Integration complexes derived from HIV vectors for rapid assays in vitro

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Of three enzymes encoded by HIV–reverse transcriptase, protease, and integrase—only the first two have been exploited clinically as inhibitor targets. Efforts to develop inhibitors of purified integrase protein have yielded many compounds, but none with clinical utility. A different source of integration activity for studies in vitro is provided by replication intermediates isolated from HIV-infected cells. These preintegration complexes (PICs) can direct integration of the endogenously synthesized viral cDNA into an added target DNA in vitro. Despite their authentic activities, assays of PICs have not been widely used due to technical obstacles, particularly the requirement for handling large amounts of infectious HIV. Here, we describe greatly improved methods for producing PICs using HIV-based vectors that are capable of establishing an integrated provirus but not a spreading infection. We also report the development of a PIC integration assay using DNA-coated microtiter plates, which speeds assays of PIC integration in vitro. We used this method to screen a library of chemicals related to known integrase inhibitors and found a new compound, quinalizarin sulfate, that displayed enhanced activity against PICs.

Keywords: HIV, integrase, AIDS, retroviral vector, quantitative PCR, TaqMan

Chemotherapy for HIV currently employs inhibitors of the viral reverse transcriptase and protease enzymes[1,2]. The third viral enzyme, integrase, has yet to be exploited as a target. Integration is required for retroviral replication and, thus, is an attractive target for inhibitor development. As no cellular homolog of HIV integrase has been described, potential inhibitors could be relatively nontoxic[3,4].

The DNA breaking and joining reactions that mediate viral cDNA integration are well understood. The integrase protein binds the blunt-ended viral cDNA and cleaves two nucleotides from the 3′ end of each strand, generating recessed 3′-hydroxyl groups (Fig. 1, steps 1 and 2). This may serve to remove nontemplated extra nucleotides occasionally added to the cDNA ends by reverse transcriptase[5,6]. Next, the 3′ ends are joined to protruding 5′ ends of staggered breaks in host DNA (step 3). The resulting gapped intermediate is then repaired, probably by host DNA repair enzymes, to yield the fully integrated provirus (steps 4 and 5)[1,4].

Integration-competent preintegration complexes (PICs) can be isolated from freshly infected cells and studied in vitro. These complexes can integrate viral cDNA into an added target DNA, yielding the integration intermediate shown in step 4 of Figure 1 (refs. 7–11). The final repair step (step 5) does not take place efficiently in the extracts studied to date. Compositional studies in the HIV-1 system reveal that, in addition to integrase, PICs contain the viral matrix and reverse transcriptase proteins[12–14], a cellular protein HMG I(Y) (ref. 15), and potentially other proteins. Protein–DNA complexes are present at the ends of the cDNA[6,16] that synapse the two termini[6]. Preintegration complexes join both of the cDNA ends to the target DNA with a defined spacing[7,9,10,17]. In contrast, reactions with purified recombinant HIV-1 integrase protein carry out mainly partial reactions involving only one cDNA end[18,19]. Although methods for assembling integration complexes from purified components are being developed[20,21] (F.D. Bushman and colleagues, unpublished data), full reconstitution of the preintegration complexes' composition or activity has not been reported.

Importantly, PICs show a more fastidious and authentic response to inhibitors than do purified recombinant integrase proteins. For example, purified integrase is inhibited by zidovudine (AZT), which clearly acts against reverse transcriptase (RT) in vivo. In contrast, PICs, are not inhibited by zidovudine[22]. Here, we describe methods employing HIV-based vectors[23,24] and a microtiter plate format that greatly simplify assays of PICs.

Results

Generating stocks of HIV-based vectors. HIV-based vectors were produced by cells engineered to contain four DNA constructions as described by Kafri et al.[24] (Fig. 2). One construction directed expression of HIV *gag-pol*, which encodes the viral enzymes including integrase and the Gag structural proteins (Fig. 2, part 1). The second directed production of the vesicular stomatitis virus G (VSV-G) envelope protein (Fig. 2, part 2). The third directed synthesis of the viral genomic RNA to be packaged, which encodes the gene for green fluorescent protein (GFP) and an HIV packaging site (Fig. 2, part 3). The fourth construction encoded tetracycline transactivator (tTA), an inducible transcriptional activator that controls the activity of parts 1 and 2 (ref. 25) (Fig. 2, part 4). Expression of all four DNA constructions in a single cell directs production of particles containing the HIV proteins, including integrase. These particles are competent to infect target cells and carry out integration but cannot support a spreading infection. The use of HIV vectors instead of live HIV greatly reduces the biohazard involved in producing PICs.

Optimal cells for production of PICs. Cell lines were screened to identify those optimal for the production of PICs (data not shown). Initially, cells were tested for their ability to support infection by the HIV-based vectors. Use of the VSV-G envelope greatly simplified this study because it permitted infection of a wide variety of target cells.

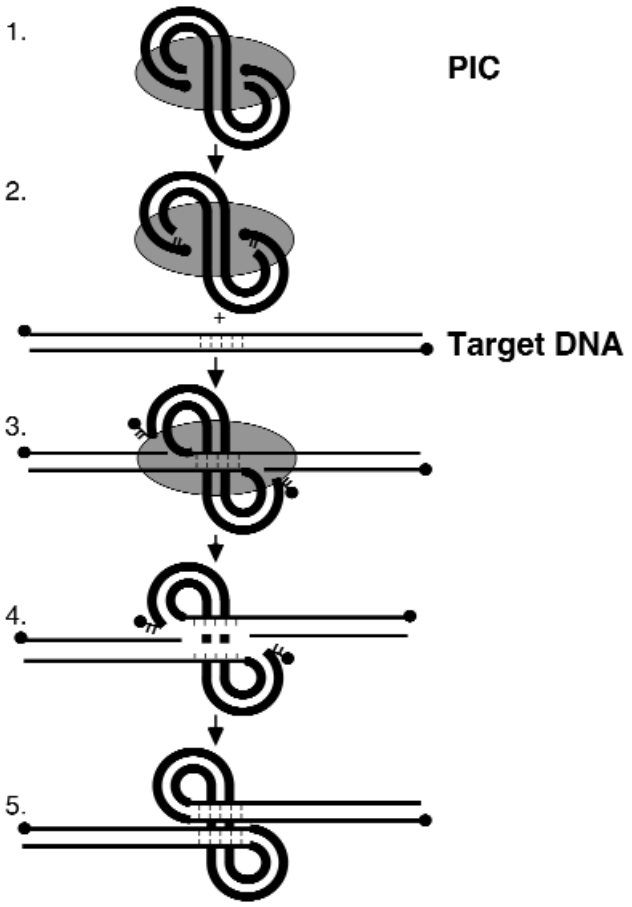


Figure 1. DNA cutting and joining reactions mediating cDNA integration. The HIV cDNA is shown by thick lines and the target DNA is shown by thin lines. The protein factors of the preintegration complex are shown by the gray oval. The solid circles represent the DNA 5′ ends. See text for an explanation.

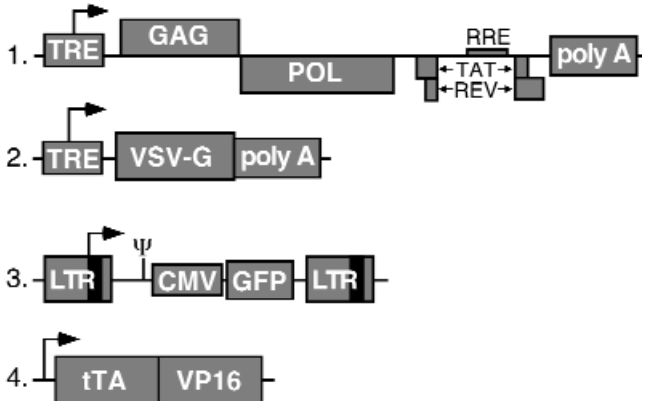


Figure 2. Diagram of the DNA constructions used for the production of HIV-based vector particles. The structural and enzymatic proteins of HIV(Gag-Pol) are expressed under control of an inducible tet-responsive promoter (TRE, part 1), as is the envelope glycoprotein of vesicular stomatitis virus (VSV-G, part 2). The packaged RNA vector encodes the gene for the green fluorescent protein (GFP) and the HIV RNA packaging signal (ψ) flanked by HIV long terminal repeats (LTRs) (part 3). The tet-vp16 fusion protein transactivates transcription from the TRE when tetracycline or the analog doxycycline are withdrawn (part 4)[24].

The titer among cell lines ranged from 1.8×10^7 IU/ml to 4×10^5 IU/ml, with 293T cells consistently displaying the highest titers. Cell lines were then screened for efficiency of production of PICs after infection with HIV-1 vectors. DNA products were assayed by Southern blot as described (Fig. 3)[8] to monitor the conversion of cDNA into integration product. All cell types tested yielded active PICs. The greatest yield was obtained with 293T cells, which produced 6.1×10^6 copies of cDNA per 10^6 cells. Evidently, multiple PICs can form in each cell, emphasizing the efficiency of infection by the HIV vectors with the VSV-G envelope. In subsequent experiments, 293T cells were used as targets.

PIC-integration into immobilized target DNA. Although the use of HIV-based vectors greatly reduced the biohazard involved in preparing PICs, the time-consuming Southern blot assay of integration products remained a significant limitation. To facilitate PIC integration assays, a method was developed for carrying out integration into immobilized target DNA in microtiter plate wells (Fig. 4).

Addition of lysates containing PICs to DNA-coated wells and incubation at 37°C for 30 min resulted in the integration of cDNA molecules into immobilized DNA (Fig. 4, parts 1 and 2). After integration, unintegrated cDNA was removed by washing. In the integration product, single-stranded DNA gaps are present on one strand at each viral–target DNA junction (Fig. 4, part 2) as a consequence of the integration mechanism (Fig. 1). Thus, HIV sequences could be eluted by denaturation (Fig. 4, part 3).

To detect HIV cDNA, released viral sequences were amplified by PCR and products analyzed by electrophoresis on native agarose gels by ethidium bromide staining. Fragments of the expected sizes were detected in integration reactions but not in controls lacking PICs or containing inhibitors (data not shown). Titration of the PIC lysate revealed that amplification products could be detected with a volume of lysate (1 µl) that contained 2×10^5–10^6 cDNA copies (data not shown). Routine reactions were carried out using 25 µl, which yielded a robust signal. In contrast, the Southern blot method required 100–200 µl of lysate.

Rapid quantification of integration by real-time fluorescence-monitored PCR. To quantify integration products more efficiently, a fluorescent real-time PCR method (TaqMan) (Perkin-Elmer Applied Biosystems) was developed[26,27]. Integration reactions were carried out in microtiter wells as described, wells were washed, and reaction products were eluted by denaturation. Samples were amplified with two primers complementary to HIV cDNA as in conventional PCR, but a third oligonucleotide probe was included that annealed on the cDNA between the two flanking primers. The probe contained a fluorescent reporter dye (FAM) at one end and a quenching group (TAMRA) at the other. Upon amplification, the

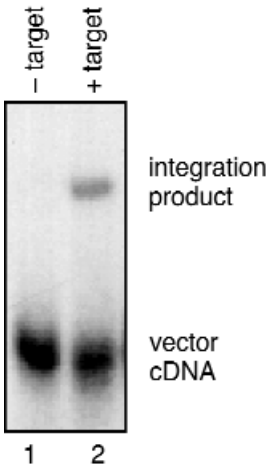


Figure 3. Integration activity of vector-based PICs analyzed by Southern blot. Lysates containing vector PICs were incubated for 30 min at 37°C, then deproteinized. Product DNAs were visualized by annealing to a labeled probe complementary to sequences in the HIV LTR. Reactions carried out in the absence (lane 1) or the presence (lane 2) of target DNA (linear pUC19 DNA) are shown.

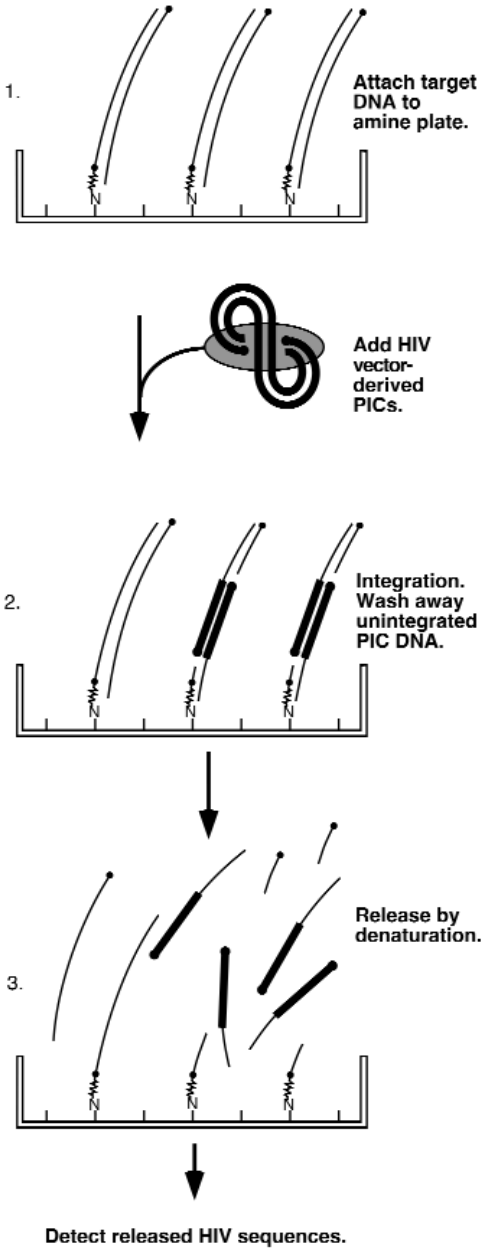


Figure 4. Diagram of a microtiter plate-based assay for detection of integration by PICs. Lysates containing HIV vector-derived PICs were added to plate wells containing immobilized target DNA and incubated to permit integration. The 3′ ends of viral cDNA become attached to target DNA due to integration (part 2). The 5′ ends of viral cDNA do not become covalently attached in reactions with PICs. Unintegrated viral cDNA is washed away. Viral sequences are then released by denaturation, as the structure of the product is such that vector cDNA is linked to the plate by hydrogen bonding only (part 3). Released viral sequences are then quantified to determine the extent of integration.

exonuclease activity of Taq polymerase degrades the annealed probe, releasing the fluorescent FAM group from the quencher and allowing FAM fluorescence to be detected. The cycle number at which the accumulation of fluorescent products is half-maximal is proportional to the number of copies of integrated DNA. Comparison of the experimental signal to a standard curve permitted quantification of the number of integrated genomes. Quantification of integration products revealed over 1,000 integration events per standard reaction, a number about 80 times higher than the background signal

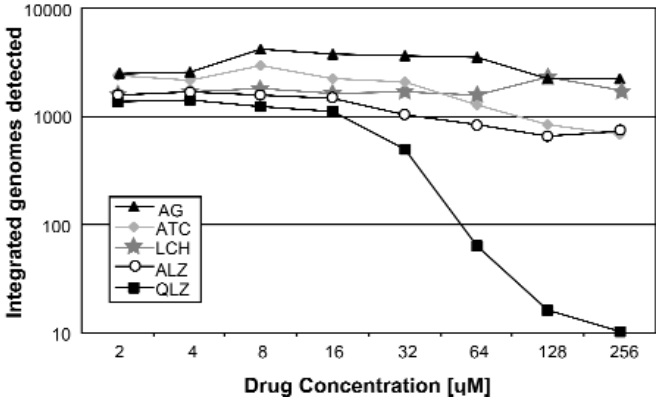


Figure 5. Quantification of integrated HIV genomes by real-time fluorescence-monitored PCR and characterization of integration inhibitors. Five compounds that inhibit recombinant HIV integrase protein in vitro were assayed for inhibition of integration by vector-derived PICs. The number of HIV-vector sequences detected in integration products is plotted on the *y*-axis. Inhibitor concentration is plotted on the *x*-axis. Standard integration reactions yielded an average of 1,253 copies, and negative control reactions yielded 15 copies. Abbreviations as in Table 1.

from inhibited control reactions. Reconstruction tests revealed that the response of the assay was linear over a range of at least 10^2–10^6 cDNA copies (data not shown).

Assaying inhibitors active against PICs. To document an application for this method, the effects of several well-studied integrase inhibitors were compared. Seven compounds were tested that are all known to inhibit purified recombinant integrase in the micromolar range[22,28,29]. Only two, quinalizarin (QLZ) and lamellarin α 20-sulfate (Lamα), are active against PICs[22] (data not shown). Inhibitors were titrated into reactions containing PICs from HIV-based vectors and products scored by quantitative PCR. A representative experiment is shown in Figure 5 and results tabulated in Table 1. As was found in previous work, QLZ and LamZ were inhibitory, but the other compounds were not, indicating that the PICs from HIV vectors respond similarly to PICs from live virus.

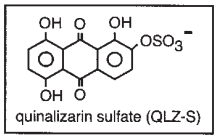
The streamlined PIC-based assay was then used to screen a library of 105 compounds with structures related to known inhibitors. The most inhibitory compound was found to be quinalizarin sulfate (QLZ-S; Table 1), a new synthetic derivative of QLZ, which inhibited PICs with an IC_{50} of 13 mM. Repeated titrations of QLZ-S, QLZ, and LamZ revealed that QLZ-S was consistently the most potent against PICs.

Discussion

We have described the use of noninfectious HIV-based vectors to develop rapid assays for viral cDNA integration. Assays with PICs mimic integration events in vivo more closely than those using recombinant integrase produced in *Escherichia coli*. However, PIC preparation has been difficult and required the use of large amounts of infectious HIV. The combination of vector-based PICs, immobilized target DNA, and real-time fluorescence-monitored PCR, reported here, greatly speeds PIC integration assays. Using this method, 96 assays can easily be carried out in about 6 h. Tests of well-characterized inhibitors reveal that PICs from replication-competent HIV[10,22,30] (data not shown) and HIV-based vectors show similar discrimination. Use of the rapid PIC assay for library screening permitted the identification of a new potent inhibitor, QLZ-S. The new assay should also facilitate study of the mechanism of integration. For example, fractions generated during purification of PICs can now be assayed during the course of the procedure.

Table 1. IC_{50} measurements for assays with vector-based PICs (determined here) or HIV-1 derived PICs (reported previously).

	IC_{50} values [μM]		
Drug	This study	Previous studies	Reference
AG	>200	>200	22
ALZ	>200	>100	22
LCH	>200	>100	30
ATC	>200	>100	22
LamZ	42	88	*
Pur	111	21	22
QLZ	46	11	22
QLZ-S	13		



quinalizarin sulfate (QLZ-S)

Compounds tested were aurintricarboxylic acid (ATC), quinalizarin (QLZ), acid green (AG), L-chicoric acid (LCH), alizarin (ALZ), purpurin (PUR), and lamellarin α 20-sulfate (Lamα). The structure of quinalizarin sulfate is shown in the inset. *Reddy et al. *J. Med. Chem.*, in press.

HIV vectors containing VSV-G envelope in place of the HIV gp120 have advantages over replication-competent HIV beyond a reduction in biohazard risk. HIV gp120 is easily lost from viral particles, causing preparations of HIV to decline in titer during storage. Particles containing VSV-G envelope, in contrast, are much more stable, allowing concentration and storage preceding generation of PICs[31]. Furthermore, the receptors for VSV-G are expressed on a broad range of cells, allowing infection of many cell types and analysis of host effects on PIC function.

Although PIC assays can be useful in assessing candidate inhibitors, we note that certain integrase inhibitors may be missed in PIC assays. Any compound that prevented the correct assembly of integrase into a PIC might not inhibit a fully assembled complex. L-Chicoric acid (LCH) may provide an example of such a compound, in that it inhibits reactions with purified integrase protein in vitro and appears to act against integrase in cell culture[32]. However, LCH does not inhibit integration by PICs (Table 1)[30]. Fully assembled PICs nevertheless represent an attractive target for inhibitors because they are long-lived intermediates[10,33].

Screening compounds against PICs offer the potential to identify new types of HIV inhibitors. Viral and cellular proteins in addition to integrase are stably associated with PICs[6,12,13,15] and may represent targets in addition to integrase. Furthermore, the additional PIC proteins may contribute to binding potential inhibitors active against integrase. If so, such compounds would be identified in assays with PICs and not in assays with purified integrase.

Experimental protocol

HIV vector particle production. The HIV vector-producing cell line SODk1CG2 used is identical to SODk1CG1 (ref. 24), except that the cell line was transduced a second time with an HIV-GFP-transducing vector to raise the copy number in the producer cell line (data not shown). Cell culture and virus titer measurements were carried out as described[24]. Culture supernatant containing vector particles was collected daily for four days. Induction was started on day 0 by removal of doxycycline, butyrate was added on day 1, and virus was harvested on days 3–6. Supernatants were concentrated by centrifugation at 200,000 *g* with a fixed-angle rotor (KOMPspin KA40.1; Composite Rotor, Mountain View, CA) for 1.5 h at 4°C. Pellets were suspended in media with 20% fetal calf serum at 1/100th of the starting volume, vortexed for 1 h at room temperature, and placed in a rotating rocker overnight at 4°C.

Preparation of preintegration complexes. Target cells (293T) were grown to 80% confluence on 150 cm^2 plates and infected in 8 ml of media containing 4 ml of concentrated viral supernatant and 10 μg/ml DEAE dextran (Sigma, St. Louis, MO). Infection was carried out at an m.o.i. of roughly 10. Virus-containing media was removed 3 h after infection and replaced with fresh media. Six hours after infection, cells were washed with buffer K (150 mM KCl, 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$), then permeabilized in 0.5 ml of buffer K per plate with 0.025% digitonin (CalBiochem San Diego, CA) for 10 min at room temperature. Lysates were centrifuged at 1000 *g*, supernatant was centrifuged at 12,000 *g*, and the supernatant was stored at -80°C (ref. 8).

Attachment of target DNA to microtiter plates. Sheared salmon sperm DNA (Stratagene, La Jolla, CA) was digested with *Eco*RI, deproteinated, precipitated, and suspended in 50 mM 1-methyl-imidazole, pH 6.2, and 20 mM NaCl at 40 μg/ml. An equal volume of 200 mM 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDC; Sigma) in 50 mM 1-methyl-imidazole, pH 6.2, and 20 mM NaCl were then added at room temperature to form the phosphorimidazolide at the 5′-phosphates. Before addition of DNA, Covalink (Corning, Acton, MA) microtiter plates containing free amines were washed three times with 50 mM 1-methyl imidazole, pH 6.2 (Sigma) and 20 mM NaCl. To attach the target DNA, plates were incubated with 50 mM 1-methyl imidazole, pH 6.2, 20 mM NaCl, 100 mM EDC, and 20 μg/ml modified DNA for 2 h at 50°C (refs. 34–38). Plate wells were washed five times with Buffer A (1 M NaCl, 20 mM HEPES, pH 7.4, 0.25% SDS, and 10 mM EDTA) at 65°C (the third wash was 20 min at 68°C), and five times with Buffer B (20 mM HEPES, pH 7.4, 0.25% SDS, and 10 mM EDTA) at 50°C (the third wash was 20 min at 50°C), and five times with Buffer K (150 mM KCl, 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$). Plates were incubated at 50°C in Buffer K containing 10 mM citraconic anhydride (Sigma) for 30 min. Plates were coated with 100 μg/ml tRNA (Boehringer Mannheim Biochemicals, Mannheim, Germany) and 0.2% bovine serum albumin (Sigma) by incubation at 4°C in Buffer K.

Rapid PIC integration reactions with immobilized target DNA. Microtiter plates containing immobilized target DNA were rinsed with Buffer K before addition of lysate. Reactions were carried out by adding 25 μl of extract containing PICs to DNA-coated plates plus 20 ml of Buffer K and 5 ml of dimethyl sulfoxide (DMSO). We included 10% DMSO (final volume, 50 ml) because it was the solvent used to dissolve many of the inhibitors tested. In inhibitor studies, 5 μl of 10× drug stocks in DMSO were incubated with lysates containing PICs for 7 min at room temperature before addition to the DNA-coated plates. Reactions were incubated for 30 min at 37°C to allow integration, the lysate solution was then removed, and any further reaction was terminated by addition of Buffer A to the wells. Plate wells were washed five times with Buffers A and B as above, followed by washing five times with Buffer C to remove SDS (20 mM KCl, 10 mM HEPES, pH 7.4, and 10 mM $MgCl_2$). Samples were eluted by the addition of 25 μl 0.04 N NaOH at 50°C for 10 min, transferred to strip PCR tubes (Phenix, Hayward, CA), and neutralized by the addition of 25 μl 0.04 N HCl and 50 mM HEPES, pH 7.4.

Detection of integration. For PCR amplification, 5 μl of integration product were added to reactions. HIV long-terminal-repeat sequences were detected with MH 301 5′-GAAGGGCTAATTCACTCCCAACGAAGACAA-3′ and MH 302 5′-CTAGAGATTTTCCACACTGACTAAAAGGGT-3′. The regimen was 95°C for 5 min, 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 20 s, followed by 72°C for 7 min. Control pUC19 DNA (100 fg) added to eluted DNA was amplified under the same conditions using MH 303 5′-TCACAATTCCACACAACATACGAGC-3′ and MH 304 5′-GTGATGCTCGTCAGGGGGGCGGAGC-3′. Fluorescence-monitored real-time PCR (TaqMan) reactions[26,27] were performed on an ABI Prism 7700 (PE Applied Biosystems) in a volume of 50 μl. Primers for amplification were MH 305F 5′-GCCGCCTAGCATTTCATCA-3′ and MH 306F 5′-CAAGCTCGATGTCAGCAGTTCTT-3′ (Retrogen, San Diego, CA). The probe for detection was MH 307F 5′-(FAM)-CCCGAGAGCTGCATCCGGAGTACTT-(TAMRA)-3′ (Perkin-Elmer). Reactions contained 300 nM primers, 100 nM probe, 5.5 mM $MgCl_2$, 1 mM dATP, dCTP, dGTP, 2 mM dUTP (Pharmacia, Uppsala, Sweden), 0.25 μl TaqGold (Perkin-Elmer), and Rox (a reference dye included to control for minor variations in reaction volume). The cycling method was 95°C for 10 min, and 40 cycles of 95°C 15 s, and 60°C for 1 min. Each point in Figure 5 was determined in at least four separate measurements.

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