An Improved Non-Radioisotopic Reverse Transcriptase Assay and Its Evaluation

Takashi NAKANO[1], Kouichi SANO[1], Fumitomo ODAWARA[2], Yukiko SAITOH[1], Toru OTAKE[3], Tsumukata NAKAMURA[1], Katsuhide HAYASHI[1], Hideo MISAKI[2] and Masuyo NAKAI[1]

[1]Department of Microbiology, Osaka Medical College
[2]Diagnostic Division, Asahi Chemical Industry Co., Ltd.
[3]Osaka Prefectural Institute of Public Health

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Abstract

We developed an improved, highly sensitive non-radioisotopic (non-RI) reverse transcriptase (RT) assay (RTA). While the original non-RI method previously reported made use of primer immobilization, our improved method was based on a primer-template immobilization procedure. We tested the template specificity, reproducibility and linearity of the new method in assays of human immunodeficiency virus type-1 (HIV-1) RT. The sensitivities of the method previously reported, the improved method and the sensitive radioisotopic (RI-) RTA were compared in assays of recombinant HIV-1 RT, partially purified HIV-1 particles, and the culture supernatant derived from HIV-1-infected cells. For each of these samples except the culture supernatant the improved method was the most sensitive. It appeared that the fetal bovine serum presented in the culture medium interfered with the assay reaction. The curve describing inhibition of the assay reaction by fetal bovine serum showed that the highest degree of sensitivity in the assay was obtained when the culture supernatant sample was diluted four times. With this degree of dilution, the sensitivity of the new method for assay of culture supernatant sample was still half that of the sensitive RI-RTA. Culture supernatants of five peripheral blood mononuclear cell samples obtained from HIV-1-seropositive carriers were assayed by both the improved method and the sensitive RI-RTA; and with each of the methods, however all virus-positive cultures could be detected. The improved non-RI RTA was considered especially useful for assay of culture supernatants for purposes of virus isolation because of its advantages of excellent sensitivity and lack of requirement for radioisotopes.

Introduction

Establishment of a method to detect human immunodeficiency virus (HIV) is of importance for monitoring the clinical status of patients, isolation of drug-resistant HIV mutants, and identification and evaluation of anti-HIV drugs both *in vivo* and *in vitro*. Practical methods for the detection of HIV making use of enzyme immunoassay (EIA)[1], reverse transcriptase (RT) assay (RTA)[2] and the polymerase chain reaction[3] have been developed. RTA has been used to detect virus particles in both clinical viral isolates and basic investigational viral cultures. Some modifications of the RTA have been devised, including the sensitive radioisotopic (RI) RTA[2], which can be used to detect as few as 97–213 HIV particles. In most

Correspondence to: Kouichi Sano, M.D.
Department of Microbiology, Osaka Medical College, 2-7 Daigaku-machi Takatsuki-shi Osaka 569, Japan

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RTAs, radiolabeled material is used to detect the product of an RT reaction. RI methods, however, require special facilities or licenses, and radioactive waste from biosafety laboratories has been a problem from both biohazardous and radiohazardous aspects[4,5]. To resolve these problems, non-radioisotopic (non-RI) RTAs have been developed[6)~13)]. RI- and most non-RI methods have required steps for the transfer and harvest of the polymerized product, which are laborious and intricate, and which may compromise the reproducibility of the assay. Our non-RI assay[13)], in which the primers are immobilized, does not require the step of harvesting the product of the RT reaction and is based on the conventional ELISA format, and is, therefore, simpler and easier to use than other RTAs. The sensitivity of our method is almost the same as that of the sensitive RI-RTA[2)] for assay of purified virions. However, it is decreased by fetal bovine serum (FBS) in the culture supernatant[14)]. It is possible to remove FBS by ultracentrifugation of the culture supernatant, but biosafety considerations make this impractical. Therefore, improvements in our assay system without substantial loss of sensitivity due to the presence of FBS are required. In this paper, we report and evaluate an improvement in non-RI RTA.

Materials and Methods

Polymerases and virus samples

Molt-4 cells infected with the HIV-1 strain LAV-1_{BRU}[15)] were cultured and the medium was changed every 3-4 day. The culture fluid was harvested on the 7th and 10th days of culture and clarified by centrifugation at 1,500 ×g for 10 min. The supernatant was passed through a Millipore MILLEX-HA filter (pore size 0.45 μm) and ultracentrifuged at 218,000 ×g for 60 min in a Hitachi 55P-72 ultracentrifuge with an RP-65 rotor. The pellet was lysed with 0.25% Triton X-100, transferred immediately to a cryotube and stored at −80°C, and used as a sample from partially purified HIV-1 particles. The partially purified HIV-1 particles, the clarified culture fluid, and the recombinant HIV-1 RT (rHIV-1 RT: Seikagaku Kogyo Co., Ltd. Tokyo) was used to determine sensitivities. In addition, two purified RTs of Rous-associated virus 2 (RAV-2; Takara Shuzo Co., Ltd., Kyoto) and avian myeloblastosis virus (AMV; Pharmacia LKB Biotechnology, Uppsala, Sweden), and DNA polymerase I of *Escherichia coli* (Klenow fragment; Takara Shuzo) were used to determine template specificity. The reagent used as the diluent in the serial dilution of the RT samples was 0.25% Triton X-100 in sterile distilled water to eliminate the effect of salt on enzymatic activities.

Culture supernatant of fresh HIV-1 isolates

Five peripheral blood mononuclear cell (PBMC) samples obtained from asymptomatic seropositive HIV carriers were cultured in RPMI 1640 medium containing 20% FBS using the method of Otake et al.[16)], and the serially collected culture supernatants (total number, 22) were stored at −80°C.

Non-radioisotopic reverse transcriptase assay

The non-RI RTA devised in this study followed in most respects the method previously reported[13)]. However, we immobilized the primer template and some other modifications were made in the original assay, as follows. First, oligo (dT) was immobilized on an aminated 96-well microtiter plate (Sumitomo Bakelite Co., Ltd, Tokyo) via the 5′-phosphate group by placing 100 μl of a 100 mM 1-methyl-imidazole-HCl buffer (pH 7.0) containing 0.2 μg of oligo $(dT)_{19\text{-}24}$ (Pharmacia LKB Biotechnology) in each well, and incubating the plate at room temperature for 24 hr. The wells were washed with a washing solution containing 0.1 M NaCl, and 0.05 M $MgCl_2$ in 50 mM Tris-HCl (pH 7.5). Then, 10 μg of poly (rA) (Pharmacia LKB Biotechnology) in 100 μl of 50 mM Tris-HCl (pH 7.5) containing 0.8 M NaCl was added to each well and the plate was incubated at 37°C for 18 hr prior to washing. Poly (dA) (Pharmacia LKB Biotechnology) was used instead of poly (rA) to determine template specificity. The primer-template-immobilized plates were dried, sealed with a plastic sheet, and stored at 4°C until use. The reaction mixture was also changed to a

final concentration of 90 mM N-2-hydroxyethyl-piperazone-N'-2-ethanesulfonic acid (pH 7.8), 9 mM $MgCl_2$, 0.45 mM DTT, 1.08 mM glutathione, 0.23% Triton X-100, 1.8% ethylene glycol, 126 mM KCl, 0.01% NaN_3, 3 μM of biotin-11-deoxyuridine triphosphate (Bio-dUTP: ENZO Inc., New York, NY USA), and 58.75 μM dTTP. Fifty microliters of a sample and 50 μl of the reaction mixture were mixed in the well, and the plate was incubated at 37°C for the designated time. The reaction was stopped by the addition of 10 μl of 5 M NaCl for 5 min, and the wells were washed five times with the washing solution described above with 0.02% Tween-20 added. One hundred microliters of streptavidin-alkaline phosphatase (SA-ALP) solution containing 83 ng SA-ALP (Jackson Immunoresearch Inc., West Grove, PA USA) per ml, 0.2 M NaCl and 2% bovine serum albumin in 50 mM Tris-HCl (pH 7.5) was then placed in each well and the plate was incubated at 37°C for 1 hr. The wells were washed five times with the washing solution containing 0.02% Tween 20. Prior to carrying out the colorimetric reaction at 37°C for 30 min, 150 μl of the substrate solution of alkaline phosphatase containing 1 mg of *p*-nitrophenyl phosphate per ml and 1 mM $MgCl_2$ in 1 M diethanolamine-HCl buffer (pH 9.5), was added to each well. The reaction was stopped by adding 50 μl of 1 N NaOH, and the absorbance at 405 nm was measured with a microtiter plate electrophotometer (NJ-2001; Japan InterMed Co., Ltd. Tokyo). Duplicate samples were assayed in each experiment and the results were expressed as mean absorbances.

Radioisotopic reverse transcriptase assay (RI-RTA)

RI-RTA was performed by the method previously reported[2] with 10 μCi of ^{3}H-dTTP (47 Ci/mmol; ICN Pharmaeuticals Inc., Irvine, CA USA) at 37°C for 18 hr.

Results

The kinetics of the RT reaction in the improved non-RI RTA are illustrated in Fig. 1. The reaction was found to be time dependent. The template specificity of the RT assays is shown in Table 1. The three kinds of RT specifically required poly (rA) as template; therefore the absorbance determined with this assay represents RNA-dependent DNA polymerase activity. On the other hand, *E. coli* DNA polymerase I had both RNA- and DNA-dependent DNA polymerase activities. We tested the reproducibility of the results obtained with the assay. Tests with 20, 5, and 1.25 μU of rHIV-1 RT were conducted 10 times; the respective coefficients of variation obtained were 3.7% (absorbance: 1.284 ± 0.048), 4.9% (0.430 ± 0.021), and 6.3% (0.193 ± 0.012). We used quantitative analyses to evaluate the assay, as shown in Fig. 2. The

Table 1 Template specificity of the improved assay

Template	Absorbance at 405 nm			
	HIV-1 RT (8 μU)	AMV RT (100 μU)	RAV-2 RT (20 μU)	DNA Polymerase I [a] (25 μU)
poly rA	0.745	1.153	1.200	1.740
poly dA	0.010	0.022	0.022	1.725

[a] *E. coli* DNA polymerase I, Klenow fragment

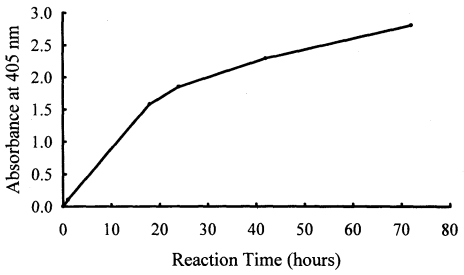

Fig. 1 Kinetics of the improved reverse transcriptase assay.
The relationship between the reaction period and absorbance is illustrated. In this assay, 3.125 μU of rHIV-1 RT was used.

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Fig. 2 Standard curve for the improved method of assay.
There was a linear relationship between the amount of reverse transcriptase and absorbance between 20 and 300 μU in the 1-hr incubation (a), and between 1.25 and 20 μU in the 18-hr incubation (b).

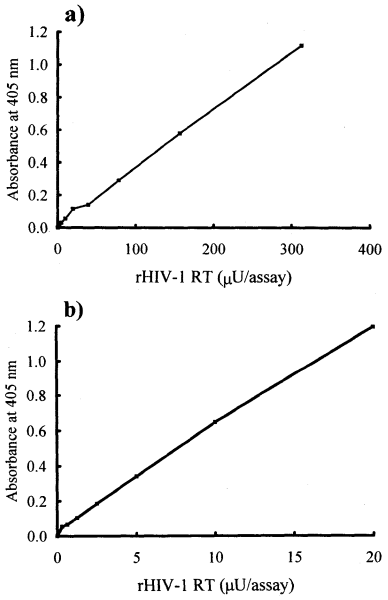

Fig. 3 The inhibitory effect of fetal bovine serum (FBS) in the non-RI assay.
The degree of inhibitory effect of FBS in the non-RI assay is indicated. In both of these assays, 0.1 mU of rHIV-1 RT was used. (—): improved method. (----): previous method.

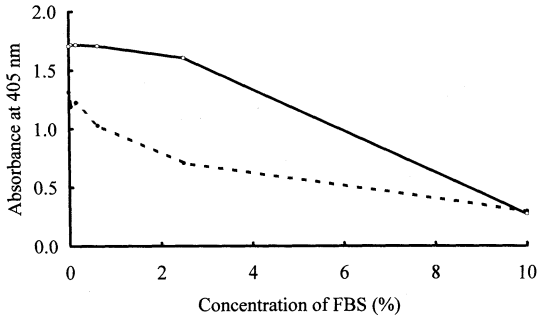

Table 2 Comparison of sensitivities of the RI methods

a. Previous and improved non-RI methods for measuring rHIV-1 RT

rHIV-1 RT (μU/assay)	Improved method (absorbance)	Previous method (absorbance)
50.00	1.851(+)[a]	1.238(+)[a]
25.00	1.744(+)	0.793(+)
12.50	1.300(+)	0.594(+)
6.25	0.682(+)	0.300(+)
3.13	0.380(+)	0.132(+)
1.56	0.173(+)	0.070(−)
0.78	0.103(+)	0.043(−)
0.39	0.045(−)	0.021(−)
0.20	0.022(−)	0.005(−)
0.10	0.004(−)	0.001(−)

[a](+) detectable; (−) not detectable. The cut-off value for positive results was 0.100.

b. Improved non-RI and RI methods for assaying the virus pellet lysate

Reciprocal of dilution	Improved non-RI (absorbance)	RI ($\times 10^4$ cpm)
100	6.750(+)[b]	208.2(+)[b]
400	6.258(+)	70.9(+)
1600	5.794(+)	19.7(+)
6400	5.176(+)	6.1(+)
25600	1.952(+)	1.4(+)
102400	0.712(+)	0.3(−)
409600	0.151(+)	0.2(−)
1638400	0.056(−)	0.1(−)
6553600	0.025(−)	0.1(−)
26214400	0.000(−)	0.0(−)

[b](+) detectable, (−) not detectable. The cut-off values for positive results were 0.100 and 1×10^4 cpm for improved non-RI and RI RTA, respectively.

absorbance was linearly related to the concentration of rHIV-1 RT between 20 and 300 μU for the 1-hr incubation, and between 1.25 and 20 μU for the 18-hr incubation. We determined the cut-off value for this assay by testing each the following five times: distilled water, phosphate-buffered saline (−), fresh culture medium (RPMI 1640 + 10% FBS) and the culture supernatant derived from uninfected U937 cell line. We decided on the cut-off value for a positive result as 0.100 above "mean + 3 S.D." for each the four samples.

A comparison of the sensitivities of the previous method and our improved method of assay for rHIV-1 RT is shown in Table 2-a. Comparison of the dilution end-points showed that the improved method was 2 dilution steps (4 times) more sensitive than the previous method. We next used the improved method to assay serial dilutions of partially purified HIV-1 particles and compared its sensitivity with that of the sensitive RI-RTA[2], as shown in Table 2-b. The numbers of virus particles were calculated from the results of the sensitive RI-RTA[2]. The cut-off value for a positive result is 10,000 cpm according to Lee et al.[2]. With the sensitive RI-RTA, as few as 97–203 virions could be detected, while with our improved non-RI RTA, as few as 6–13 virions could be detected.

The influence of FBS in testing for rHIV-1 RT was examined in both the previous and improved non-RI assays (Fig. 3). The improved assay was less influenced by the FBS concentration than the previous assay, especially when the concentration was 1.25–2.5%. Much of the residual activity in this assay was retained when FBS was diluted, and it therefore appears that dilution of the culture supernatant containing FBS would enable measurement with a higher degrees of sensitivity.

To determine the optimal dilution of the culture fluid for use in the improved assay, we tested serially diluted samples of culture supernatant containing various numbers of HIV-1 particles, (data not shown) and found that fourfold dilution resulted in the highest degree of sensitivity in assays of culture supernatants containing 10–20% FBS. We diluted samples fourfold in subsequent non-RI RTAs of culture supernatants (the sample dilution procedure). Culture supernatants that contained 2333–4900 HIV-1 particles/50 μl were serially diluted wit RPMI 1640 medium containing 10% FBS, and subjected to both the improved non-RI RTA by the sample dilution procedure and the sensitive RI-RTA, and the results were compared (Table 3). The RI-RTA was 2 dilution steps (4 times) more sensitive than the non-RI RTA without sample dilution in the detection of HIV-1 particles in the culture medium containing 10% FBS, while the RI-RTA was 1 dilution step (twice) more sensitive than the non-RI RTA by the sample dilution procedure.

The 22 culture supernatants derived from PBMCs from five seropositive individuals were tested with the improved nbn-RI RTA and the sensitive RI-RTA. A comparison of the results obtained with the two assays is shown for each individual in Table 4. With the sensitive RI-RTA, 16 (72.7%) supernatants were

Table 3 Comparison of sensitivities in assaying culture supernatant

Reciprocal of[a] dilution	RI-RTA ($\times 10^4$cpm)	Improved non-RI RTA	
		direct assay (absorbance)	by the sample dilution procedure[b] (absorbance)
1	19.6(+)[c]	0.431(+)[c]	0.815(+)[c]
2	11.2(+)	0.278(+)	0.370(+)
4	6.4(+)	0.162(+)	0.247(+)
8	3.2(+)	0.094(−)	0.144(+)
16	1.8(+)	0.054(−)	0.082(−)
32	0.9(−)	0.027(−)	0.059(−)
64	0.5(−)	0.008(−)	0.035(−)
128	0.4(−)	0.017(−)	0.036(−)
256	0.3(−)	0.000(−)	0.002(−)
512	0.2(−)	0.012(−)	0.022(−)

[a]Original sample containing 10% FBS was serially diluted with RPMI 1640 medium containing 10% FBS.

[b]In the sample dilution procedure, samples were diluted four times with 0.25% Triton X-100, and assayed by the non-RI method. Details in the text.

[c](+) Detectable ; (−) not detectable. The cut-off values for positive results were 1×10^4 cpm and 0.100 for RI and improved non-RI RTA, respectively.

Table 4 Assays of fresh HIV-1 isolates from five PBMC specimens

Specimen No.	Days in culture	RI-RTA ($\times 10^4$ cpm)	Improved non-RI RTA[a] (absorbance)
5-2-29	7	0.4(−)[b]	0.011(−)[b]
	14	132.1(+)	3.552(+)
	21	119.2(+)	2.556(+)
	28	79.4(+)	2.489(+)
	35	90.0(+)	2.532(+)
5-1-8	7	0.1(−)	0.027(−)
	12	0.1(−)	0.016(−)
	19	3.1(+)	0.124(+)
	26	4.8(+)	0.110(+)
5-26-6	7	0.3(−)	0.031(−)
	14	17.4(+)	1.816(+)
	21	6.6(+)	0.906(+)
5-6-9	7	0.3(−)	0.011(−)
	14	1.5(+)	0.071(−)
	21	6.5(+)	0.831(+)
	28	7.5(+)	0.614(+)
	35	4.3(+)	0.393(+)
	42	5.1(+)	0.419(+)
5-9-7	7	0.4(−)	0.002(−)
	16	6.2(+)	0.196(+)
	21	7.0(+)	0.241(+)
	28	11.8(+)	0.456(+)

[a]Improved non-RI RTA by the sample dilution procedure. Details in the text.
[b](+) detectable; (−) not detectable. The cut-off values for positive results were 1×10^4 cpm and 0.100 for RI and non-RI RTA, respectively.

positive, while with the non-RI RTA, 15 (68.1%) were positive. Only one culture supernatant was RI-RTA positive and non-RI RTA negative, but 5/5 individuals were found to be virus-positive in both assays.

Discussion

Since we consider the culture supernatant to be the most practically usable sample for RTA, we attempted to improve our non-RI RTA system. In our new method, both primer and template are immobilized, and improvements have also been made in the contents of the reaction mixture. The new method displayed template specificity, high reproducibility, and linearity for use as a semi-quantatitative method of analysis equal to those of the previous non-RI RTA. In assays of rHIV-1 RT, the new method was found to be more sensitive than the previous one. In the assays of partially purified HIV-1 particles, the previous non-RI RTA was twice as sensitive as the sensitive RI-RTA developed by Lee et al.[13], while the improved non-RI RTA was 16 times more sensitive than the RI-RTA. The improved non-RI RTA was thus eight times more sensitive than the previous method for detecting partially purified HIV-1 particles.

We previously reported that the sensitivity of the primer-immobilized non-RI RTA was decreased in the presence of FBS[14]. Attempting to interpret this finding, we proposed that FBS might interfere with the hybridization between primer and template. In the improved method both primer and template were therefore immobilized prior to the addition of a sample, and we prevented its interference at low concentrations of FBS. Fourfold dilution was found to be appropriate for the assay of culture fluid containing 10-20% FBS. With this dilution, sensitivity was slightly lower than that of the sensitive RI-RTA. As a practical application of the new method, we successfully assayed the supernatants from

PBMC cultures of clinical samples, and this method of assay was considered to be of practical use.

Since the number of biosafety facilities which are permitted to use radioactive materials is limited and the necessity to reduce radioactive waste is worldwide, our improved non-RI RTA is considered a practically applicable system for the assay of culture supernatants. There are two commercially available RTA kits not requiring radioactive materials, one from Boeringer-Mannheim and the other from DuPont. These assays use both biotinylated dUTP and digoxygenine-labeled dUTP to capture and detect newly synthesized DNA, respectively. Since biotin molecules were captured by immobilized streptavidin on microwells, free biotin present in the culture medium inhibited the binding of reaction-product competitively (in preparation).

Nocito et al.[10] reported a non-RI RTA immobilizing template primer. In their protocol, the template primer complex was immobilized via an -NH group on the surface of a microtiter plate. It was shown that about 32 mU of AMV RT yielded absorbance of 1.000 in a 1-hr assay. Our assay required only 1.5 mU to yield the same absorbance. Consequently, the sensitivity of the method appeared to be about 20 times less than that of ours. A likely reason for the lower sensitivity of their method is that in it the 5′-terminal of both primer and template were bound to the well.

It has been stated that AC-EIA, the other non-RI method for the detection of HIV particles, is, because of its sensitivity, the best method for the detection of HIV particles in culture media[17]. Recently an AC-EIA kit for HIV-1 p24 was devised by Abbott especially for assay of serum or plasma samples (HIV core EIA "Abbott" II) and it is not recommended for assay of culture fluid according to the instruction manual in Japan. It appears, therefore, that our method is ideal for laboratories HIV culturing and not permitted to use RI material.

The complete RT reaction in the wells appeared to include a binding step, in which an RT molecule attaches to a primer-template complex, and a polymerization step. In our method, these two steps can be differentiated. A similar analysis of the RT reaction had been made by Gronowitz et al.[18] on the basis of findings obtained with the use of template-primer immobilized macrobeads and [^{125}I] IUTP. Our method has the advantages of a high degree of sensitivity and lack of requirement of radioisotopes, and it may enable further study of the mechanisms of action of RT inhibitors in conventional laboratories.

Retroviral contamination of biological products has been a significant problem, particularly in the case of Chinese hamster ovary cells[19], and manufacturers are required to certify the lack of retroviral contamination in bio-products[20]. RTAs to be used for the detection of retroviral contamination must be as sensitive as possible. Our method of assay, which has the advantage of not requiring radioactive materials, appears to be the most useful for determining whether retroviral contamination is present in a biological product or not.

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非放射性逆転写酵素活性測定法の改良とその評価

[1] 大阪医科大学微生物学教室
[2] 旭化成工業株式会社診断薬研究部
[3] 大阪府立公衆衛生研究所

中野　隆史[1]　佐野　浩一[1]　小田原史知[2]
斎藤由紀子[1]　大竹　　徹[3]　中村　積方[1]
林　　克英[1]　美崎　英生[2]　中井　益代[1]

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我々は，96穴プレートにプライマーを固相化した非放射性逆転写酵素（RT）活性測定法を開発してきた．この方法は今までの放射性高感度RT測定法（RI-RTA）に比べ，手技が容易であり，感度もほぼ同様であるという特徴を持っていたが，fetal bovine serum（FBS）の含まれる試料では感度が安定しない欠点があった．そこで今回，その問題点を改良するため，テンプレート・プライマーを固相化した方法を開発，評価した．

本法は，逆転写反応を測定するのに十分な鋳型特異性を持っており，さらに高い再現性をもっていた．本法をプライマー固相化法と比較したところ，試料としてリコンビナントHIV-1 RTを用いて約4倍，またLeeらのRI-RTAと比較した場合HIV-1 lysateで約16倍，いずれも本法が最も高い感度を示した．また，FBSを10～20%含む培養上清試料を使用した場合，試料を0.25%Triton X-100で4倍に希釈したとき最も高い感度が得られ，その感度はRI-RTAとほぼ同じであった．その条件で臨床分離株の検出を試みた．HIV抗体陽性者より得られた末梢血単核球サンプル5例の分離培養について，RI-RTA，及び本法で5例すべての培養陽性試料の検出が可能であった．