ASIAN PACIFIC JOURNAL OF ALLERGY AND IMMUNOLOGY (2001) 19: 139-144

Application of HIV-1-Green Fluorescent Protein (GFP) Reporter Viruses in Neutralizing Antibody Assays

Prasert Auewarakul, Verapong Paungcharoen, Suda Louisirirotchanakul and Chantapong Wasi

SUMMARY **We made reporter HIV-1 DNA constructs carrying green fluorescent protein (GFP) gene and exchangeable *env* of subtype E. The recombinant constructs were used to produce infectious reporter viruses, which induced infected cells to emit green fluorescent light and rendered them easily detectable at single cell level. Because the *env* in this construct can be easily exchanged, viruses with different antigenic epitopes can be made. We used these reporter viruses to set up a neutralizing antibody assay based on fluorescence reduction by flow cytometric measurement. The result of this new assay correlated with the standard infectivity reduction assay using primary isolates. Because this new assay is faster and much less costly than the standard assay using a p24 endpoint and can be performed in peripheral blood mononuclear cells (PBMC), it provides a useful tool for analysis of HIV-1 immune responses.**

Conventional detection of HIV-1 infection in cell culture can be done either by morphological changes of infected cells, p24 antigen assay, or reverse transcriptase assay. Detection of morphological changes such as cell ballooning and syncytium formation is not sensitive and can be applied only to cytopathic strains of HIV-1. The p24 antigen and reverse transcriptase assays measure the production and release of viruses into supernatant. They therefore do not provide information on the number of infected cells. Although, the latter two methods are sensitive and quantitative, they are expensive and time consuming. In order to detect and quantitate the infection without using these assays, reporter systems for HIV-1 infection have been developed. These systems can be generally divided into 2 categories. The first one uses cell lines carrying reporter genes under the control of a *tat*-activatable promotor such as HIV-1 LTR. Upon infection by HIV-1, Tat protein is made and the protein will transactivate the reporter gene resulting in production of reporter gene product.[1-3] Another strategy for the reporter system is to put a reporter gene into the HIV-1 genome.[4,5]

GFP is a fluorescent protein from a jellyfish (*Aequorea victoria*). It absorbs ultraviolet or blue light and emits bright green light.[6] It has been widely used as a reporter gene because of its favorable properties including being active in a wide host range, requiring no additional co-factor, and having no deleterious effect on cell function.[7] GFP gene has been inserted into HIV-1 genome replacing *nef* to produce a reporter virus.[4] The HIV-1 GFP reporter virus has been an important tool in HIV research. The limitation of the currently available reporter viruses lies, however, on the heterogeneity of HIV-1. They are not suitable for studies, in which the viral heterogeneity is an important issue, for example, neutralizing antibody studies. The major determinants of the viral immunological and phenotypical properties lie in the *env* region. We, therefore, constructed a reporter

From the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
Correspondence: Prasert Auewarakul

virus with a built-in strategy for *env* exchange. We used *env* clones from Thai subtype E viruses to construct reporter viruses in order to demonstrate the feasibility of this approach.

HIV-1 neutralizing antibody assays have a variety of endpoint measurements, including syncytia or plaque formation, reverse transcriptase activity, viral p24 core protein production, Tat-dependent expression of reporter genes, and viral DNA.[8-10] Among these, p24 production measured by antigen-captured enzyme-linked immunosorbent assay (ELISA) is the most widely used method. This assay requires several cycles of cell washing to remove the input virus, as well as residual antibody against p24 in serum that may interfere with p24 measurement resulting in erroneous neutralization results. Tat-dependent expression of reporter genes, such as green fluorescent protein (GFP), has been employed to setup systems for detection of HIV infection in engineered cell lines.[1-3] These cell lines have been used to provide the fluorescence reduction as an alternative endpoint for HIV neutralizing antibody assays.[9] Although, this method has advantages of being simple, less time consuming, and much less costly than the standard p24 production endpoint assay, its limitation lies in the target cell to be used. It has to be performed in specially engineered cell lines that harbor the reporter GFP gene under the control of a Tat-dependent promoter. Because cell types used in neutralization assays affect the viral sensitivity to neutralizing antibodies,[11-13] the inability of the assay to perform in peripheral blood mononuclear cells (PBMC) is its major drawback. To overcome this problem, we developed a GFP-fluorescence reduction based neutralization assay that can be performed in PBMCs.

MATERIALS AND METHODS

DNA construction

The HIV-1 construct, HXB2-AscNotI, with restriction sites for *env* swapping was provided by Dr. Tun Hou Lee. It was generated by introducing the endonuclease restriction sites *Asc* I and *Not* I into a prototype infectious molecular clone of HIV-1 subtype B, HXB2-RU3,[14] immediately up- and down-stream to the *env* open reading frame (ORF), respectively. The introduction of the *Asc* I site resulted in a frame shift-mutation of the *vpu* ORF. Therefore, the HXB2-AscNotI was *vpu*-negative. A fragment containing a GFP gene was PCR amplified from the pGFP plasmid (Clontech) using upstream and downstream primers that contained *Not* I and *Xho* I restriction sites, respectively. This amplified fragment was then cloned into HXB2-AscNotI replacing the *Not* I-*Xho* I fragment of the *nef* ORF to obtain the construct HXB2-GFP. The *env* clones were PCR-amplified from HIV-1 primary viruses (#406 and #449) that had been isolated from infected pregnant mothers at Siriraj Hospital in 1995.[15] The isolates had been shown to be syncytial inducing (SI) in the MT-2 cell line and belong to subtype E by full length *env* sequencing. The *env* amplification was done by nested PCR using the primers envA (GGCTTAGGCATCTCCTATGGCAGGAAGAA) and envN (CTGTCAATCAGGGAAGTAGCCTTGTGT) as outer primers,[16] and *Asc* I (GGCGCGCCATGAGAGTGAAGGAGACACAGA) and *Not* I (GCGGCCGCTTATAC(G)CAAAGC(T)CCTTTCTA) as inner primers. The PCR condition was as previously described.[16] The PCR added the *Asc* I and *Not* I sites to the amplified product, which enabled the subsequent swapping into HXB2-GFP to generate HXB2-GFP-406 and HXB2-GFP-449.

Production of replication competent virus

The constructs HXB2-GFP-406 and HXB2-GFP-449 were transfected into MT-2 cells using DMRIE-C transfection reagent (Gibco BRL, MD, USA) following the manufacturer's instruction. Briefly, lipid-DNA complex was prepared by mixing 4 μg of DNA resuspended in 500 μl of RPMI-1640 medium and 8 μl of DMRIE-C reagent resuspended in 500 μl of RPMI-1640 per 35 mm^3 tissue culture dish. Then, 2 x 10^5 MT-2 cells were washed twice with 1x PBS and incubated with the lipid-DNA complex for 5 hours at 37°C in 5% CO_2. After incubation, 2 ml of RPMI-1640 medium supplemented with 15% fetal calf serum (FCS) was added. The culture was maintained in RPMI-1640/15% FCS and monitored daily for fluorescent cells by fluorescent microscope. At 2 weeks after transfection, the virus supernatant was centrifuged at 500 x *g* for 10 minutes to obtain cell-free virus and stored in aliquots at -70°C.

Neutralization assay using HIV-GFP viruses

HIV-1 seropositive sera for neutralizing antibodies testing was randomly collected from routine samples sent for HIV-1 testing at the Virology Laboratory, Siriraj Hospital. The sera were heat-inactivated at 56°C for 30 minutes and stored in aliquot at -20°C until tested. The target cells for the neutralization assay were prepared as follows: PBMCs were separated from HIV-1 sero-negative blood by cen-

trifugation on Ficoll density cushion and cultured in RPMI-1640/10% FCS. $CD8^+$ cells were depleted from the PBMC population by immunomagnetic separation using CD8 immunomagnetic beads (Dyna Tech). Before infection, $CD8^+$-depleted PBMCs were stimulated by 10 μg/ml of phytohemagglutinin (PHA) for 3 days.

The tested sera and normal human serum (NHS) were diluted in 15% FCS supplemented RPMI-1640 medium, 10 IU/ml of interleukin-2 (IL-2), starting with a dilution of 1:20 followed by three five-fold serial dilutions. Chimeric-GFP viruses were titrated and the amount that gave 100-200 fluorescent cells/20,000 cells was used in the neutralization assay. The test was run in triplicates. Equal volumes of diluted sera preparation and viruses were mixed and incubated at 37°C for 1 hour. Then, 110 μl of the virus-antibody mixture was added into duplicated wells containing 1 x 10^5 cells/well of pre-stimulated $CD8^+$-depleted PBMCs. The infected cells were maintained in the presence of 10 IU/ml of IL-2. After overnight incubation at 37°C in 5% CO_2, the supernatant was carefully discarded, 150 μl of RPMI-1640/10% FCS/10 IU/ml IL-2 was added and the cultures were incubated at 37°C in 5% CO_2 for 3 days. After 3 days, the cells were fixed with 300 μl of 3% paraformaldehyde and analyzed on a FACScan flow cytometer using CellQuest software. The neutralization activity was presented as % fluorescence reduction, which was calculated from the formula: (number of fluorescent cells with NHS – number of fluorescent cells with tested serum)/number of fluorescent cells with NHS.

Neutralization assay using a p24 production endpoint

The standard infectivity reduction assay (IRA) for neutralizing antibody detection was performed as described.[17] Seventy-five microliter of serial 5-fold dilutions of virus stock were pre-incubated with the 75 μl of 1:20 diluted tested sera or NHS control in quadruplicates for 1 hour at 37°C in 96 well, U-plate, prior to the addition of 75 μl of 1 x 10^5 PHA-stimulated PBMCs from two seronegative donors. The culture was washed on days 1, 2 and 3 and supernatant was collected on day 7 for p24 antigen captured-ELISA as previously described.[17] The neutralizing activity of antibodies is expressed as virus neutralization index (*V*ni) which is the ratio of the 50% tissue culture infectious dose ($TCID_{50}$) using Spearman-Karber's formula in the presence of NHS and the $TCID_{50}$ in the presence of tested sera. *V*ni < 3 was considered negative, while 3-9 indicated weak, and ≥ 10 indicated strong neutralizing activity.

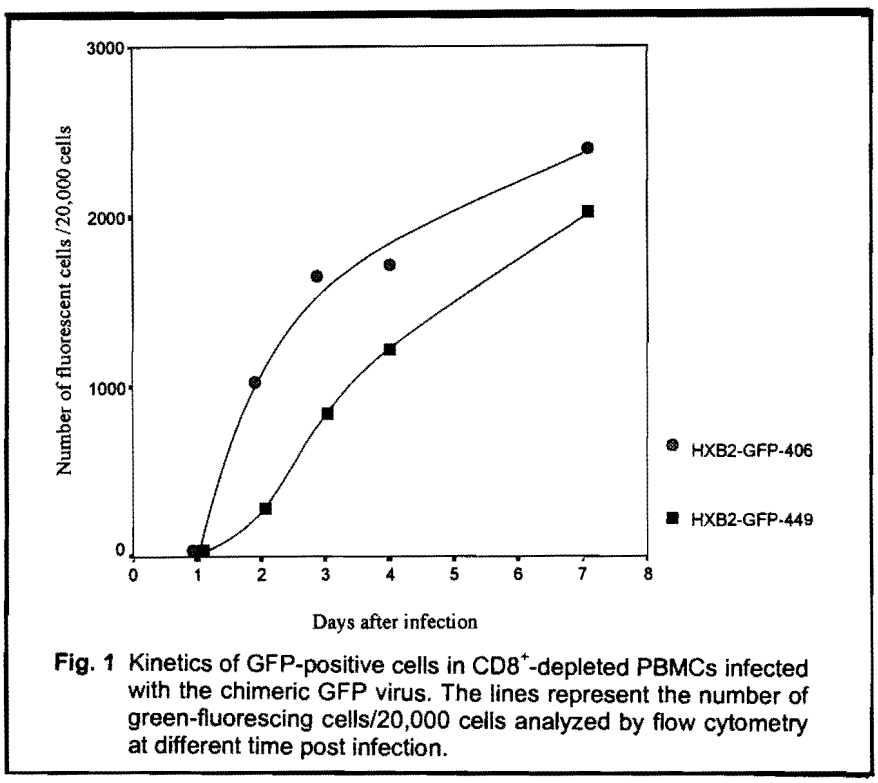

Fig. 1 Kinetics of GFP-positive cells in $CD8^+$-depleted PBMCs infected with the chimeric GFP virus. The lines represent the number of green-fluorescing cells/20,000 cells analyzed by flow cytometry at different time post infection.

RESULTS

Green fluorescence was found in 1-5% of MT-2 cells transfected with the HXB2-GFP-406 and –449 at day 2 after transfection. The number of fluorescent cells increased thereafter to reach the peak in 2 weeks when about 80% of the cells were positive. When viral supernatant from transfected MT-2 cells was used to infect $CD8^+$-depleted PBMCs, the number of fluorescent cells increased rapidly during the first three days after infection. Thereafter they continued to increase at a slower rate until day 7 (Fig. 1). After day 7, the number of positive cells declined while auto-fluorescing dead cells were increasingly prominent. In both MT-2 and PBMCs, the green fluorescence was observed in both individual cells with normal morphology and in syncytium (data not shown). This indicated that the expression of GFP started at a very early phase

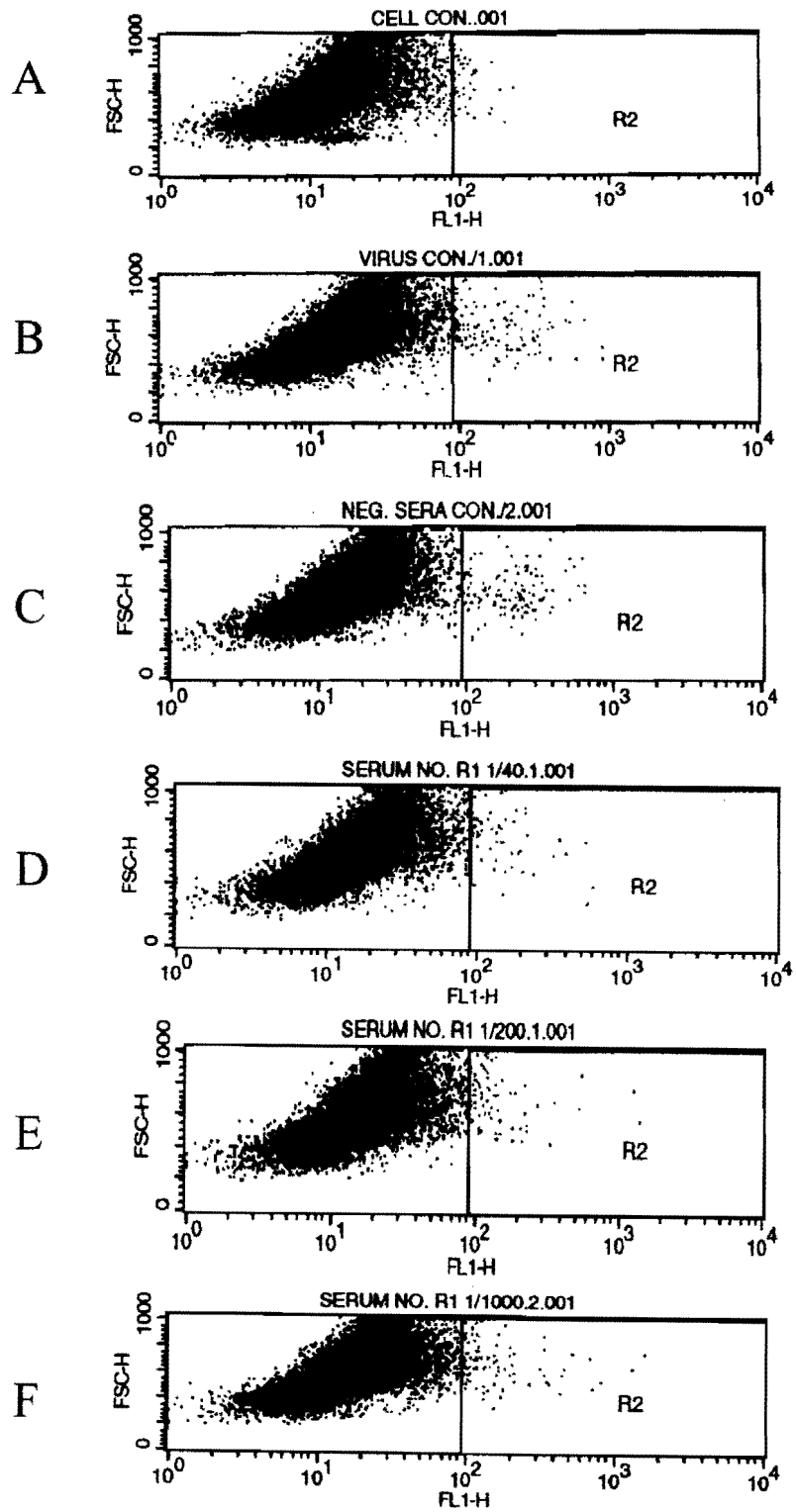

Fig. 2 A typical flow cytometric analysis of fluorescent reduction assay. Each dot plot represents uninfected cell control (A), virus control (B), serum control (C), tested serum at dilution 1:40, 1:200, and 1:1,000 (D, E, and F, respectively). Each dot in the right windows of the plots represents one cell with positive green fluorescence.

in the viral replication cycle when cytopathic effects were not yet apparent, and that the GFP expression continued into late phases when syncytium had been formed.

Fig. 2 shows a typical neutralization analysis by GFP-fluorescence reduction. Uninfected control cells, which were processed in parallel, were analyzed to account for autofluorescent cells, which were always found to be minimal. Virus controls were taken from infection samples without pre-incubation with serum to verify the infectivity titer of the virus stocks. While pre-incubation with NHS (serum control) did not alter the number of fluorescent cells compared to the virus controls, pre-incubation with tested sera with neutralizing activity resulted in a reduction of fluorescent cells in a concentration-dependent manner.

The two primary isolates, ie. #406, #449 were selected because they showed different sensitivity to neutralization by sero-positive sera. As shown in Table 1, #406 was resistant to neutralization by almost all sera, while significant neutralization activity against #449 was seen in some sera. For #406, only one serum showed significant neutralization with *V*ni of 31.5, while the other 11 sera showed negative (*V*ni < 3) or borderline (*V*ni 3-9) neutralization. For #449, seven out of 12 sera showed positive neutralization with *V*ni ≥ 10, and the other 5 sera showed negative (*V*ni < 3) or borderline (*V*ni 4-9) neutralization.

The new neutralizing antibody assay showed good agreement with the standard assay using p24 as endpoint. Out of 24 virus-serum pairs, 21 showed concordant results (Table 1). The three discordant results were as follows. One serum

Table 1 The viral neutralization index (*Vni*) of primary isolates by IRA, % fluorescence reduction (%NT) and fold enhancement of GFP-viruses at the serum dilution of 1:40

Virus	Serum number	*Vni* (primary isolate)	% NT (GFP virus)	Fold enhancement
406	1	4.3	0	-
406	2	4.3	0	2.4
406	5	5.2	0	2.2
406	8	0.7	0	5.3
406	10	1.3	0	5.2
406	11	31.5	0	4.2
406	12	2.7	0	1.4
406	13	1	0	2.2
406	14	2.3	0	2
406	P14	3.6	0	3.8
406	P18	0.2	0	3
406	P23	5.1	0	2.9
449	2	44	79	-
449	11	1.6	17	-
449	12	0.4	71	-
449	P23	14	80	-
449	R1	14.9	72	-
449	R2	10.4	88	-
449	R3	10.4	75	-
449	R4	10.4	58	-
449	R5	5.1	29	-
449	R6	2.9	44	-
449	R7	2	0	-
449	R8	21.7	34	-

(#11) neutralized the primary isolate 406 in IRA but enhanced HXB2-GFP-406 in the new neutralization assay. Another serum (#R8) strongly neutralized the primary isolate 449 in IRA with *Vni* of 21.7 but showed only weak neutralization against HXB2-GFP-449 with a 34% fluorescence reduction, and serum #12 was negative against the primary isolate 449, but neutralized HXB2-GFP-449 with a 71% fluorescence reduction. In agreement with IRA, HXB2-GFP-406 was resistant to neutralization. It might represent strains with global neutralization resistant phenotypes, which have been proposed to be more susceptible to antibody-mediated enhancement.[15] While none of the sera enhanced the neutralization-sensitive HXB2-GFP-449, almost all sera enhanced the neutralization-resistant HXB2-GFP-406.

DISCUSSION

An HIV-1 molecular construct carrying reporter gene that can be detected easily *in situ* is of great benefit for HIV research. It can be used for detection of infection in living cells as well as the inhibition of infection as in neutralizing antibody assays or antiviral agent testing. GFP is a reporter that can be detected in living cells without any manipulation or processing. The currently available HIV-1 GFP reporter construct is a molecular clone of HIV-1 subtype B. Because the *env* in this clone cannot be exchanged, its use for neutralizing antibody assays is limited. We therefore constructed an HIV-1 GFP reporter virus in which the *env* can be exchanged. In this construct, we inserted an *env* of subtype E, which is the prevalent virus in Thailand. The reporter virus was replication-competent and could be detected easily in T-cell lines as well as in PBMCs. Because the *env* in this construct can be easily exchanged, viruses with different antigenic epitopes as well as different cell tropism can be made. These viruses will be beneficial for biological and immunological studies of HIV-1.

Although cultured primary isolates are more homogeneous than *in vivo* viruses, they still have some degree of heterogeneity. Therefore, the cloned *env* could not represent all the quasispecies in the primary isolates. This might lead to the discordant result in 3 virus-isolate pairs. For a primary isolate that contains both neutralizing antibody sensitive and resistant variants, molecular cloning might randomly pick up a clone from a minority variant resulting in different phenotypes between the bulk quasispecies and the clone. While this might be a weak point of the method, it provides the advantage of the ability to study the phenotype of a single clone and the results of any sequence changes on the phenotype.

In this study we demonstrated the feasibility of a new HIV-1 neutralizing antibody assay using reporter GFP viruses. Compared to the standard assay using p24 antigen endpoint, this new

assay is faster, does not require excessive washing to remove the input virus or serum and does not require expensive assays to measure the endpoint. This assay provides an inexpensive alternative tool for studying the immune responses to HIV-1.

ACKNOWLEDGEMENTS

This work was supported by a research grant from the Ministry of University Affairs, Thailand and the Japan Foundation for AIDS Prevention. The HXB2-AscNot I construct was kindly provided by Dr. Tun Hou Lee of the Harvard AIDS Institute.

REFERENCES

1. Dorsky DI, Wells M, Harrington RD. Detection of HIV-1 infection with a green fluorescent protein reporter system. J Acquir Immune Defic Syndr Hum Retrovirol 1996; 13: 308-13.
2. Dorsky DI, Harrington RD. An indicator cell assay for T-cell tropic, macrophage-tropic, and primary isolates of HIV-1 based on green fluorescent protein. J Acquir Immune Defic Syndr 1999; 22: 213-20.
3. Kar-Roy A, Dong W, Michael N, Li Y. Green fluorescent protein as a transcriptional reporter for the long terminal repeats of the human immunodeficiency virus type 1. J Virol Methods 2000; 84:127-38.
4. Page KA, Liegler T, Feinberg MB. Use of a green fluorescent protein as a marker for human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses 1997; 13: 1077-81.
5. Muthumani K, Montaner LJ, Ayyavoo V, Weiner DB. Vpr-GFP virion particle identifies HIV-infected targets and preserves HIV-1 Vpr function in macrophages and T-cells. DNA Cell Biol 2000; 19: 179-88.
6. Chalfie M. Green fluorescent protein. Photochem Photobiol 1995; 62: 651-56.
7. Cubitt AB, Heim R, Adams SR, *et al.* Understanding, improving and using green fluorescent proteins. Trends Biochem Sci 1995; 20: 448-55.
8. Golding H, D'Souza MP, Bradac J, Mathieson B, Fast P. Neutralization of HIV-1. AIDS Res Hum Retroviruses 1994; 10: 633-43.
9. Cecilia D, Kewalramani VN, O'Leary J, *et al.* Neutralization profiles of primary human immunodeficiency virus type 1 isolates in the context of coreceptor usage. J Virol 1998; 72: 6988-96.
10. Achkar JM, Wang XH, Nyambi P, Gorny MK, Zolla-Pazner S, Bandres JC. Polymerase chain reaction-based assay for antibody-mediated neutralization of HIV-1 reveals a population of nonneutralized virus undetected by conventional p24 assay. J Acquir Immune Defic Syndr 2000; 24:203-10.
11. Montefiori DC, Collman RG, Fouts TR, *et al.* Evidence that antibody-mediated neutralization of human immunodeficiency virus type 1 by sera from infected individuals is independent of coreceptor usage. J Virol 1998; 72: 1886-93.
12. Zolla-Pazner S, Sharpe S. A resting cell assay for improved detection of antibody-mediated neutralization of HIV-1 primary isolates. AIDS Res Hum Retroviruses 1995; 11; 1449-57.
13. Zolla-Pazner S, Lubeck M, Xu S, *et al.* Induction of neutralizing antibodies in T-cell line-adapted and primary human immuodeficiency virus type 1 isolates with a prime-boost vaccine regimen in chimpanzees. J Virol 1998; 72: 1052-59.
14. Fisher AG, Collalti E, Ratner L, Gallo RC, Wong-Staal F. A molecular clone of HTLV-III with biological activity. Nature 1985; 316: 262-65.
15. Auewarakul P, Louisirirotchanakul S, Sutthent R, *et al.* Analysis of neutralizing and enhancing antibodies to human immunodeficiency virus type 1 primary isolates in plasma of individuals infected with env genetic subtype B and E viruses in Thailand. Viral Immunol 1996; 9:175-85.
16. Gao F, Yue L, Craig S, *et al.* Genetic variation of HIV type 1 in four World Health Organization-sponsored vaccine evaluation sites: generation of functional envelope (glycoprotein 160) clones representative of sequence subtypes A, B, C, and E. AIDS Res Human Retroviruses 1994; 10: 1359-68.
17. Beddows S, Louisirirotchanakul S, Chiengsong-Popov R, Easterbrook PJ, Simmonds P, Weber JN. Neutralization of primary and T-cell line adapted isolates of human immunodeficiency virus type 1: role of V3 specific antibodies. J Gen Virol 1998; 79: 77-82.