Development of a New Methodology for Screening of Human Immunodeficiency Virus Type 1 Microbicides Based on Real-Time PCR Quantification

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Potential topical retroviral agents or vaginal microbicides against human immunodeficiency virus type 1 (HIV-1) include nonnucleoside reverse transcriptase inhibitors (NNRTIs). To be successful, such agents have to be highly active against cell-free virions. In the present study, we developed a new real-time PCR-based assay to measure the natural endogenous reverse transcription (NERT) activity directly on intact HIV-1 particles in the presence of reverse transcriptase (RT) inhibitors. We further evaluated the permeability to nevirapine (NVP) and efavirenz (EFV) and their retention within nascent viral particles. We also demonstrated the NVP and EFV inhibitory effects on NERT activity and the impact of resistance mutations measured directly by this new strategy. Furthermore, the results showed a clear correlation between NERT activity and classical infectivity assays. The 50% inhibitory concentrations (IC_{50}) of NVP and EFV were demonstrated to be up to 100-fold higher for cell-free than for cell-associated virions, suggesting that cell-free virions are less permeable to these drugs. Our results suggest that NVP and EFV penetrate both the envelope and the capsid of HIV-1 particles and readily inactivate cell-free virions. However, the characteristics of these NNRTIs, such as lower permeability and lower retention during washing procedures, in cell-free virions reduce their efficacies as microbicides. Here, we demonstrate the usefulness of the NERT real-time PCR as an assay for screening novel antiretroviral compounds with unique mechanisms of action.

The human immunodeficiency virus (HIV)/AIDS pandemic is continuously spreading at a rate of over 15,000 new infections every day. Heterosexual transmission is the leading mode of HIV type 1 (HIV-1) infection worldwide, with women particularly vulnerable (33). In the absence of an effective prophylactic anti-HIV therapy or vaccine, current efforts are aimed at developing anti-HIV agents, such as retroviral drugs or topical microbicide formulations. Accordingly, the World Health Organization has established a research priority for the development of effective anti-HIV vaginal microbicides (22).

Substances that can be applied directly inside the vagina or the rectum before sexual intercourse to prevent the transmission of HIV, and possibly other infections, were proposed in 1990 and widely researched as one alternative to condoms (8, 29). More than 60 candidate agents have already been identified to have in vitro activity against HIV, with 6 either in or about to enter phase III efficacy trial testing (2). More recently, rapid advances in our understanding of the biology of HIV transmission and infection have led to the development of microbicides that specifically target mechanisms of HIV transmission without harming the body’s natural defenses (26).

Ideally, a retroviral agent should fulfill several requirements: it should act directly on the virion, it should act at replication steps prior to integration of viral DNA into the infected host cell genome, it should be adsorbed by uninfected cells in order to provide a barrier to infection by residual active virions, and finally, it should be effective at noncytotoxic concentrations readily attainable in vivo (6).

Potential retroviral or vaginal microbicides include inhibitors of HIV surface glycoprotein and CD4 receptor interaction (3, 15, 17), virus envelope-disrupting agents (18, 25), coreceptor antagonists (11, 12), virus-cell fusion inhibitors (4), and viral-uncoupling inhibitors (7). Inhibitors of reverse transcription, such as nonnucleoside reverse transcriptase inhibitors (NNRTIs), could also be used as retrovirucides (30). Since their discovery, NNRTIs have become one of the cornerstones of highly active antiretroviral therapy. Currently, three NNRTI agents, efavirenz (EFV), nevirapine (NVP), and delavirdine, are commercially available (21).

To screen for potential reverse transcriptase (RT) inhibitor compounds that fulfill the requirements for an effective microbicide, a sensitive and high-throughput assay would be useful. The natural endogenous reverse transcription (NERT) activity is based on intravirion DNA synthesis in the absence of permeabilizing agents. This has been termed NERT to distinguish it from the somewhat artificial process that takes place in standard ERT assays (36). NERT is made possible by the amphipathic domains of the gp41 transmembrane protein, which render the HIV-1 envelope permeable to a range of small molecules, such as deoxynucleoside triphosphates (dNTPs) (37). NERT is an active process that is responsive to the virion microenvironment. Virions isolated from seminal plasma, which contains high levels of dNTPs, contained much higher levels of full-length or nearly full-length intravirion reverse transcripts than did virions isolated from the peripheral blood of the same patients (36). Moreover, the ability of purified virions to infect initially quiescent T cells and nonproliferating cells, such as macrophages, was significantly

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increased by preincubation of the virions with seminal plasma, indicating that NERT may be an integral part of the viral life cycle (36).

We developed a NERT assay based on real-time PCR quantification (NERT real-time PCR) in order to evaluate the effects of NNRTIs on intact cell-free virions. The methodology developed here was highly sensitive and demonstrated good correlation with other techniques commonly used for microbicidal screening. Our results demonstrate the usefulness of the NERT real-time PCR as a sensitive and efficacious assay for screening novel antiretroviral compounds with unique mechanisms of action.

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MATERIALS AND METHODS

Plasmids. Plasmids encoding the HIV-1 genome were from the wild-type pNL-A-3 (1) and the mutant Zöüst (32). The mutant ZöüstRTmut was obtained by site-directed mutagenesis at position 702. This mutation is known to decrease the susceptibility of HIV-1 to the nonnucleoside analogue NVP (23). The following primers were used in this site-directed mutagenesis: 5′-AGA AGT AGT TAT CTG TCA ATA CAT GGA TG-3′ (F) and 5′-CAT CCA TGT ATT GAC AGA TAA CTA TTT CT-3′ (R). Each mutation was separately introduced into the respective plasmid with the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions.

An env deletion variant of Zöüst, designated Zöüst-env, was obtained by oligonucleotide site-directed mutagenesis as described above. A frameshifting mutation was introduced into the env gene to prevent the expression of the gp160 glycoprotein. The following primers were used for site-directed mutagenesis: 5′-ATT GAT GAC CTG TAG TTA ATG CAG ACA ATC T-3′ (Forward) and 5′-ATT GAT GAC CTG TAG TTA ATG CAG ACA ATC T-3′ (Reverse). All constructs were sequenced and checked by digestion mapping to confirm the presence of the desired mutation and plasmid integrity. The plasmid SVA-MLV-env, encoding the amphotrophic murine leukemia virus (MLV) envelope glycoprotein, has been described elsewhere (20).

Cell culture and preparation of viral stocks. The HeK-293 cell line (26) was a kind gift from B. M. Peterlin (University of California—San Francisco). HeLa-MAGI cells were obtained from the NIH AIDS Research and Reference Reagent Program (catalog numbers 4666 and 4624, respectively). HeLa cells were cultured in Dulbecco’s modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA). MT4 cells were obtained from the NIH AIDS Research and Reference Reagent Program (catalog no. 120) (9). The cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA). All media were supplemented with 10% fetal bovine serum (FBS), 50 mg/ml of streptomycin, and 50 U/ml penicillin antibiotics (Invitrogen, Carlsbad, CA). All cell lines were maintained at 37°C with a 5% CO2 atmosphere. Virions were generated by plasmid transfection into Hek-293 cells by Lipofectamine-2000 protocols (Invitrogen, Carlsbad, CA). Briefly, cells were seeded in six-well plates (Corning, New York, NY) at a concentration of 2 × 105. The next day, the medium was replaced with a mixture of DNA and liposomes (2 μg DNA-2 μg Lipofectamine-2000) in OptiMEM (Invitrogen, Carlsbad, CA) was added. The cells were incubated at 37°C with a 5% CO2 atmosphere for 5 h. After this period, the medium was removed and replaced by fresh DMEM supplemented with FBS and antibiotics. To produce pseudotyped viral stocks, the ZöüstRTmut DNA was transfected together with equal proportions of the SV-A-MLV-env expression vector (1 μg each), following the procedure described above. The cell supernatants were harvested 72 h after transfection, centrifuged for 15 min at 20,000 rpm, and filtered through a 0.45-μm-pore-size filter to remove cellular debris. Virus production was quantified by measuring p24 antigen levels. Infected cells showing cytopathic effects were washed three times with PBS, and the numbers of viable cells were assayed, and data sets in which the linear correlation coefficient of the assay was less than 0.98 were not included for further analysis. The 50% inhibitory concentration (IC50) for each NNRTI, which is the concentration required for 50% reduction in RT activity (IC50 for each of these assays was calculated from the data using SigmaPlot software (version 9.0)).

Viruloinfectivity assay. The relative infectivity of viral particles was determined with MAGI cells (13) at 105 cells were seeded the day before the assay in 96-well plates. Each well was infected with 100 μl of serial dilutions of each virus aliquot and incubated for 5 h at 37°C. The medium containing free viruses was removed, the cells were washed in 1× PBS, and fresh DMEM was added. The plates were incubated at 37°C for 48 h. Further, the supernatants were removed, and the cells were fixed with 0.2% glutaraldehyde and 1% formaldehyde in 1× PBS at room temperature for 5 min. The cells were then washed twice with 1× PBS and stained with a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) solution: 0.4 mg/ml of X-Gal, 4 mM potassium ferricyanide, 4 mM potas-
sodium ferrocyanide, and 2 mM of MgCl₂ in 1× PBS. The staining was allowed to continue for 50 min at 37°C, and then the cells were washed twice with 1× PBS. The cells were inspected by light microscopy, and the results were scored as the total number of blue foci in each plate. This number was multiplied by the original viral dilutions to calculate the actual viral titer.

Statistical methods. Results are presented as means of three independent experiments with the standard deviations associated. Groups were compared by analysis of variance. Paired Student's t tests were utilized to compare the differences between the IC₅₀s for the two NNRTIs used. All tests were two tailed. A P value of 0.05 or less was considered statistically significant. The nonlinear regressions between the plots in the graphics were calculated with SigmaPlot software, version 9.0. We used the equation of Hill for the sigmoidal function. The significances between different dose-response curves were calculated using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

NERT-based real-time PCR assay. It was demonstrated that without detergent the addition of dNTPs to isolated HIV-1 particles could stimulate intravirion RT activity, leading to virions carrying −ssDNA intermediates and more complete negative-strand molecules. In our study, intact virions were incubated with increasing concentrations of dNTPs at 37°C for 120 min, and the reactions were terminated by stop solution addition and boiling. −ssDNA products were quantified by real-time PCR amplifications using specific oligonucleotides and a fluorescent probe (Fig. 1). NERT reactions carried out without dNTP addition did not produced appreciable levels of −ssDNA, while the endogenous RT activity increased proportionally to the increase in the dNTP concentration (Fig. 2). Interestingly, even without the addition of exogenous dNTPs, some level of −ssDNA was synthesized, suggesting the presence of small amounts of dNTPs inside viral particles. However, in the presence of increasing concentrations of dNTPs, the amounts of −ssDNA detected in NERT-based real-time PCR assays were significantly higher. The synthesis of

![Schematic representation of real-time PCR strategy and primers used to detect the NERT −ssDNA synthesis. We used the −ssDNAs synthesized during NERT reactions as the templates for real-time PCR assays. The absolute quantification of NERT activity and detection of its inhibition by NVP and EFV were performed using the SSF1-SSR1 oligonucleotide pair and the ssDNA probe for the 181-bp fragment corresponding to −ssDNA (for details, see Materials and Methods).](http://aac.asm.org/)

![NERT activities from intact viral particles exposed to increasing concentrations of dNTPs. Aliquots of Z6gpt, Z6gpt/env⁻, and MLV pseudotyped Z6gpt/env⁻ viruses were exposed to increasing concentrations of dNTPs, and the NERT activity was measured by real-time PCR. The absolute quantification of −ssDNA synthesis was obtained by comparison with a DNA standard curve. A plasmid containing the Z6gpt infectious clone was serially diluted in the NERT stop solution (10⁴ to 10⁷ copies of DNA) and subjected to real-time PCR amplification. The results are means of triplicate experiments, and the standard deviations are shown by the bars.](http://aac.asm.org/)
ssDNA reached very high levels with 400 μM of dNTPs (10,000fold higher than with the control without dNTPs) and did not increase any further with a concentration of 1 mM of dNTPs (Fig. 2). This is in agreement with the levels of −ssDNA synthesis observed by Zhang and coworkers (36) using equivalent concentrations of dNTPs. These results demonstrated that our new methodology is very sensitive for directly measuring the endogenous RT activity in intact viral particles without the use of permeabilizing agents, as used in classical ERT reactions (5). Furthermore, since levels of −ssDNA amplification were very satisfactory with 400 μM of dNTP, we choose this concentration for further experiments.

We also observed that the yield of −ssDNA could be reduced if the viral stocks were frozen and thawed many times. For this reason, all viral stocks were kept at 4°C, which preserved the NERT activity for at least 4 weeks.

HIV-1 envelope renders virions permeable to dNTPs. Using this new methodology, we further investigated the role of HIV-1 envelope for the permeability of virions to dNTPs. A Z6gpt/env− DNA clone was transfected into Hek-293 cells either alone or together with plasmids carrying envelope genes from either HIV-1 or MLV. Virions were harvested from the cell supernatants 48 h after transfection, and the levels of p24 were measured by enzyme-linked immunosorbent assay—p24 antigen. About 100 ng of p24 was used in the NERT reaction. As shown in Fig. 2, NERT activity was over 10,000-fold higher in virions harboring the HIV-1 envelope (Z6gpt) than in virions lacking the env gene (Z6gpt/env−). These results suggest that viral particles containing HIV-1 envelope glycoproteins were more permeable to dNTPs than viral particles lacking envelope glycoproteins. Moreover, the significant increment of permeability to dNTPs was observed only for the HIV-1 envelope glycoproteins, since the use of MLV envelope glycoproteins did not make pseudotyped virions more permeable to dNTPs, as shown by the low −ssDNA synthesis levels found when the MLV env pseudotyped virions were used in the experiment (Fig. 2).

Membrane permeabilities of virions and cells to NVP and EFV. We evaluated the effects of NVP and EFV on −ssDNA synthesis by NERT-based real-time PCR quantification. These specific inhibitors of RT can bind directly to the hydrophobic pocket within the enzyme, causing a conformational change that disrupts the catalytic site and blocks its DNA polymerase activity (23). Wild-type virions obtained from Hek-293 transfections were exposed to increasing concentrations of NVP or EFV for 2 h prior to NERT reactions. NERT was performed as described above, and the synthesis of −ssDNA was measured by real-time PCR. Identical aliquots of the initial virion inputs, not exposed to NNRTI, served as positive controls for −ssDNA synthesis in order to quantify the inhibitory effects of NVP and EFV on endogenous RT activity. The basal levels of −ssDNA synthesis in controls not exposed to dNTPs during NERT reactions were subtracted from the obtained values for all other samples. The IC_{50}s were calculated for both NVP (Fig. 3A) and EFV (Fig. 3B). NVP at 10 to 250 μM decreased the total levels of −ssDNA from 75% and up to 98%, respectively (Fig. 3A). This is in agreement with previous studies demonstrating NERT inhibition by NVP measured by radioactive semiquantitative PCR (10). For EFV, a concentration of 50 nM was effective in inhibiting NERT by 50% (Fig. 3B).

In order to compare the permeabilities to NVP and EFV in cell-free viruses with those in cell-associated viruses, the aliquots of virions exposed to NVP and EFV were also tested for HeLa MAGI infection. We observed that the IC_{50}s for NVP (Fig. 3A) and EFV (Fig. 3B) were 0.05 μM and 10 nM, respectively. The IC_{50}s are in agreement with those reported in the literature (34, 35). Importantly, the two assays showed similar effects of NNRTI inhibition on viral infectivity and in NERT assays, demonstrating good correlation between the two strategies. However, both NVP and EFV compounds were...
more effective in viral infectivity analyzed by HeLa MAGI cells than in −ssDNA synthesis evaluated by NERT reactions. In fact, the IC_{50} obtained for both NVP and EFV were significantly lower when measured by viral infectivity than for NERT reactions (Fig. 3A and B). The IC_{50} for cell-free viruses were demonstrated to be up to 100-fold higher for NVP and 10-fold higher for EFV than for HeLa MAGI cell infections (Fig. 3A and B). These differences were statistically significant and suggest the effect of membrane composition on drug permeability. Since lower concentrations of both NVP and EFV were required to inhibit cell-associated viruses than for cell-free viruses, we conclude that cellular membranes could have higher permeability to NNRTI ingress than viral membranes.

Effects of resistance mutations on NERT reactions in virions exposed to NNRTIs. Using this system, we then tested the effects of NNRTI treatment in intact viral particles carrying the primary RT mutation Y181C, which confers high levels of resistance to NVP (24). The Y181C mutation was introduced by site-directed mutagenesis into the Z6gpt infectious clone to generate the Z6gptRT^{Y181C} mutant. Aliquots of both viruses, Z6gpt and Z6gptRT^{Y181C}, were exposed to increasing concentrations of NVP for 2 h prior to NERT reactions. Synthesis of −ssDNA in the wild-type virus was completely inhibited with a concentration of 6.5 ± 4.0 μM of NVP, whereas the Z6gptRT^{Y181C} mutant was resistant to NVP at concentrations up to 256 ± 48 μM (means and standard deviations of IC_{50} determined by real-time PCR based on three independent experiments; P < 0.0001). These results demonstrated the potential of our quantitative NERT-based real-time PCR assay in screening for drugs that affect RT activity, as well as mutations related to resistance to such drugs.

Retention of NNRTIs in virions budded from infected cells in the presence of drugs. A microbicide needs to be active against the first steps of the virus replication cycle and to be incorporated into nascent virions during assembly and budding to prevent infection of new cells. Here, we evaluated the incorporation of NVP and EFV into nascent viral particles. MT4 cells were infected with NL4-3 virions by “spinoculation.” The infection was monitored by microscopic assessment until the start of cytopathic effects (syncytium formation). Infected cells were washed to remove viral particles still attached to the cell membrane and seeded in the presence of increasing concentrations of NVP and EFV. Virions released in the presence of these drugs were recovered after overnight exposure. The residual compounds still present in the supernatants were removed by dialysis. With this treatment, the unique NNRTIs remaining in these preparations were found only inside viral particles, which were incorporated during virion budding. To test for NNRTI effects, aliquots of dialyzed and nondialyzed supernatants were evaluated by NERT-based real-time PCR and HeLa MAGI cell infectivity. We further compared NERT reactions and the infectivities of the nascent virions prior to and after dialysis. Control experiments showed that the level of residual drug present on these dialyzed samples was below that required to affect the infectivity of virus that had not been previously exposed to the drug (data not shown). Inhibition of RT activity by NVP was less pronounced in viruses dialyzed (IC_{50} = 12 μM) after being harvested from cells exposed to NVP than in their nondialyzed (IC_{50} = 2.0 μM) counterparts (a sixfold drop after dialysis; P = 0.00103). In contrast, EFV-

![Figure 4](http://aac.asm.org/Downloaded from http://aac.asm.org)

FIG. 4. Retention of NVP and EFV in viruses budded in the presence of the NNRTIs. MT4 cells infected with NL4-3 virus were washed to remove attached viruses and exposed to increasing concentrations of NVP (A) and EFV (B). The viruses budded in the presence of these NNRTIs were collected and dialyzed to remove all excess drugs in the medium, leaving only the NNRTIs associated with the RT molecules. The same aliquots of dialyzed and nondialyzed viruses were subjected to NERT analysis. The absolute quantification of −ssDNA synthesis in each aliquot was calculated as a percentage relative to the positive controls not exposed to NNRTI treatment. The basal values of −ssDNA synthesis in negative controls without dNTPs were subtracted from all other values of NERT reactions. The IC_{50} are shown for each NNRTI in both situations (before and after dialysis). These results were obtained from two independent experiments, and the means and standard deviations are shown. The nonlinear regressions were calculated using the equation of Hill for the sigmoidal function of SigmaPlot software (version 8.0), and the significances between different dose-response curves were calculated using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).
that the NERT-based real-time PCR assay is an efficacious tool for quantifying RT activity inside particles, and more importantly, such activity correlates very well with virion infectivity ($r^2 = 0.8821; P < 0.05$).

**DISCUSSION**

In this work, we successfully evaluated the use of real-time PCR for measuring intravirion DNA synthesis (the endogenous RT activity) in intact HIV-1 particles. Furthermore, RT inhibition by widely used NNRTIs in this new assay correlated well with RT inhibition measured by classical infectivity assays. This is the first time that endogenous RT activity measured directly inside viral particles was correlated with virion infectivity, and our results indicated that there is a clear linear correlation between these two variables. The real-time PCR detection of NERT activity is advantageous, since it is highly sensitive and less time-consuming than the classical infectivity assays used to measure inhibition of viral replication by antiretroviral compounds. Therefore, this new methodology is suitable for large-scale and rapid screening of potential new RT inhibitors to be used, for example, as microbicides.

The HIV-1 envelope composition renders viral particles permeable to dNTPs, which favors RT activity while still inside the virion environment. The results obtained here confirmed the findings of Zhang et al. (37), demonstrating that the amphipathic domain of the gp41 transmembrane glycoprotein is responsible for the passage of dNTPs from the outside medium to the interior of the virions. Due to this, the increase in the basal levels of RT activity in response to an increase in the dNTP concentration was not detected in nonenveloped or even pseudotyped HIV-1 particles carrying the MLV envelope proteins. These results were important to demonstrate the specificity of our assay.

Blockage of intravirion DNA synthesis has been demonstrated to occur in the presence of both nucleoside analog inhibitors (ddTTP, ddATP, and AZT) and NNRTIs (10). Real-time PCR detection of NERT activity was equally sensitive to the presence of NNRTIs. Furthermore, this is the first time that the effect of an RT resistance mutation was demonstrated in a NERT assay. We have also observed that a drug concentration over the IC$_{50}$ calculated in cells is required to inhibit NERT activity, and for the first time, we could obtain an absolute quantification of this phenomenon. The increase in the drug concentration required to completely inhibit NERT was dependent on the drug analyzed, up to 100-fold for NVP and 10-fold for EFV; however, in both cases, the viral-membrane composition can play an important role in virion permeability to antiretroviral drugs compared to the infected-cell situation.

Other important factors are the binding and retention of NNRTIs in the RT molecules inside viral particles budded in the presence of an RT inhibitor. Typically, NNRTIs are hydrophobic molecules and readily traverse membrane barriers, such as that surrounding the HIV core. However, this property would also facilitate the NNRTI's efflux from the virion in the absence of sufficient extravirion levels of these inhibitors. In our experiments, we compared EFV and NVP retention inside the viral particle by dialysis procedures. NVP was more easily washed off by the dialysis process than EFV. The values of NERT inhibition for virus budded in the presence of EFV were not different before and after the wash procedure, suggesting that this NNRTI remains bound to the RT molecules inside the viral particles. These results agree with reports that named NNRTIs as rapid-equilibrium and tightly binding inhibitors (16, 28). NVP is a rapid-equilibrium inhibitor and requires an excess of the drug over the enzyme concentration (27). On the other hand, EFV is a tightly binding inhibitor of HIV-1 RT (14). Tightly binding inhibitors exhibit unique properties in their interactions with enzymes, which distinguish them from rapid-equilibrium inhibitors (31). EFV binds rapidly to RT but, once bound, dissociates only very slowly. Thus, RT remains inhibited for prolonged periods of time after being bound, even in the absence of significant levels of unbound inhibitor or the wash procedure to remove the excess inhibitor (14). Therefore, we suggested that tightly binding inhibition might be an important criterion for the NNRTI microbicide screening, and compounds classified as tightly binding, such as EFV, have great potential as microbicides.

Although both NVP and EFV fulfill the basic requirements for use as microbicides, our results for both the concentration...
required for NERT inhibition and the permeability to virions indicate that EFV is potentially a better candidate than NVP.

Of note, this is the first time that a highly sensitive method for NERT detection has been used to directly evaluate the impacts of antiretroviral drugs, validating the usefulness of the NERT assay methodology developed here. The method described in the present study could contribute to blocking subsequent cell infection. Furthermore, our results are the first report of a direct impact of resistant variants, which can be present in semen. The method developed here can be used to directly evaluate the impacts of RT mutations previously described in the antiretrovirals commonly used in AIDS treatment on the microbicide candidates for which we do not have previous knowledge of resistance mutations.

This work shows the usefulness of the NERT real-time PCR in screening novel antiretroviral microbicide compounds with unique mechanisms of action.

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