Acta Medica Okayama

Volume 49, *Issue* 2 1995 *Article* 2

APRIL 1995

Detection of reverse transcriptase activity by enzyme-linked immunosorbent assay in human immunodeficiency virus type 1.

Noriko Tomita* Masayuki Miyahara† Hiromasa Satoh‡

Kazuo Suzuki** Koichi Kitajima†† Kanji Miyamoto‡‡

*Okayama Red Cross Blood Center,

†Okayama Red Cross Blood Center,

‡Okayama Red Cross Blood Center,

**Biomedical Research Center,

††Okayama University,

‡‡Biomedical Research Center,

Detection of reverse transcriptase activity by enzyme-linked immunosorbent assay in human immunodeficiency virus type 1.*

Noriko Tomita, Masayuki Miyahara, Hiromasa Satoh, Kazuo Suzuki, Koichi Kitajima, and Kanji Miyamoto

Abstract

An enzyme-linked immunosorbent assay (ELISA) using biotin-labelled oligo-dT primer and digoxigenin (Dig)-dUTP was designed to measure the reverse transcriptase (RT) activity of human immunodeficiency virus type 1 (HIV-1). The ELISA system involves the selective detection step of a newly synthesized cDNA by two specific bindings, biotin-streptavidin binding and alkaline phosphatase (AP)-conjugated anti-Dig-Dig binding, and the enzymatic amplification step to increase coloring generated by AP. This method was used to measure the activity of RT in the culture supernatants of peripheral leukocytes obtained from four anti-HIV-1-positive persons cocultivated with those from four anti-HIV-1-negative persons. RT activity was detected in all of four anti-HIV-1-positive culture supernatants but not in those cultivated with anti-HIV-1-negative supernatants alone. Thus, our improved ELISA for detection of HIV-1 appears to be sensitive enough and useful for routine laboratory work. This non-radioactive method will also be useful for detecting other retroviruses and for screening of RT inhibitors.

KEYWORDS: human immunodeficiency virus, reverse transcriptase, ELISA

---

*PMID: 7542428 [PubMed - indexed for MEDLINE]

ACTA MED OKAYAMA 1995; 49(2): 69-73

Detection of Reverse Transcriptase Activity by Enzyme-Linked Immunosorbent Assay in Human Immunodeficiency Virus Type 1

Noriko TOMITA, Masayuki MIYAHARA, Hiromasa SATOH, Kazuo SUZUKI[a], Koichi KITAJIMA[b] and Kanji MIYAMOTO*[b]

Okayama Red Cross Blood Center, Okayama 700, [a]Biomedical Research Center, Olympus Corporation, Tokyo 192 and [b]School of Health Sciences, Okayama University, Okayama 700, Japan

An enzyme-linked immunosorbent assay (ELISA) using biotin-labelled oligo-dT primer and digoxigenin (Dig)-dUTP was designed to measure the reverse transcriptase (RT) activity of human immunodeficiency virus type 1 (HIV-1). The ELISA system involves the selective detection step of a newly synthesized cDNA by two specific bindings, biotin-streptavidin binding and alkaline phosphatase (AP)-conjugated anti-Dig-Dig binding, and the enzymatic amplification step to increse coloring generated by AP. This method was used to measure the activity of RT in the culture supernatants of peripheral leukocytes obtained from four anti-HIV-1-positive persons cocultivated with those from four anti-HIV-1-negative persons. RT activity was detected in all of four anti-HIV-1-positive culture supernatants but not in those cultivated with anti-HIV-1-negative supernatants alone. Thus, our improved ELISA for detection of HIV-1 appears to be sensitive enough and useful for routine laboratory work. This non-radioactive method will also be useful for detecting other retroviruses and for screening of RT inhibitors.

Key words: human immunodeficiency virus, reverse transcriptase, ELISA

Human immunodeficiency virus type 1 (HIV-1) appears to be the etiologic agent of acquired immune deficiency syndrome (AIDS) (1-5). The genome of HIV-1 encodes structural proteins such as *gag*, *pol*, *env* and regulatory proteins. The *pol* protein has reverse transcriptase (RT) activity. RT activity is most widely used for detecting and quantifying retroviruses since retroviruses need RT to replicate (6). The conventional isotopic RT assay has high sensitivity, but requires much time and labor. In addition, its application is limited because it involves the use of radioactive labels.

We have devised an enzyme-linked immunosorbent assay (ELISA) to measure the RT activity of HIV-1. In this method, the RT activity is quantified as the amount of cDNA which was synthesized by RT using polyA as a template, biotin-labelled oligo-dT as a primer, and digoxigenin (Dig)-dUTP (instead of RI-labelled dTTP) as a substrate. The cDNA strand has biotin and Dig at each side, so it reacts with streptavidin (SA)-coated microwells at the biotin site and with alkaline phosphatase (AP) conjugated-anti-Dig at the Dig site. In addition, the method involves an enzymatic amplification step for coloring generated by AP. We used this ELISA system to measure the RT activity in the culture supernatants of peripheral leukocytes obtained from four anti-HIV-1-positive persons cocultivated with those from four anti-HIV-1-negative persons.

Subjects and Methods

Sample preparation. Blood was obtained from four anti-HIV-1 positive persons. These sera were anti-HIV-1-positive by the particle agglutination test (serodia HIV: Fujirevio, Tokyo, Japan) and Western blotting (Bio Rad, LA, USA). Leukocytes from 10-20 ml of peripheral blood were separated on a Ficoll-Conray gradient, and then cultured at 1×10^6/ml in 25 cm^2 tissue culture flasks with RPMI-1640 medium containing 15% fetal calf serum, interleukin-2 and antibiotics. At the same time, 1×10^6/ml of phytohemagglutinin-stimulated leukocytes from anti-HIV-1-negative healthy volunteers were added to the tissue culture flasks. These cells were incubated at 37°C in a humidified atmosphere (5% CO_2) for about 30

* To whom correspondence should be addressed.

days. On various days after cocultivation, 1 ml aliquots of the culture supernatants were collected for the RT assay. The culture supernatants were placed in 1.5 ml tubes and centrifuged at 5,000 rpm for 5 min at 4°C. The supernatants were transferred to new 1.5 ml tubes and centrifuged at 15,000 rpm for 50 min at 4°C to sediment the viruses. The viral pellet was solubilized in 20 μl of NTET solution [100 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA and 0.1% Triton-X100] on ice. A 10 μl aliquot of the virus lysate was used for the ELISA RT assay.

RT reaction. Ten microliters of NTET solution containing RT sample was transferred to a new microtube. Forty microliters of the RT reaction mixture [6.25 μM Dig-dUTP (Boehringer Mannheim, Germany), 25 mM KCl, 6.25 mM $MgCl_2$, 62.5 mM This-HCl (pH 7.8), 1.25 mM dithiothreitol, 2.5 μg/ml polyA, and 446.4 pmol/ml biotin-labelled oligo-dT (Promega, Madison, WI, USA)] was added to each tube. After 30 min incubation at 37°C, the reaction mixture was boiled for 10 min to inactivate RT compeletely.

Detection of RT products. Microwell module plates (Nunc Roskilde, Denmark) were coated with streptavidin (10 mg/ml in 10 mM acetate buffer at pH 5.4) by incubation at 4°C for 16 h. The coated plates were kept at 4°C until use. Immediately before use, the plates were washed three times with STET buffer [10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA and 0.01 % Tween20]. After the wells were blocked with blocking solution (Boehringer Mannheim) for 30 min at room temperature, the blocking solution was aspirated and wells were washed three times with STET buffer. Ten microliters of the reaction mixture was transferred to the SA-coated well, followed by the addition of 90 μl of buffered saline (0.75 M NaCl, 75 mM sodium citrate). The wells were incubated at 37°C for 30 min, and then washed three times with STET buffer. To the well 100 μl of AP-conjugated anti-Dig (Boehringer Mannheim) diluted 1:5000 in STE buffer [10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA] was added and then incubated for 30 min at 37°C. To remove the free conjugates, the wells were washed six times with STET buffer and four times with TBS [0.05 M Tris-HCl (pH 7.5, 0.15 M NaCl]. The final enzyme reaction was carried out by adding 50 μl of substrate (ELISA amplification system: GIBCO BRL, MD, USA). The wells were incubated at 25°C for 15 min, followed by the addition of 50 μl of amplifier (ELISA amplification system: GIBCO BRL). After the wells were incubated again for 15 min at 25°C, the reaction was stopped by adding 50 μl of 0.3 M H_2SO_4. The absorbance at 492 nm was measured with a microplate reader.

Standard curve for RT activity. Recombinant reverse transcriptase from HIV-1 (HIV-1 RT) (Eiken Chemical, Tokyo, Japan) (7) and avian myeloblastosis virus-reverse transcriptase (AMV-RT) (Du Pont, Boston, MA, USA) were used for standard curve. These RTs were diluted from 10 mU/μl to 0.02 mU/μl with NTET solution. Ten microliters of the solution were used for the RT reaction.

Specificity of RT assay. T7 DNA polymerase (Pharmacia Biotech, Uppsala, Sweden), Vent (exo-) DNA polymerase (MILLIPORE Bedford, MA, USA), Tth DNA polymerase (TOYOBO, Osaka, Japan) and Taq DNA polymerase (Perkin-Elmer, Norwalk, CT, USA) were used in RT reaction instead of RT to check the specificity of the assay.

Isotopic RT assay. Ten microliters of NTET containing AMV-RT was added to a microtube. Forty microliters of the reaction mixture [25 mM KCl, 6.25 mM $MgCl_2$, 62.5 mM Tris-HCl (pH 7.8), 1.25 mM dithiothreitol, 3.6 μg/ml polyA, 2.5 μg/ml oligo-dT_{12-18} (Pharmacia LKB, Uppsala, Sweden) and 5 mCi [^{3}H] dTTP (1 mCi/ml, specific activity 30 Ci/mmol; Amersham, UK)] was added to each tube. After 1 h incubation at 37°C, 50 μl of reaction mixture was spotted onto 3MM filters (Whatman, Madstone, England). The filters were dried and then washed twice (1 h each wash) with 0.1 M sodium pyrophosphate in 5% trichloroacetic acid (TCA), and (1 h each wash) with 5% TCA, respectively, and then washed with 95% alcohol for 10 min. After drying, the filters were placed into scintillation vials. Five milliliters of Scintiverse 2 (Fisher, USA) was added to each vial and then radioactivity levels were measured with a liquid scintillation counter.

Measurement of p24 core antigen of HIV. The p24 core antigen of HIV in the same culture supernatants was measured by HIV antigen enzyme immunoassay (EIA) (Abbott Laboratories, IL, USA).

Results

Standard curve for RT activity. The standard curves using the ELISA RT assay are shown in Fig. 1-a and -b. The ELISA assay gave approximately linear result up to 10 mU of HIV-RT and 5 mU of

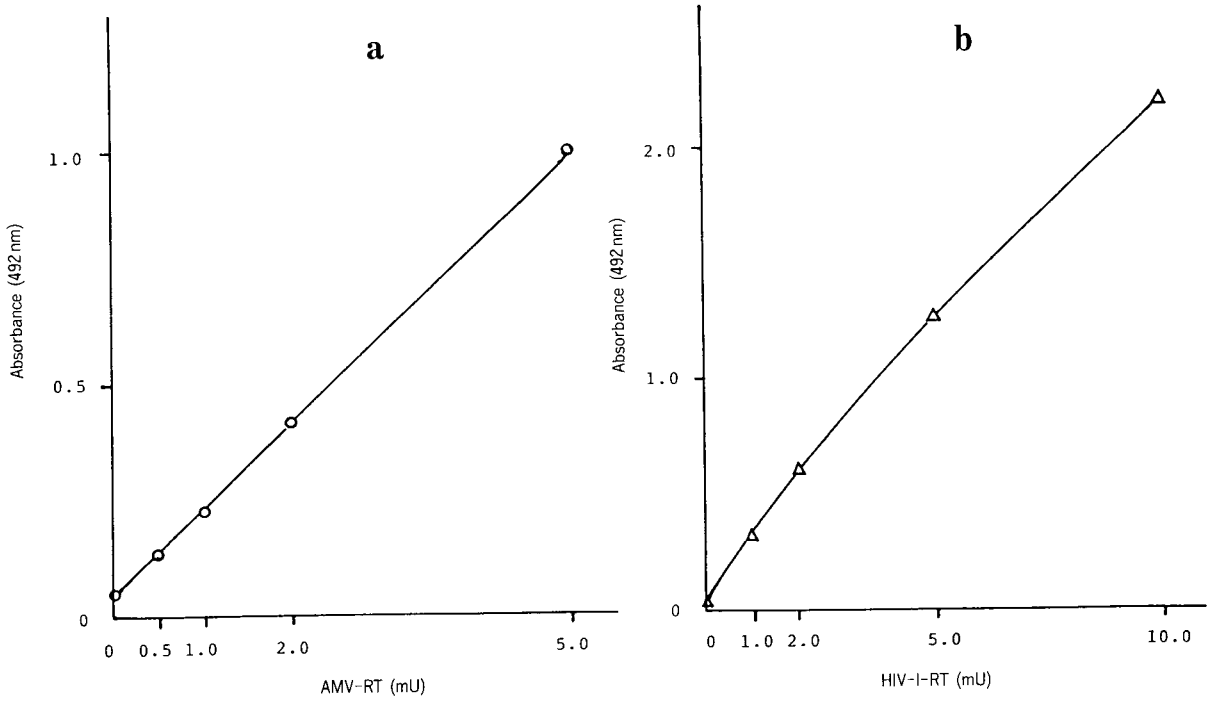

Fig. 1 Standard curves for the enzyme-linked immunosorbent assay (ELISA) of reverse transcriptase (RT) obtained using five concentrations. **a**: Avian myeloblastosis virus (AMV)-RT (0-5 mU). **b**: Human immunodeficiency virus type 1 (HIV-1)-RT (0-10 mU).

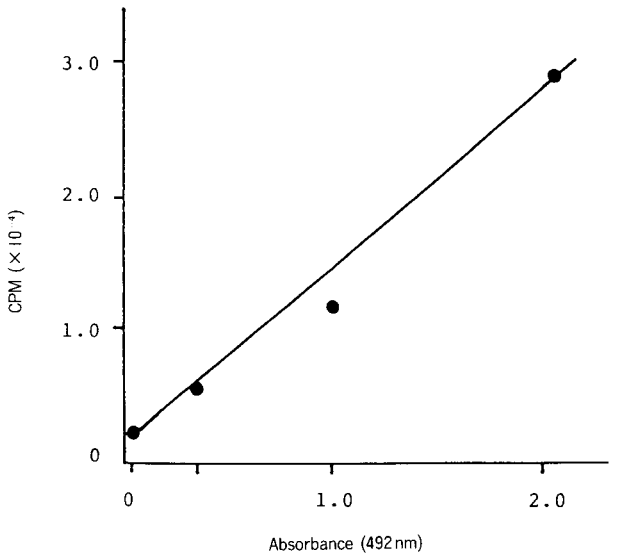

Fig. 2 Correlation between isotopic RT assay and ELISA RT assay. Correlation between isotopic RT assay and ELISA RT assay was indicated with data obtained at four concentrations (0~10 mU/RT reaction) of AMV-RT. CPM: Count per min. RT, ELISA, AMV: See Fig. 1.

AMV-RT.

Correlation of ELISA RT assay and isotopic RT assay. The correlation of the ELISA RT and the isotopic RT assay is shown in Fig. 2 using the data from 0 to 10 mU of AMV-RT. As shown in Fig. 2, there was a good correlation between the two assays ($r \fallingdotseq 0.974$).

Specificity of RT assay. Five kinds of DNA polymerases were used to check the specificity of the RT detection method. All of them showed some incorporation of Dig-dUTP (Table 1). Tth DNA polymerase in particular indicated showed substrate specificity.

RT activity of culture supernatant. The RT activity was detected in all of four supernatants cocultivated with the anti-HIV-1-positive and anti-HIV-1-negative leukocytes after 2-4 weeks of cocultivation, but not in those cultivated with anti-HIV-1-negative leukocytes alone (Fig. 3).

p-24 core antigen of HIV. P-24 core antigen was detected in all of four supernatants cocultivated with the anti-HIV-1-positive samples and anti-HIV-1-negative samples whenever the RT activity was detected (data not

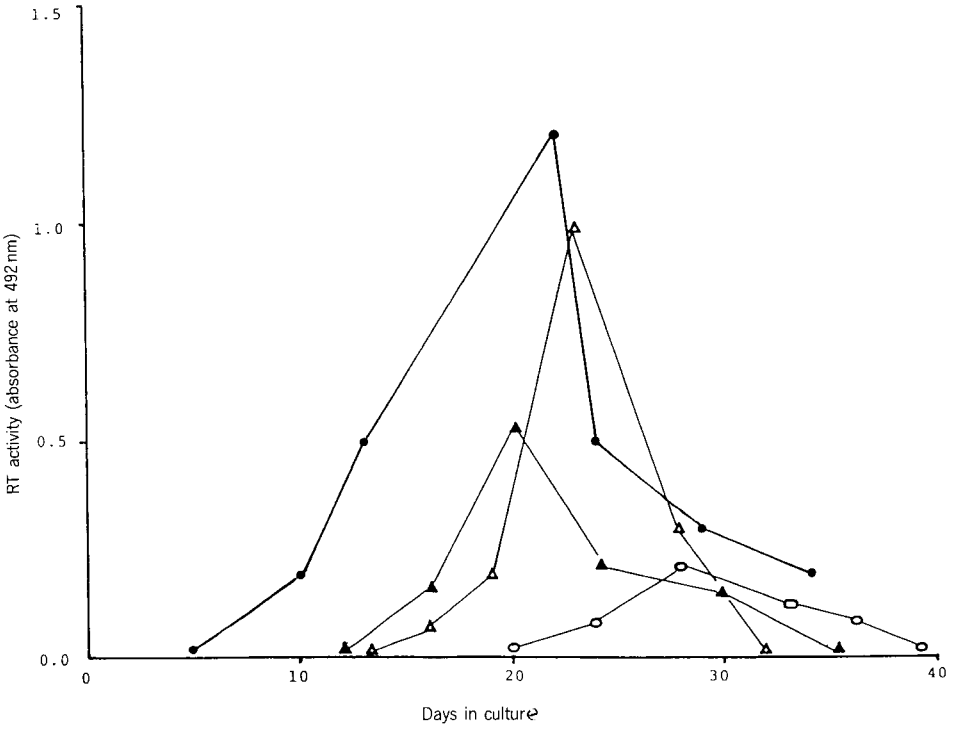

Fig. 3 Kinetics of HIV-1 replication. RT activity is indicated by absorbance at 492nm. Each mark (○●△▲) indicates four HIV-1-positive subjects. The mean of the reagent control of the ELISA RT assay was 0.043.

Table I Reverse transcriptase (RT) reactivities of the DNA polymerases

Concentration	DNA polymerase				
	HIV-RT	T7	Vent(exo-)	Tth	Taq
0.1 mU/RT reaction	0.080	0.058	0.051	0.063	0.089
1 mU/RT reaction	0.327	0.053	0.048	0.167	0.091
10 mU/RT rection	2.121	0.420	0.045	0.968	0.069
100 mU/RT reaction	n.t.	2.026	0.058	2.150	1.931

DNA polymerases [HIV-RT, T7, Vent(exo-), Tth, Taq] were used for RT reaction. Values of RT activity in DNA polymerases are indicated by absorbance at 492nm. n.t. = not tested

shown).

Discussion

Since RT is detected only in retroviruses, measurement of RT activity is extensively used for detecting and quantifying retroviruses. With the expansion of HIV infection, RT assays have been frequently used to confirm HIV infection and for studying HIV inhibitors (1-5, 8). The conventional isotopic RT assay has high sensitivity but has some difficulties in handling as already described. Recently, several methods for detecting RT without use of radioisotopes (RI) were developed (7, 9, 10), but they also require complicated procedures or special equipment.

Here we have improved an ELISA which has high sensitivity and is relatively easy to perform in general laboratories which have no RI rooms or luminometers.

The ELISA RT assay system reported by Suzuki *et al.* (10) consists of an RT reaction step using biotin-labelled oligo-dT and digoxigenin-dUTP and a cDNA detection step using chemiluminescene measured by a luminometer. On the other hand, our ELISA RT assay system involves a unique amplification step instead of chemiluminescene.

In the ELISA amplification step, the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) is dephosphorylated by bound AP to reduced nicotinamide adenine dinucleotide (NADH). NADH activates a secondary enzyme system which involves a redox cycle driven by diaphorase and alcohol dehydrogenase. In this cycle, NADH reduces a tetrazolium salt (iodonitrotetrazolium violet) to form an intensely colored formazan dye in the presence of diaphorase and NADH itself is oxidized to form NAD^+. At the same time, NAD^+ is reduced to NADH and ethanol is oxidized to acetaldehyde by alcohol dehydrogenase. The rate of reduction of the tetrazolium salt is directly proportional to the concentration of NADH originally formed by AP. In these steps, the sensitivity is enhanced more than tenfold over the conventional coloring generated by AP like *p*-nitrophenyl prosphate. The sensitivity of the assay system was so high that 0.5 mU AMV-RT/RT reaction (Fig. 1-a) could be detected, which is almost equal to the conventional isotopic RT assay for AMV-RT (Fig. 2), and does not require the use of special equipment such as a luminometer. In addition, the procedure is so rapid and easy that the assay can be complated less than 2.5 h after the initial 30 min incubation for the RT reaction. Concerning specificity, Tth DNA polymerase seemed to possess the RT activity because its absorbance was as high as half of HIV RT (Table 1). Furthermore, Tth has been reported to have RT activity in the presence of Mn^{++} (11).

In practice, this method detected HIV-RT from all of the four anti-HIV-1 positive samples used in this study. The p24 HIV antigen was also detected in all these samples. This means that the RT activities detected here were almost certainly derived from HIV. HIV isolates have been reported to be divided into two major types, those with rapid/high and those with slow/low *in vitro* replication (12). One of the HIV isolates (Fig. 3) tested here is considered to be the rapid/high type, because it showed large and many syncytia.

In conclusion, the ELISA assay system described here will be useful for the detection of HIV-1 in carriers or patients and for investigating RT inhibitors without the need for radioactive materials.

References

1. Barre Sinoussi F, Chermann JC, Rey F, Nugeyre MT, Chamaret S, Gruest J, Dauguet S, Axler Blin C, Vezinet Brun F, Rouzioux C, Rosenbaum W and Montagnier L: Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science (1983) **220**, 868-871.
2. Gallo RC, Salahuddin SZ, Popovic M, Shearer GM, Kaplan M, Haynes BF, Palker TJ, Redfield R, Oleske J, Safai B, White G, Foster P and Markham PD: Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science (1984) **224**, 500-503.
3. Levy JA, Hoffman AD, Kramer SM, Landis JA, Shimabukuro JM and Oshiro LS: Isolation of lymphocytopathic retrovirus from San Francisco patients with AIDS. Science (1984) **225**, 840-842.
4. Montagnier L, Gruest J, Chamarest S, Dauguest C, Axler C, Gruest J, Nugeyre MT, Barre Sinoussi F, Chermann JC, Brunet JB, Klatzmann D and Gluckman JC: Adaptation of lymphadenopathy-associated virus (LAV) to replication in EBV-transformed B lymphoblastoid cell lines. Science (1984) **225**, 63-66.
5. Sarngadharan MG, Popovic M, Bruch L, Schupbach J and Gallo RC: Antibodies reactive with human T-lymphotropic retroviruses (HTLV-III) in the serum of patients with AIDS. Science (1984) **224**, 506-508.
6. Hoffman AF, Banapour B and Levy JA: Characterization of AIDS-associated retrovirus reverse transcriptase and optimal conditions for its detection in virions. Virology (1985) **147**, 326-335.
7. Saitoh A, Iwasaki H, Nakata A, Adachi A and Shinagawa H: Overproduction of human immunodeficiency virus type I reverse transcriptase in *Escherichia coli* and purification of the enzyme. Microbiol Immunol (1990) **34**, 509-521.
8. Mitsuya H, Weinhold KJ, Furman PA, Clair MHS, Lehrman SN, Gallo RC, Bolognesi D, Barry DW and Broder S: 3'-Azido-3'-deoxythymidine (BWA509u): An antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type III/lymphoadenopathy-associated virus *in vitro*. Proc Natl Acad Sci USA (1985) **82**, 7096-7100.
9. Eberle J and Seibl R: A new method for measuring reverse transcriptase activity by ELISA. J Virol Methods (1992) **40**, 347-356.
10. Suzuki K, Craddock BC, Kano T and Steigbibel RT: Chemiluminescent enzyme-linked immunoassay for reverse transcriptase, illustrated by detection of HIV reverse transcriptase. Anal Biochem (1993) **210**, 277-281.
11. Myers TW and Gelfand DH: Reverse transcription and DNA amplification by a thermus themophilus DNA polymerase. Biochemistry (1991) **30**, 7661-7666.
12. Fenyo EM, Morfeld Manson L, Chiodi F, Lind B, von Gegerfelt A, Albert J, Olausson E and Asjo B: Distinct replication and cytopathic characteristics of human immunodeficiency virus isolates. J Virol (1988) **62**, 4414-4419.

Received October 10, 1994; accepted December 26, 1994.