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Application of a Commercial Kit for Detection of PCR Products to Quantification of Human Immunodeficiency Virus Type 1 RNA and Proviral DNA

HSIANG JU LIN,* MICHAIL HAYWOOD, AND F. BLAINE HOLLINGER

Division of Molecular Virology, Baylor College of Medicine, Houston, Texas

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Quantitative tests for human immunodeficiency virus type 1 (HIV-1) RNA in plasma and proviral DNA in peripheral blood mononuclear cells (PBMC) provide valuable information on the status of HIV-1 infection. This paper describes tests that were carried out with commercially available materials and an enzyme-linked immunosorbent assay reader for detecting spectrophotometric changes. Samples consisted of 100 μl of plasma or 200,000 PBMC. The procedure involved sample preparation, PCR-based amplification with the primer pair SK39 (biotinylated at the 5′ end) and SK38, hybridization of the cDNA PCR product to an RNA probe, capture of the RNA-DNA hybrid on a solid phase by means of strepavidin, binding to an alkaline phosphatase-conjugated antibody directed against RNA-DNA hybrids, and incubation with *p*-nitrophenylphosphate. Spectrophotometric changes were recorded at four intervals over a period of 20 h. The inclusion of HIV-1 RNA or proviral DNA standards in each run was an integral part of the procedure. The dynamic ranges afforded by these assays—500 to 1 million RNA copies per ml and 10 to 5,000 proviral DNA copies per 10^6 PBMC—were applicable to most plasma specimens and to all PBMC specimens from HIV-1-infected patients. Correlations of log-transformed HIV-1 RNA and proviral DNA concentrations with those found by reference methods were, respectively, 0.88 and 0.80. The between-run coefficients of variation for the detection method were ≤25% (range, 9.1 to 24.7) and ≤15% (range, 10.9 to 15.1), respectively, for HIV-1 RNA and proviral DNA. The reproducibility of the overall procedure for HIV-1 RNA in plasma (including sample preparation, amplification, and detection) was given by a duplicate standard deviation of $\log_{10}$ copies per ml of 0.11. Thus, the method was sufficiently precise to allow the detection of fourfold changes in plasma HIV-1 RNA concentrations, with a power of 0.95.

Detection of low concentrations of viral nucleic acids requires over 10^{13}-fold amplification of a target region (14). In general, this objective can be achieved by two approaches. Nested PCR assays consisting of two rounds of PCR achieve the desired degree of amplification, because each round of 20 to 30 cycles results in $>10^6$-fold amplification (14). The products of nested PCR assays can be made visible by ethidium bromide staining after their separation by electrophoresis. This approach is attractive in its simplicity, but it does not readily lend itself to the purpose of precise quantitation. The use of a single round of PCR followed by detection of the PCR products utilizing nonenzymic or enzymic reactions can provide the necessary sensitivity (10, 11, 17, 18). For example, PCR products hybridized to an acridinium ester-labelled probe may be detected by the chemiluminescence formed in the presence of alkali and hydrogen peroxide (8). With another method, PCR products labelled with biotin are bound to strepavidin-enzyme conjugates; after incubation with a chromogenic substrate, the PCR products are detected colorimetrically (8, 11). Quantification of PCR products is achieved by comparing the strengths of the signals from the standards and test samples. Techniques that are not based on PCR have also been developed. The branched DNA method utilizes a series of hybridization reactions between the target region and oligonucleotides, followed by hybridization of alkaline phosphatase-labelled probes to the branched DNA and enzymic hydrolysis of a chemiluminescent substrate (10, 16). The NASBA technique is based on the use of T7 RNA polymerase, RNase H, and a reverse transcriptase (9).

Thus, a number of methods have been developed for quantification of human immunodeficiency virus type 1 (HIV-1) nucleic acid (8–11, 13, 16–18). The novel feature of the method described in this report is its ability to cover a 5-log range without dilution of the sample. The method makes use of commercially available reagents and an enzyme-linked immunosorbent assay (ELISA) reader. It lends itself particularly to use in laboratories that wish to perform quantitative HIV-1 RNA assays without incurring large expenditures.

MATERIALS AND METHODS

Patient blood specimens. Whole blood was collected in tubes containing anticoagulant acid-citrate-dextrose. Peripheral blood mononuclear cells (PBMC) were separated by means of Ficoll-Hypaque gradients and stored as pellets consisting of 2×10^6 cells (8). All specimens and standards were stored at −180°C. Clinical specimens from subjects seropositive for HIV-1 were collected after informed consent was obtained.

Materials. The Sharp Signal System products used in this study were gifts from Digene Diagnostics, Inc. (Silver Spring, Md.). The probe-primer set consisted of the RNA probe, which was an RNA transcript of approximately 244 nucleotides in length that was specific for HIV-1 in positions 1395 to 1638 in HIV isolate SF2 (GenBank accession number KO2007); primer SK39 biotinylated at the 5′ end; primer SK38; and a positive assay control. The kit for detection of biotinylated PCR products consisted of the negative assay control, probe diluent, sample diluent, denaturation reagent, *p*-nitrophenylphosphate, wash buffer, two 96-well microplates with strepavidin-coated wells, and an enzyme-antibody conjugate directed at RNA-DNA hybrids. The conjugate is prepared by linking alkaline phosphatase to a mouse monoclonal antibody specific for RNA-DNA hybrids. The antibody is not base specific but recognizes the specific structure of the

* Corresponding author. Mailing address: Division of Molecular Virology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030. Phone: (713) 798-3009. Fax: (713) 798-3490. Electronic mail address: lin@bcm.tmc.edu.

TABLE 1. Between-run imprecision of the assay for PCR products[a]

Specimen	*n*	Mean[b]	Coefficient of variation (%)
Plasma 1	13	10,600	9.1
Plasma 2	13	171,000	18.1
Plasma 3	15	627,000	24.7
PBMC 1	17	82	13.2
PBMC 2	17	583	15.1
PBMC 3	16	1,477	10.9

[a] Replicate samples of every standard and each of the six specimens were amplified as described in Materials and Methods. Three to five amplified samples of each standard or specimen were pooled to obtain sufficient material for testing. In each run, two 5-μl aliquots from each pooled sample were used in the assay of PCR products.

[b] Values for the plasma specimens are expressed in copies of HIV-1 RNA per milliliter of plasma, and values for the PBMC specimens are expressed in copies of HIV-1 proviral DNA per 10^6 PBMC.

RNA-DNA helix; it exhibits almost no cross-reactivity to other nucleic acids such as single-stranded RNA, single-stranded DNA, and double-stranded DNA.

The RNA and DNA standards, RNA extraction reagent, PBMC lysis buffer, and the reagents for PCR-based amplification that contained the Sharp Signal System primers were made in this laboratory.

Overview of extraction, amplification, and detection. Single samples of RNA and DNA standards and clinical PBMC specimens were prepared. Two 100-μl samples of each clinical plasma specimen were prepared for reverse transcription (RT)-PCR. Each preparation was amplified in duplicate, and one detection assay was carried out on each amplified sample. A single 96-well plate could be used to test up to 19 clinical plasma specimens for HIV-1 RNA or up to 37 PBMC specimens for proviral DNA. Two wells were used for each standard or PBMC specimen, and four wells were used for each clinical plasma specimen.

HIV-1 RNA and DNA standards. RNA standards consisted of plasma spiked with cell-free supernatant fluid from an HIV-1 culture. The assignment of copy number concentrations to these standards was based on different quantitative assays performed independently at five laboratories (10). Serial dilutions were prepared with seronegative plasma to obtain standards containing from 500 to 10^6 copies per ml. HIV-1 DNA standards containing 2, 5, 10, 20, 50, 100, 200, 500, and 1,000 copies per 200,000 cells were prepared from the established cell line 8E5/LAV (4, 8).

Plasma HIV-1 RNA sample preparation. The guanidinium thiocyanate-phenol mixture (3) and carrier gel were obtained from the Molecular Research Center (Cincinnati, Ohio). The RNA extraction reagent was prepared by mixing 37.5 ml of the guanidinium thiocyanate-phenol mixture, 0.5 ml of the microcarrier gel, 7.5 ml of water, and 50 ng of yeast tRNA. After thorough shaking, 900-μl aliquots were dispensed into sterile 2-ml screw-cap polypropylene tubes, and 100 μl of plasma was added to each tube. The mixture was vortexed vigorously for 30 s and could be processed immediately or stored at −30°C for at least 12 weeks. Frozen samples were vortexed for 10 s after thawing. After 200 μl of chloroform was added, the tubes were shaken by hand 60 times and centrifuged at 2,900 × *g* for 10 min at 4°C, resulting in the separation of the aqueous phase (600 μl) from the organic phase. Exactly 450 μl (representing 75 μl of plasma) of aqueous phase was mixed with 450 μl of isopropanol. After 30 to 60 min at −30°C, the RNA and carrier gel were pelleted at 13,000 × *g* for 15 min at 4°C. The pellet was washed twice with 1-ml portions of ice-cold 75% ethanol. In order to remove all the fluid except that contained within the pellet, the inside of the tube was swabbed with a sterile cotton-tipped applicator. The moist pellet was dissolved at 37°C for 30 min with 185 μl of gel solvent, which was composed of 0.01 M Tris (pH 8.3 at 25°C), 0.05 M KCl, 0.2% Nonidet P-40, 1.5 mM dithiothreitol, and RNase inhibitor (0.5 U/μl). After storage at 4°C overnight, the samples were warmed to 37°C for 15 min and used for RT-PCR.

HIV-1 proviral DNA sample preparation. PBMC lysis buffer was added to the cell pellet to a concentration of 4 million cells per ml. The lysate was incubated at 60°C for 1 h and then held at 98°C for 15 min (17). PBMC lysis buffer was composed of 0.1 M Tris-HCl (pH 8.3), 0.05 M KCl, 0.45% Nonidet P-40, 0.45% Tween 20, and proteinase K (0.5 mg/ml).

PCR-based amplification of HIV-1 RNA and DNA. Fifty microliters of sample was mixed with 50 μl of PCR reagent, which was composed of 0.01 M Tris HCl (pH 8.3), 0.05 M KCl, 7.4 mM $MgCl_2$, 0.4 mM (each) deoxynucleoside triphosphate, 1 μM (each) primer, and *Taq* DNA polymerase (0.05 U/μl). For RT-PCR, the reagent contained, in addition, 1.5 mM dithiothreitol, RNase inhibitor (0.5 U/μl), and Moloney murine leukemia virus reverse transcriptase (0.4 U/μl). The two enzymes were obtained from Boehringer Mannheim Corporation (Indianapolis, Ind.), and the RNase inhibitor was obtained from Pharmacia Biotech Inc. (Piscataway, N.J.). RT was carried out at 42°C for 1 h. Amplification of either RNA or DNA was carried out with the Perkin-Elmer thermal cycler (model 9600), with 38 cycles at 94°C for 30 s, 55°C for 30 s, and 60°C for 2 min. Amplified samples stored at 4°C remained stable for at least 3 months.

Assay of PCR products. The procedure for detection of PCR products was carried out by following the manufacturer's directions. In brief, 5 μl of amplified sample was used for detection. cDNA PCR products were denatured, hybridized to RNA probe, and captured on strepavidin-coated wells in a microtiter plate format. Incubation was carried out with an alkaline phosphatase-conjugated antibody that was directed at RNA-DNA hybrids. After removal of unbound antibody, substrate was added, and the plate was incubated at 37°C for 20 h. A_{405} and A_{650} values were recorded by an ELISA reader at 0.75, 1.5, 3, and 20 h. The absorbance values, calculated as the difference between A_{405} and A_{650}, were corrected for the substrate blank and the cutoff level. Standard curves were constructed on log-log paper, and the points were connected with the aid of a flexible curve.

Absorbance data obtained for the four wells representing one HIV-1 RNA assay were used as follows. The two values for the duplicate amplifications set up from each preparation were averaged to obtain one result; if results from the two preparations were discordant (i.e., one positive and one negative), the entire assay was repeated, starting with RNA extraction. If the results of the two amplifications set up from one PBMC pellet were discordant, the test was repeated with a second pellet (8).

Statistical analyses. With the exception of the data presented in Table 1, log transformations of HIV-1 RNA and proviral DNA values were carried out prior to performance of the statistical analyses (10). Correlation coefficients were calculated by using Microsoft Excel, version 5.0, and linear regression analysis performed by SlideWrite (Advanced Graphics Software, Inc., Sunnyvale, Calif.). To evaluate the reproducibility of the HIV-1 RNA quantification method, the duplicate standard deviation (SD) of log copies was calculated. Log-transformed results obtained with the duplicate samples taken from each specimen were used to determine the duplicate SD of each pair of results, and the reproducibility was obtained by pooling these values over different pairs (10).

RESULTS

Between-run imprecision of detection of HIV-1 RNA and proviral DNA amplification products. The analytical performance of the kit for detection of PCR products was evaluated by analysis of PCR products amplified from HIV-1 RNA in plasma and proviral DNA from PBMC in samples at low, medium, and high concentrations (Table 1). Quantitation was performed against standards that were likewise amplified. The imprecision, expressed as the coefficient of variation, ranged from 9 to 25% for RNA quantification and from 11 to 15% for proviral DNA.

HIV-1 RNA standard curves. Figure 1 shows standard curves for the determination of HIV-1 RNA concentrations in plasma. The minimal number of RNA copies per RT-PCR that

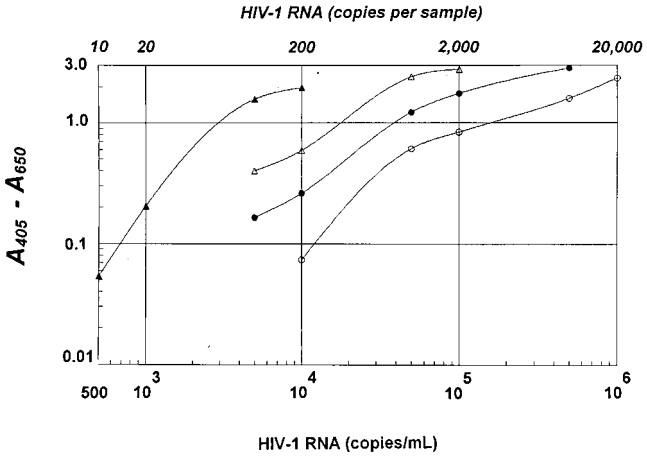

FIG. 1. Standard curves for the quantitation of plasma HIV-1 RNA. Absorbance values (the difference between A_{405} and A_{650}) of the standards were read at intervals during incubation of enzyme-conjugated antibody with substrate: ○, 0.75 h; ●, 1.5 h; △, 3 h; ▲, 20 h. Absorbance values were corrected for substrate blanks and cutoff values. The lower *x*-axis scale gives the RNA concentration of the standards. The upper *x*-axis scale gives the number of RNA copies present in the RT-PCR mixture.

TABLE 2. Plasma HIV-1 RNA concentrations determined at different time intervals during incubation of enzyme-conjugated antibody with substrate

Specimen no.	Plasma HIV-1 RNA concentration (copies/ml) at indicated time (h)			
	0.75	1.5	3	20
1	950,000	—[a]	—	—
2	780,000	—	—	—
3	330,000	280,000	—	—
4	160,000	180,000	—	—
5	100,000	85,000	95,000	—
6	50,000	50,000	47,000	—
7	10,500	10,600	10,500	—
8	—	9,000	9,500	9,300
9	—	—	—	3,700
10	—	—	—	750

[a] —, no result was obtained at this interval because the absorbance value was either below the cutoff level or ≥ 3.0.

could be detected was 10, representing 20 μl of the 500 copies-per-ml standard. The significance of this observation was that although the assay of PCR products required only 5 μl of amplified product, smaller volumes (i.e., <50 μl) of sample and PCR reagent could not be used without reducing the limit of detection.

The log-log plot of absorbance versus RNA concentration approached but did not reach linearity because of several factors. With a relatively large number of PCR cycles, amplification ceased to be exponential. The kinetics of some reactions involved in the detection method, such as binding of antigen to antibody, may have been nonlinear. In the absence of a clearly defined relationship between substance concentration and absorbance, the standard curves were drawn by spline approximation, a procedure that joins sections of a curve, each of which can be fitted to a different low-order polynomial (15).

Standards or test samples with absorbance readings that were ≥3.0 were not used; thus, the standard curves (such as the one at 20 h) appeared truncated. There was no need for extrapolation, since any RNA concentration within the dynamic range could be obtained from at least one of the four standard curves. Clinical specimens with absorbance values higher than the highest standard were diluted 10-fold prior to repetition of the amplification and detection procedures.

Quantification of HIV-1 RNA in plasma. Table 2 shows the precision of values determined for 10 representative specimens at different time intervals. The differences between the highest and lowest values found for a given specimen ranged from 0 to 18%, with a mean of 8.8%, with no observed bias toward values obtained at earlier or later time intervals. When RNA concentrations were determined at two or three time intervals (specimens 3 to 8), the mean of the observed values was reported.

To validate the method, we analyzed 15 coded patient specimens which had previously been determined to be positive by another PCR-based method (Roche Molecular Systems, Alameda, Calif.) (10). HIV-1 RNA concentrations obtained with the Roche method ranged from 2,300 to 457,400 copies per ml. Linear regression of the results obtained with the Digene method with values obtained with the Roche method is presented in Fig. 2. Correlation between the two methods was excellent: $r = 0.88$.

Reproducibility of HIV-1 RNA quantification. The overall imprecision of the HIV-1 quantification method was estimated by calculating the pooled duplicate SDs of 72 plasma specimens assayed on 13 different runs. This measure of imprecision included the variation introduced by all steps of the procedure, including sample processing, RT-PCR, and detection of PCR products. To be able to detect fourfold differences in plasma HIV-1 RNA concentration with a power of 0.95, the pooled duplicate SD of log copies must be no greater than 0.50 (10). The pooled duplicate SD obtained was 0.11, which fell within the target value.

Quantification of HIV-1 proviral DNA. Standard curves for HIV-1 proviral DNA (not shown) differed from those for HIV-1 RNA in two respects. The limit of detection was 2 proviral HIV-1 DNA copies per PCR amplification. The largest copy number per amplification was 1,000, representing 5,000 proviral DNA copies per 10^6 PBMC. Table 3 presents results of the quantification of proviral DNA in 20 subjects and a comparison of 15 of these results against assays performed by the Gen-Probe method (8, 17) that were obtained in a previous study (10). The regression of y (Digene) on x (Gen-Probe) was given by the equation $y = 0.81x + 0.77$. The coefficient of correlation between HIV-1 DNA values obtained by the Digene method versus those obtained by the Gen-Probe assay was 0.80. Although this degree of correlation was acceptable, there were twofold or greater differences in some of the datum pairs. These discrepancies may reflect differences in the two methodologies.

Table 3 illustrates a second point. Proviral DNA concentrations, expressed as copies per 10^6 PBMC, showed a statistically insignificant degree of correlation with the CD4 counts ($r = -0.33$). This was the result of two opposing effects. With disease progression, HIV-1 infected a larger proportion of $CD4^+$ cells, but this change was offset by a decrease in the concentration of $CD4^+$ cells. Consequently, the observed range of HIV-1 proviral copy numbers across all specimens was relatively narrow. When the results were expressed as proviral DNA copies per 10^6 $CD4^+$ cells, they ranged from 150 in subject 20 to 62,000 in subject 9, and the correlation of these values with the CD4 counts was significant ($r = -0.72$).

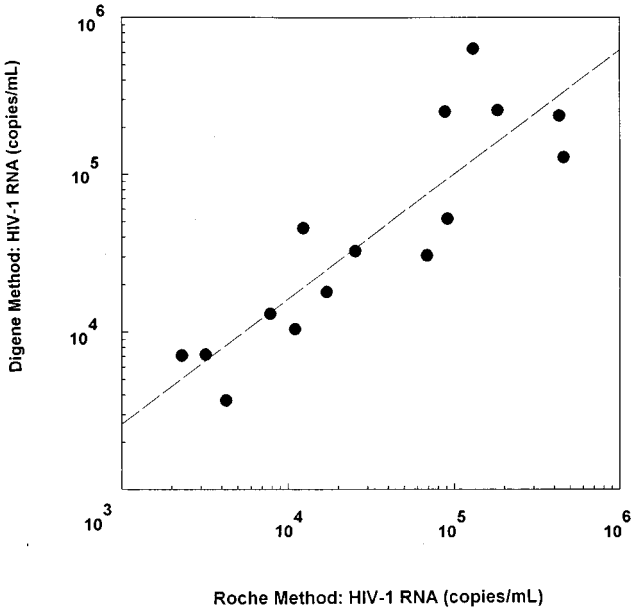

FIG. 2. Comparison of plasma HIV-1 RNA concentrations obtained with the Digene method and those obtained with the Roche procedure. Linear regression was given by the equation $y = 1.49x + 0.66$.

TABLE 3. Quantitation of HIV-1 proviral DNA in PBMC

Subject no.	HIV-1 proviral DNA (copies/10^6 PBMC)		$CD4^+$ cells/μl
	Digene method	Gen-Probe method	
1	2,800	NA[a]	285
2	2,600	1,600	515
3	2,400	630	706
4	1,700	350	74
5	1,600	1,200	234
6	1,500	1,500	46
7	1,400	930	165
8	1,300	NA	430
9	910	850	12
10	780	990	47
11	650	250	494
12	640	680	841
13	620	290	723
14	510	390	1,522
15	430	920	14
16	250	120	27
17	180	NA	291
18	120	NA	440
19	90	NA	441
20	75	50	1,382

[a] NA, not available.

DISCUSSION

The measurement of plasma HIV-1 RNA levels appears to hold many advantages over other virologic tests for HIV-1 infection (6). In contrast to HIV-1 p24 antigen, plasma HIV-1 RNA levels are measurable in the vast majority of HIV-1-infected patients in various stages of the disease (13). HIV-1 RNA in plasma is detectable in acute infection, in the ensuing stage prior to seroconversion, and in the relatively long period preceding the development of AIDS. Although the same could be said of HIV-1 proviral DNA in PBMC, plasma HIV-1 concentrations can change dynamically in response to treatment (7), in contrast to cellular proviral DNA (2). Unlike the observed changes in CD4 count, the decrease in the concentration of HIV-1 RNA in response to a specific course of antiretroviral therapy has been reported to be predictive of overall survival and of survival free of AIDS-defining events, showing the prognostic significance of such quantitative assays (19). There appear, then, to be several reasons for measuring plasma HIV-1 levels in patients, whether for patient care, clinical trials, or basic research. One drawback to applying measurements of HIV-1 RNA levels to these purposes has been the relatively high cost of some test systems. In this connection, the product evaluated in this study may offer the advantage of relatively low cost. For laboratories already equipped with ELISA readers and thermocyclers, no expenditures for capital equipment are needed.

We recognize some limitations of the present method. The technology does not allow the addition of an internal standard or control to the clinical specimens as a means of detecting substances that might interfere with PCR-based amplification. Second, the RNA extraction and PCR procedures are technically demanding.

The accurate and precise measurement of plasma HIV-1 RNA levels depends on several factors, including sample preparation (3, 12), the efficiency of RT (1, 5), and the degree of amplification compatible with the detection method. The prescribed method for RNA extraction utilizes a neutral guanidinium isothiocyanate reagent, which acts as a preservative, and a gel as carrier. The broad dynamic range for HIV-1 RNA quantification was achieved by working within narrow limits. The sample volume for RT-PCR was chosen so that an HIV-1 RNA concentration of 500 copies per ml was detectable. The number of PCR cycles was carefully selected so that the lowest standard remained detectable in the large majority of instances without flattening the high end of the standard curve.

Quantification also requires accurate standards. Proviral DNA standards were prepared from 8E5/LAV cells that contain one copy of defective proviral DNA per cell (4). We were confident of the correctness of the DNA copy number standards, since it depended essentially on the accuracy of the cell count of the 8E5/LAV cell culture. There was more uncertainty in regard to the HIV-1 RNA standards, because there is no reference preparation or measurement. However, the HIV-1 RNA standards employed in this study appeared to yield the correct order of magnitude. Analyses of RNA and DNA curves showed that to obtain the same absorbance reading for DNA at a given time interval, 5 to 10 times as many nominal RNA copies were required. Thus, a minimum requirement of 10 nominal RNA copies was required for production of a detectable signal, compared with that of 2 copies for the HIV-1 proviral DNA assay. Since the efficiency of RT has been estimated at about 10 to 25% (1, 5), the differences in the signals generated by the DNA standards and those produced by the RNA standards could be accounted for by the inefficiency of the RT step.

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