV in 39 and 44 samples,

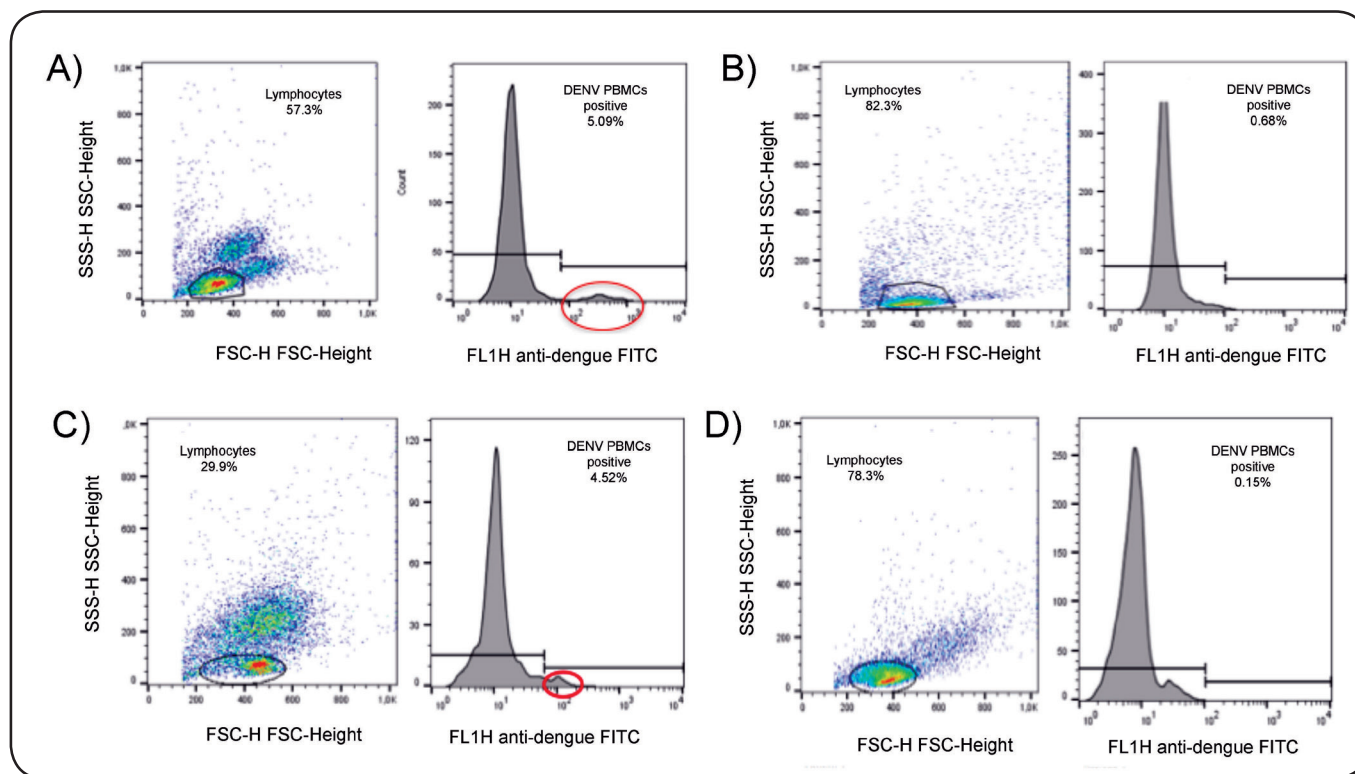


FIGURE 1: FACS-based diagnosis of dengue virus in PBMCs infected *in vitro* with dengue virus **A)** uninfected PBMCs, **B)** PBMCs of patients infected with dengue virus, **C)** and PBMCs of patients negative for dengue virus **D).** **DENV:** dengue virus; **PBMCs:** peripheral blood mononuclear cells; **SSS-H:** side scatter height; **SSS-C:** side scatter; **FSC-H:** forward scatter height; **FL1H:** intensity (height) in the flow cytometry channel 1; **FITC:** fluorescein isothiocyanate; **FACS:** flow cytometry assay.

respectively (**Figure 2A**). In addition, the Cohen's kappa coefficient showed a substantial agreement among the three methods (**Figure 2A**). Unfortunately, 18 samples could not be tested by real-time RT-PCR. Additionally, RT-nested PCR revealed that 95.5% (42/44) of the patients were infected with DENV-1 and 4.5% (2/44) with DENV-2 (**Figure 2B**). All samples with a positive diagnosis of DENV showed more than 1.6% infected cells in the respective PBMCs by FACS (**Figure 1C** and **Figure 1D**). Interestingly, the 19 DENV-positive samples detected by the NS1 immunochromatographic assay had infected PBMCs, as identified by FACS, but showed less than 1.5% infected cells.

The patients infected with DENV-2 presented an average ratio of 3.6% DENV infected PBMCs on the first day of febrile symptoms, but this ratio decreased to 2.0% in the samples collected on the fifth day after the initiation of clinical signs. The inverse correlation between the percentage of DENV infected PBMCs and the number of days after onset the disease was significant based on the Spearman correlation coefficient, which showed an r value of -0.96 (p -value = 0.0043). Interestingly, the subpopulations of PBMCs infected by DENV were predominantly T cells (CD3+ and CD4+) at an average of 73.7%, which also include the 32.1% of T helper cells. On the other hand, DENV positivity was detected at 8.5% in the B cells, 8.0% in the NK cells, and at 1.9% and 0.6% in the cytotoxic T cells and monocytes, which were the minority cell populations infected with DENVs, respectively.

DISCUSSION

Flow cytometry is used to detect and quantify viral antigens on the surface or within infected cells of human immunodeficiency virus (HIV), orthohantavirus, herpes simplex virus, rotavirus, and other viruses¹³⁻¹⁷. Additionally, this approach has been used to diagnose and titer flaviviruses of importance in veterinary and human public health, such as bovine viral diarrhea virus and DENV¹⁸⁻²⁰. In this study, we showed that the DENV in clinical samples of patients with acute febrile symptoms can be rapidly detected using PBMCs by FACS, as demonstrated by the observed 29.1% DENV positivity that includes two patients with DENV-1 and 42 patients with DENV-2. This data demonstrates that FACS can detect all genotypes of DENV, in addition to DENV-4, which was used in the *in vitro* infection of PBMCs. This approach can be useful because PBMCs are the primary target cells in DENV infection^{21,22}.

Our study showed that the subpopulations of PBMCs infected by DENV were predominantly T cells, especially T helper cells, and a representative proportion of B cells. This fact has also been reported in previous studies, which have demonstrated that T and B cells are the target cells in dengue infection²³⁻²⁶. Another hypothesis suggests that monocytes are the primary targets in DENV infection and that T and B cells are not infected^{27,28}. However, we did not observe this in our study, and in the contrary found that the smallest subpopulation

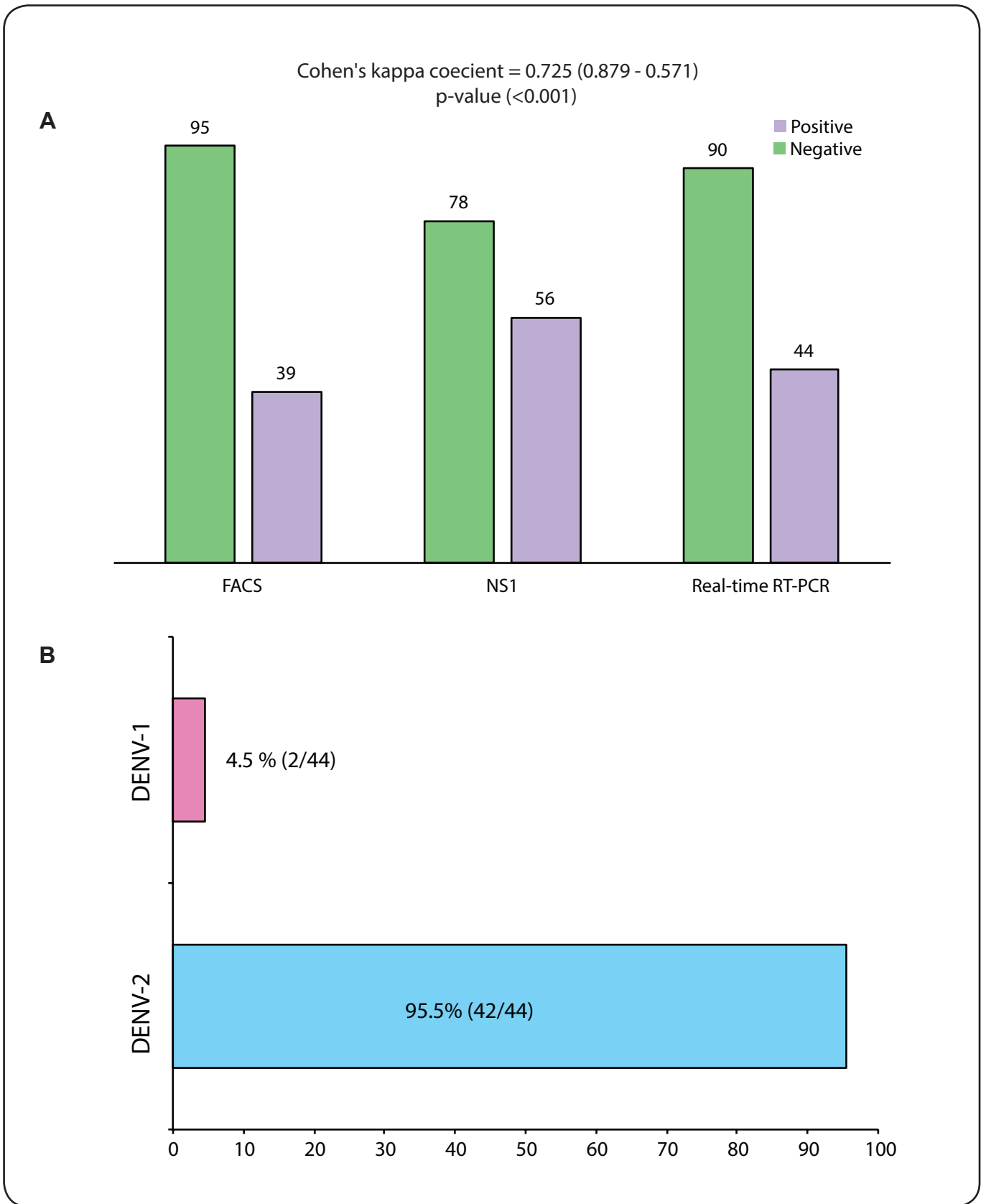


FIGURE 2: Frequency of dengue virus positivity in patients by FACS, NS1 and RT-PCR. **A)** Numbers of negative and dengue positive samples are highlighted in green and purple, respectively. **B)** The serotype of dengue-positive samples, dengue serotype-1 and 2, are highlighted in pink and blue, respectively. **DENV:** Dengue virus; **FACS:** flow cytometry assay; **NS1:** non-structural protein 1; **RT-PCR:** reverse transcriptase polymerase chain reaction.

of PBMCs infected by DENV was monocytes, making up only 0.6% of the infected cells. Our results reinforce that T and B cells are the major target cells in dengue infection. However, the time and severity of infection, primary or secondary nature of infection, and genetic and immunity factors may affect the subpopulations cells of PBMCs^{25,26,29-31}. In addition, further studies analyzing the mononuclear cells in primary and secondary dengue infections, as well as at varied severities of the infection may be important for elucidating the mechanism of dengue pathogenesis.

Results from all the diagnostic methods employed in this study showed a substantial agreement among them. The FACS approach to diagnose DENVs in PBMCs showed results similar to those obtained by RT-PCR with a concordance of 97.7%. However, the FACS approach provides a quantification of the infected PBMCs that can be correlated to the DENV load in patients³². On the other hand, the FACS approach diagnosed a lower number of DENV-positive patients compared to the NS1 assay. This could be probably due to the multiple antigenic determinants that are expressed, both intracellularly and on the cell surface, in the NS1 protein, which is involved in enhancing viral RNA replication and also in the release of infectious viral particles¹⁹. Additionally, the temporal increase in antiviral immunity, including the appearance of neutralizing antibodies, causes reduced DENV viremia and probably decreases the number of infected cells in blood³³.

In summary, the FACS approach using highly reactive antibodies that are specific to the antigenic components of DENVs may be quite useful for the diagnosis in outbreak, and surveillance of DENV in Brazil. This assay can also be implemented in public and private laboratories that have a large number of flow cytometers available for monitoring patients infected with HIV. Additionally, this assay enables quantification of the number of PBMCs infected by DENVs; thus, it can be easily implemented to monitor viral activity and as well as to measure viral load in pathogenesis studies.

Conflict of interest

The authors declare that there is no conflict of interest.

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