JOURNAL OF CLINICAL MICROBIOLOGY, July 2009, p. 2232–2242 Vol. 47, No. 7
0095-1137/09/$08.00+0 doi:10.1128/JCM.01739-08
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Novel Recombinant Virus Assay for Measuring Susceptibility of Human Immunodeficiency Virus Type 1 Group M Subtypes To Clinically Approved Drugs▽

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Received 9 September 2008/Returned for modification 14 March 2009/Accepted 21 April 2009

Combination therapy can successfully suppress human immunodeficiency virus (HIV) replication in patients but selects for drug resistance, requiring subsequent resistance-guided therapeutic changes. This report describes the development and validation of a novel assay that offers a uniform method to measure susceptibility to all clinically approved HIV type 1 (HIV-1) drugs targeting reverse transcriptase (RT), protease (PR), integrase (IN), and viral entry. It is an assay in which the antiviral effect on infection within a single replication cycle is measured in triply transfected U87.CD4.CXCR4.CCR5 cells, based on homologous recombination between patient-derived amplicons and molecular proviral clones tagged with the enhanced green fluorescent protein (EGFP) reporter gene and from which certain viral genomic regions are removed. The deletions stretch from p17 codon 7 to PR codon 98 in pNL4.3-ΔgagPR-EGFP, from PR codons 1 to 99 in pNL4.3-ΔPR-EGFP, from RT codons 1 to 560 in pNL4.3-ΔRT-EGFP, from IN codons 1 to 288 in pNL4.3-ΔIN-EGFP, and from gp120 codon 34 to gp41 codon 237 in pNL4.3-Δenv-EGFP. The optimized experimental conditions enable the investigation of patient samples regardless of viral subtype or coreceptor use. The extraction and amplification success rate for a set of clinical samples belonging to a broad range of HIV-1 group M genetic forms (A-J, CRF01-03, CRF05, and CRF12-13) and displaying a viral load range of 200 to >500,000 RNA copies/ml was 97%. The drug susceptibility measurements, based on discrimination between infected and noninfected cells on a single-cell level by flow cytometry, were reproducible, with coefficients of variation for resistance ranging from 7% to 31%, and were consistent with scientific literature in terms of magnitude and specificity.

Despite continued improvements in treatment of human immunodeficiency virus type 1 (HIV-1)-infected patients, therapy failure still occurs, often leading to drug resistance development, which necessitates a change in regimen. At this moment, clinicians have at their disposal 22 drugs, targeting the viral protease (PR), the reverse transcriptase (RT), the integrase (IN), the transmembrane glycoprotein (gp41), or the interaction between the surface glycoprotein (gp120) and the cellular coreceptor CCR5. The choice of which drugs to include in the next regimen is based upon the likelihood of the drugs being active against the mutant virus present in the patient with incomplete suppression of replication (11).

Currently, genotypic drug resistance testing is used more frequently within the clinical setting of patient follow-up due to practical reasons, such as a shorter turnaround time, easier implementation within laboratories, and lower cost, but also due to the fact that no clinical trial has yet provided sufficient evidence for supporting phenotypic over genotypic drug resistance testing. Nevertheless, the interpretation of genotypic drug resistance testing can be very complex, which makes phenotypic drug resistance testing in certain settings very useful and even necessary (48).

Increasing numbers of drug resistance mutations have been identified within *gag*, *pol*, and *env*, reflecting the genetic flexibility of HIV-1. These mutations can directly boost the ability of the virus to specifically replicate in the presence of the drug (major mutations) or indirectly aid the virus by increasing its replication capacity in general, whether in the presence or absence of the drug (minor or compensatory mutations). In general, the major mutations are found at the drug binding sites within the targeted protein (or the viral protein that interacts with the cellular target), whereas the others can be found at distinct sites within the target protein or even other proteins. In this respect, PR inhibitor (PI) mutations have been observed not only within the PR but also at several sites within its Gag substrate (13, 28).

The use of genotypic data to determine if certain drugs are still active against patient-derived virus is further complicated by the presence of natural polymorphisms in non-B strains and by baseline subtype-dependent combinations of mutations that occur during treatment, leading to discordances between different interpretation algorithms (43, 51). Several studies have revealed novel mutations or differences in the prevalence of known mutations in non-B subtypes (1, 3). Others have demonstrated the impact of the genetic background on subtype dependencies in drug susceptibility and resistance development (2, 6, 7). In this respect, phenotypic drug resistance test-

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▽ Published ahead of print on 29 April 2009.

ing is a very useful tool to determine the effects of specific mutations or combinations thereof in their respective backbones and to improve genotypic drug resistance interpretation in the long run.

For newly approved drugs, the relationships between genetic changes in the target region and the clinical response still need to be established. Phenotypic assays can determine whether or not the new drug is active against the patient-derived virus and could be included in the next regimen (23). They also allow the investigation of potential cross-resistance for novel drugs with similar mechanisms of action to those of drugs included in previous regimens (8).

In this paper, we describe a novel assay based on the creation of recombinant replication-competent viruses that enables determination of the impact of mutations and combinations of mutations on susceptibility toward all clinically approved HIV-1 inhibitors. The recombinant viruses are tagged with the enhanced green fluorescent protein (EGFP) reporter gene, which allows for discrimination between infected and noninfected cells on a single-cell level by flow cytometry, which provides a high sensitivity. The assay was designed such that it offers a uniform methodology for all currently approved inhibitors, regardless of subtype or cellular tropism of the viral strains tested.

MATERIALS AND METHODS

Antiviral drugs. Zidovudine (AZT), abacavir (ABC), didanosine (DDI), efavirenz (EFV), lamivudine (3TC), stavudine (D4T), nelfinavir (NFV), ritonavir (RTV), nevirapine (NVP), and enfuvirtide (ENF) were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). Raltegravir (RAL), maraviroc (MVC), and AMD3100 were obtained from Merck & Co. (Rahway, NJ), Pfizer (Groton, CT), and AnorMed (Langley, British Columbia, Canada), respectively. Elvitegravir (EVG) and (*R*)-9-(2-phosphonylmethoxypropyl)adenine (PMPA; also called tenofovir [TDF]) were kindly provided by Gilead Sciences (Foster City, CA).

Cells. Human embryonic kidney cells (293T cells) were purchased from the ATCC (through LGC Standards, Teddington, United Kingdom) and cultivated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (Perbio Science, Erembodegem, Belgium), 20 μg/ml gentamicin (Invitrogen), and 75 mM $NaHCO_3$ (Invitrogen). U87.CD4.CXCR4, U87.CD4.CCR5, and U87.CD4.CXCR4.CCR5 cells (35) were cultured in DMEM containing 10% fetal calf serum supplemented with 0.2 mg/ml Geneticin (Invitrogen) and 1 μg/ml puromycin (Sigma-Aldrich, Bornem, Belgium). The cell cultures were maintained in a humidified CO_2-controlled atmosphere and subcultivated every 2 to 3 days by digestion with trypsin-EDTA (Invitrogen).

Plasmids. The hemigenomic plasmids p83-2 and p83-10 (16), containing the 5′ half and the 3′ half of the HIV-1 NL4.3 genome, respectively, as well as pT66I (containing the T66I mutation in the NL4.3 integrase [45]), were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH) from Ronald Desrosiers and Vinay K. Pathak, respectively.

The molecular clone pNL4.3-EGFP, containing the gene encoding EGFP between *env* and *nef* without affecting expression of any HIV gene, and pNL4.3-Δenv-EGFP, displaying a deletion within the *env* gene (53), were provided by M. Quiñones-Mateu of The Cleveland Clinic Foundation (Cleveland, OH). The molecular clone pNL4.3-Δenv-EGFP contains a deletion between nucleotides 6404 and 8458.

Viruses. (i) Reference viruses. For optimization of amplification procedures, a dilution series of the HIV-1 lab strain III_B (kindly provided by R. C. Gallo, National Institutes of Health, Bethesda, MD) was used.

A CXCR4-tropic (X4) strain was obtained by transfecting pNL4.3-EGFP into 293T cells as described below. The CCR5-tropic (R5) strains Ada and BaL were originally obtained from the NIAID AIDS Research and Reference Reagent Program (Bethesda, MD). The dual-tropic (R5/X4) HIV-1 strain HE was isolated from a Belgian AIDS patient (33).

(ii) Validation samples. Plasma samples were obtained from patients attending the AIDS Reference Centers at the University Hospitals Leuven (a kind gift of E. Van Wijngaerden).

Plasmids containing the HIV-1 wild-type RT or *env* sequence were generated by amplifying the RT region from p83-2 or the *env* region from p83-10, BaL, or HE, using PfuTurbo DNA polymerase (Stratagene, Amsterdam, The Netherlands), and subsequently cloning the sequence by use of a Topo XL PCR cloning kit (Invitrogen). These constructs were used as templates in site-directed mutagenesis experiments to generate the following reference plasmids: p83-10-gp41-D36G (wild type for ENF), p83-10-gp41-D36G-V38M, envBaL-gp41-V38M, envHE-gp41-V38M, RT-Q151M, RT-K70R, RT-K65R, and RT-A62V-S68G-V75I-I77L-F116Y-Q151M. All nucleotide sequences were verified.

Quantification of virus and infected cells. (i) Viral load. The plasma viral load was determined using Versant HIV-1 RNA 3.0 assay (bDNA assay; Bayer HealthCare, Brussels, Belgium) and Abbott RealTime HIV-1 assay (Abbott Molecular, Louvain-La-Neuve, Belgium).

(ii) Viral core antigen. HIV-1 core antigen (p24 Ag) in the supernatant was analyzed with an Alliance HIV-1 p24 antigen enzyme-linked immunosorbent assay kit (Perkin-Elmer Life and Analytical Sciences, Brussels, Belgium).

(iii) Viral titer. A viral stock was titrated by plating 20,000 U87.CD4.CCR5.CXCR4 cells per well in a 96-well plate 2 h in advance and infecting them afterwards with 1/2 serial viral dilutions (in triplicate). Twenty-four hours after infection, the supernatant was removed, and cells were washed with phosphate-buffered saline (PBS), trypsinized, and fixed with 2% paraformaldehyde in PBS. Subsequently, EGFP expression was quantified by flow cytometry.

(iv) Cells infected with EGFP-tagged virus. The percentage of EGFP-expressing cells was determined using a FACSCantoII flow cytometer (BD Biosciences, Erembodegem, Belgium) equipped with a high-throughput sampler. EGFP was excited using a 488-nm-wavelength argon-ion laser, and EGFP expression was detected using a 530/30-nm-band-pass filter. Data were analyzed using FACS-Diva v5.0.2 software (BD Biosciences). Forward- versus side-scatter plots were used to exclude dead cells and debris from analysis. Acquisition was stopped when 10,000 gated events were counted. Analytical gates were set in such a manner that fewer than 0.1% of uninfected cells were within the EGFP-positive region.

Extraction. (i) RNA extraction. For clinical samples and their III_B reference, 1-ml plasma samples or virus supernatant was ultracentrifuged at 37,100 × *g* for 1 h to pellet the virus, and samples were extracted subsequently using the extraction procedure from the Viroseq HIV-1 genotyping system (Abbott Molecular, Louvain-La-Neuve, Belgium).

For recombinant viruses, 20 μl of virus supernatant was incubated with 560 μl AVL lysis buffer (Qiagen, Venlo, The Netherlands) for 10 min at room temperature. Subsequently, viral RNA was precipitated with isopropanol, washed with 70% ethanol, and resuspended in 50 μl of RNase-free water (Sigma-Aldrich).

(ii) DNA extraction. Plasmid DNA was purified using a QIAprep Spin miniprep kit (Qiagen) or an Endofree plasmid maxi kit (Qiagen) when DNA was used for transfection experiments.

Primer design. The PCR and sequencing primers were designed using sequence alignments for several HIV-1 group M strains (19). Primers were developed and analyzed using Oligo software (Medprobe, Oslo, Norway). The primers were synthesized by Invitrogen.

cDNA synthesis and amplification. Ten microliters of a 50-μl RNA extract was reverse transcribed and amplified in a one-step RT-PCR, using the SuperScript III one-step RT-PCR system with Platinum *Taq* high-fidelity polymerase (Invitrogen) in the presence of 10 U of Protector RNase inhibitor (Roche Diagnostics, Vilvoorde, Belgium). Inner PCR products were obtained by adding 1 to 5 μl outer PCR product to an inner PCR mix, using the Expand high-fidelity PCR system (Roche Diagnostics). Primers and cycling conditions are displayed in Tables 1 and 2, respectively. Amplification products were separated in a 1% agarose gel and visualized by ethidium bromide staining. The images were processed on a video imager (ImageMaster VDS; GE Healthcare, Diegem, Belgium).

Sequencing. PCR products for population sequencing were purified with Microspin S-400 (GE Healthcare). Sequencing was performed using an ABI Prism BigDye Terminator v3.1 ready reaction cycle sequencing kit (42, 47, 49, 50). The sequencing reactions were run on an ABI 3100 genetic analyzer (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands). The sequences were analyzed using Sequence Analysis, version 3.7, and SeqScape, version 2.0 (Applied Biosystems).

Transfection, subcultivation, and harvest of recombinant viruses. On the day before transfection, 700,000 293T cells were subcultivated in a 5-cm dish with 5 ml of DMEM. Two micrograms of purified PCR product (QIAquick PCR purification kit; Qiagen) was mixed and coprecipitated with 10 μg of XbaI-digested

TABLE 1. Primers used for amplification of different HIV-1 regions

Amplified region	Inner or outer region	Primer name	Primer sequence (5′–3′)	Position[b]	Sense or antisense	Reference
Gag-PR	Outer	KVL064	GTTGTGTGACTCTGGTAACTAGAGATCCCTCAGA	570–603	Sense	49
	Outer	KVL065	TCCTAATTGAACYTCCCARAARTCYTGAGTTC	2797–2828	Antisense	49
	Inner	KVL066	TCTCTAGCAGTGGCGCCCGAACAG	626–649	Sense	49
	Inner	KVL067	GGCCATTGTTTAACYTTTGGDCCATCC	2597–2623	Antisense	49
PR[a]	Outer	KVL064	GTTGTGTGACTCTGGTAACTAGAGATCCCTCAGA	570–603	Sense	49
	Outer	KVL065	TCCTAATTGAACYTCCCARAARTCYTGAGTTC	2797–2828	Antisense	49
	Inner	AV75	TGTACTGAGAGACAGGCTAATTTTTTAGGG	2065–2094	Sense	
	Inner	KVL067	GGCCATTGTTTAACYTTTGGDCCATCC	2597–2623	Antisense	49
RT	Outer	AV190-1	GCTACAYTAGAAGAAATGATGACAGCAT	1810–1838	Sense	42
	Outer	CR1	GATTCTACTACTCCTTGACTTTGGGGATTGTAGGGAA	4687–4651	Antisense	42
	Inner	KVL098	GGAAGCTCTATTAGAYACAGGAGCAGAT	2312–2339	Sense	
	Inner	KVL099	CTGGACTACAGTCTACTTGTCCATG	4380–4404	Antisense	
IN	Outer	KVL068	AGGAGCAGAAACTTWCTATGTAGATGG	3854–3880	Sense	50
	Outer	KVL069	TTCTTCCTGCCATAGGARATGCCTAAG	5955–5981	Antisense	50
	Inner	KVL070	TTCRGGATYAGAAGTAAAYATAGTAACAG	4013–4041	Sense	50
	Inner	KVL084	TCCTGTATGCARACCCCAATATG	5243–5265	Antisense	50
env	Outer	EnvA	GCCTTAGGCATCTCCTATGGCAGGAAGAA	5953–5981	Sense	14
	Outer	KVL009	GCCAATCAGGGAAGWAGCCTTGTGT	9135–9159	Antisense	47
	Inner	EnvB	AGAAAGAGCAGAAGACAGTGGCAATGA	6198–6224	Sense	14
	Inner	HIV-8726-R	TTGTACTACTTCTATAACCCTATCTGT	8690–8716	Antisense	

[a] The inner PCR for amplification of PR can also be performed on the RT outer PCR product.
[b] Positions according to pNL4.3 sequence (GenBank accession no. AF324493).

proviral vector. DNA and $CaCl_2$ were gently mixed and added to an equal volume of 2× HEPES-buffered saline. After 20 min of incubation at room temperature, the mixture was added to the 293T cells. The medium was refreshed with 5 ml DMEM after overnight incubation. Transfection was monitored through fluorescence microscopy as described previously (5). Two days after transfection, 5 ml supernatant was transferred to freshly seeded U87.CD4.CCR5.CXCR4 cells. Cell cultures were monitored for EGFP expression by fluorescence microscopy. Virus supernatants were harvested by low-speed centrifugation and stored in 1-ml aliquots at −80°C for further use.

Drug susceptibility testing. One hundred microliters of medium containing 20,000 U87.CD4.CCR5.CXCR4 cells was seeded within each well of a 96-well tray. Two hours later, triplicate fivefold serial dilutions of the drugs (RT inhibitors, IN inhibitors, and entry inhibitors) were performed within the tray, followed by the addition of 100 μl of virus-containing medium to reach a final volume of 200 μl/well. Virus was prediluted based upon viral titration experiments to obtain 10% EGFP-expressing U87.CD4.CCR5.CXCR4 cells in each well, without any inhibition of antiviral drugs. For MVC and AMD3100, infection was not carried out until half an hour after the addition of the compound to allow for interaction of the compounds with CCR5 and CXCR4, respectively. Twenty-four hours after infection, the percentage of EGFP-expressing cells was determined by flow cytometry. The 50% inhibitory concentration (IC_{50}) was calculated according to the method of Reed and Muench (37).

For PI susceptibility testing, 100 μl medium containing 15,000 U87.CD4.CCR5.CXCR4 cells was seeded within each well of a 96-well tray. Two hours later, 100 μl of virus-containing medium was added. Another 3 h later, supernatant was replaced with 200 μl medium containing fivefold dilutions of the PI. Forty hours after infection, the supernatant was transferred to U87.CD4.CCR5.CXCR4 cells seeded at a density of 20,000 cells/well 2 h earlier. Twenty-four hours after the second infection round, the percentage of EGFP-expressing cells was determined by flow cytometry. The IC_{50} was calculated according to the method of Reed and Muench (37).

RESULTS

Construction of molecular proviral clones. The plasmids pNL4.3-ΔgagPR-EGFP, pNL4.3-ΔPR-EGFP, pNL4.3-ΔRT-EGFP, and pNL4.3-ΔIN-EGFP were generated by deleting specific re-

TABLE 2. Thermal cycling profiles for amplification of different HIV-1 regions

PCR round	Cycling profile for amplified region				
	gag-PR	PR	RT	IN	*env*
Outer RT-PCR	55°C for 30 min 94°C for 2 min 94°C for 15 s 57°C for 30 s and 68°C for 2 min (40 times) 68°C for 5 min 4°C (infinite)	55°C for 30 min 94°C for 2 min 94°C for 15 s 57°C for 30 s and 68°C for 2 min (40 times) 68°C for 5 min 4°C (infinite)	55°C for 30 min 94°C for 2 min 94°C for 15 s 61°C for 30 s and 68°C for 3 min (40 times) 68°C for 5 min 4°C (infinite)	55°C for 30 min 94°C for 2 min 94°C for 15 s 53°C for 30 s and 68°C for 2 min 30 s (40 times) 68°C for 5 min 4°C (infinite)	55°C for 30 min 94°C for 2 min 94°C for 15 s 55°C for 30 s and 68°C for 3 min 30 s (40 times) 68°C for 5 min 4°C (infinite)
Inner PCR[a]	95°C for 2 min 95°C for 15 s 58°C for 30 s and 68°C for 2 min 30 s (40 times) 72°C for 10 min 4°C (infinite)	94°C for 2 min 94°C for 15 s 57°C for 30 s and 72°C for 1 min (40 times) 72°C for 7 min 4°C (infinite)	94°C for 2 min 94°C for 15 s 54°C for 30 s and 72°C for 2 min (40 times) 72°C for 7 min 4°C (infinite)	94°C for 2 min 94°C for 15 s 53°C for 30 s and 72°C for 1 min 30 s (40 times) 72°C for 7 min 4°C (infinite)	94°C for 2 min 94°C for 15 s 55°C for 30 s and 68°C for 2 min 30 s (40 times) 68°C for 5 min 4°C (infinite)

[a] Starting from cycle 11, the extension time was elongated by 5 s for each cycle, and the annealing temperature for PR amplification was decreased to 55°C.

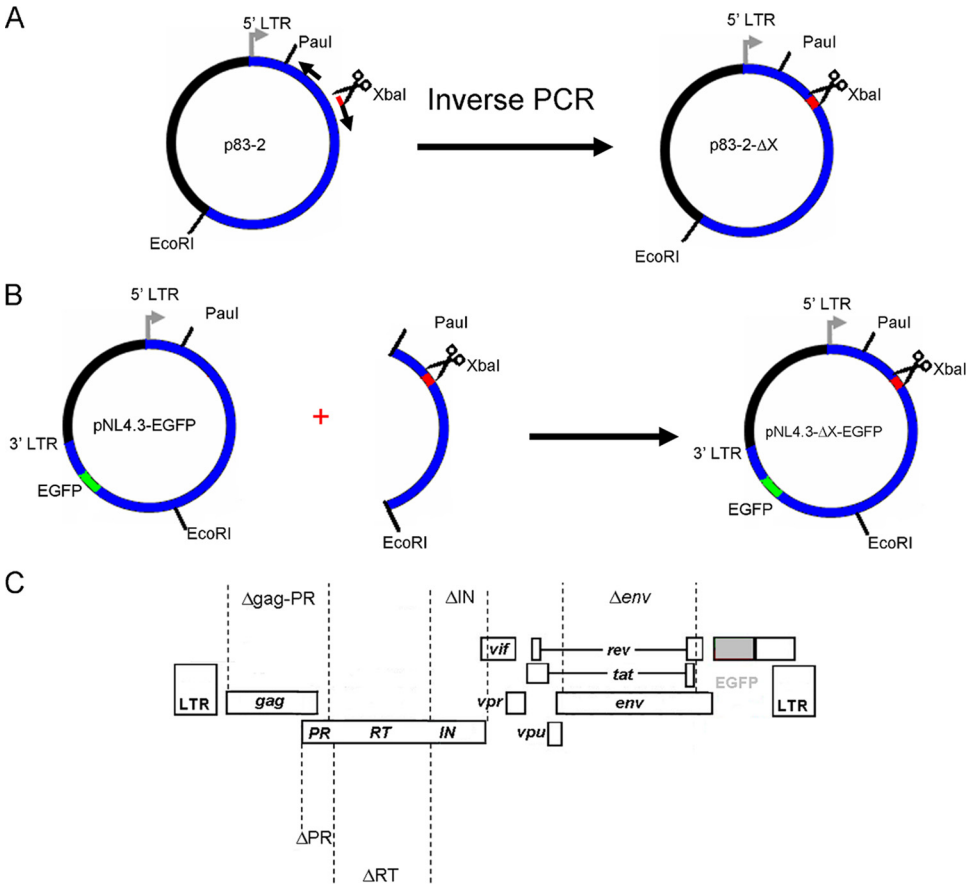

FIG. 1. Construction of molecular proviral clones containing deletions and the EGFP reporter gene. ΔX indicates a deletion of *gag*-PR, PR, RT, or IN. The deletions were generated in the 5′-hemigenomic molecular clone p83-2 by inverse PCR and self-ligation (A), followed by subcloning into pNL4.3-EGFP (B). (C) Schematic representation of generated vectors. (Adapted from reference 53 with permission of the publisher.)

gions from the p83-2 plasmid by inverse PCR using PfuUltra high-fidelity DNA polymerase (Stratagene) (Fig. 1A). The primers were designed such that each encoding fragment was removed and an XbaI restriction enzyme recognition site was inserted in the deleted region. The following combinations of primers were used to generate the deletions: for the *gag*-PR region, 5′-**TCTAGA**TTTT*CCC*ATTAGTCCTA TTGAGACTGTACCAG-3′ (positions 2546 to 2577 in pNL4.3 [GenBank accession no. AF324493]) and 5′-CGAC GCTCTCGCACCCAT*CTC*TG-3′ (positions 785 to 807); for the PR region, 5′-**TCTAGA***CCC*ATTAGTCCTATTGA GACTGTACCAGTA-3′ (positions 2550 to 2579) and 5′-*G AA*GCTAAAGGATACAGTTCCTTGTCTATCG-3′ (positions 2222 to 2252); for the RT region, 5′-**TCTAGA***TTT*TT AGATGGAATAGATAAGGCCCAAGAAGAA-3′ (positions 4230 to 4262) and 5′-*AAA*ATTTAAAGTGCAGCCAATCTGAG TCAACAG-3′ (positions 2517 to 2549); and for the IN region, 5′-*CAC*ATGGAAAAGATTAGTAAAACACCATATGTATAT TTC-3′ (positions 5097 to 5135) and 5′-**TCTAGA***TAG*TACTTTCC TGATTCCAGCACTGACCA-3′ (positions 4201 to 4229). The XbaI restriction enzyme recognition site is shown in bold, and the first codons upstream and downstream of the *gag*-PR, PR, RT, and IN coding regions are shown in italics. Parental DNA was digested with DpnI (Fermentas), and the PCR fragments were self-ligated using T4 DNA ligase (Invitrogen). The PauI-EcoRI fragments of the deleted p83-2 plasmids were subcloned into pNL4.3-EGFP (Fig. 1B). The full-length HIV-1 sequences of all molecular proviral clones were verified.

No mutations in comparison to the parental DNA were observed, and sequencing confirmed deletions going from p17 amino acid 7 to PR amino acid 98 in pNL4.3-ΔgagPR-EGFP, from PR amino acids 1 to 99 in pNL4.3-ΔPR-EGFP, and from IN amino acids 1 to 288 in pNL4.3-ΔIN-EGFP. Sequencing confirmed the deletion of amino acids 1 to 560 of RT in pNL4.3-ΔRT-EGFP but also revealed the presence of one mutation in comparison to the parental DNA. At position 2528, a G-to-T mutation was present, which would change a glutamine to a histidine at position 94 in PR. Since this mutation was close to the XbaI restriction site (positions 2550 to 2556), it was within the part of the virus that was derived from the PCR amplicon after recombination (as verified by sequencing of recombinant virus [data not shown]) and thus did not interfere with susceptibility testing. pNL4.3-Δenv-EGFP displayed a deletion from gp120 amino acid 34 to gp41 amino acid 237 (53).

Amplification performance. The primers were designed to enable the amplification of divergent strains from different HIV-1 group M subtypes and the amplification of fragments sufficiently long to enable homologous recombination with the respective molecular proviral clones (at least 100 bp of over-

TABLE 3. Sensitivities of amplification assays for *gag*-PR, PR, RT, IN, and *env* from clinical HIV-1 samples[a]

Viral load (RNA copies/ml)	Success rate (% [no. of successes/no. of attempts])					
	Gag-PR (1,998 bp)	PR (559 bp)	RT (2,093 bp)	IN (1,253 bp)	Env (2,519 bp)	Total
200–1,000	100 (1/1)	100 (3/3)	100 (3/3)	90 (9/10)	100 (11/11)	96 (27/28)
1,000–5,000	89 (8/9)	90 (12/13)	90 (7/8)	89 (17/19)	100 (5/5)	91 (49/54)
5,000–10,000	100 (6/6)	100 (9/9)	100 (5/5)	89 (8/9)	100 (1/1)	97 (29/30)
10,000–100,000	100 (15/15)	100 (21/21)	100 (26/26)	100 (27/27)	100 (2/2)	100 (91/91)
>100,000	100 (8/8)	100 (5/5)	100 (9/9)	100 (9/9)	100 (3/3)	100 (34/34)
Total	97 (38/39)	98 (50/51)	98 (50/51)	95 (70/74)	100 (22/22)	97 (230/237)

[a] Samples belonged to the genetic forms A (12%), B (17%), C (9%), D, (8%) F (6%), G (8%), H (1%), J (4%), CRF01_AE (8%), CRF02_AG (11%), CRF03_AB (3%), CRF05_DF (1%), CRF12_BF (5%), and CRF13_cpx (5%) or were a unique recombinant form (2%), as determined by the Rega subtyping tool (9; http://www.bioafrica.net/subtypetool/html/). The failed amplifications of the PR and RT regions and one in the IN region were performed on the same sample, belonging to CRF03_AB, and we believe that this can be attributed to poor quality of the extracted RNA (50). Since no more sample was available, we could not reextract the RNA. The other failed amplifications belonged to subtype B (*gag*-PR), subtype C, CRF01_AE, and CRF02_AG (IN).

lap) (Table 1). Cycling conditions were optimized with a dilution series of HIV-1 IIIB in PBS, ranging from 1,000,000 to 10 RNA copies/ml (Table 2). The optimized nested PCR procedures were specific, generating only a single amplification band, and they were very sensitive (detecting 10 to 100 RNA copies/ml).

Subsequently, they were each validated on a set of clinical samples belonging to a broad range of HIV-1 group M subtypes (A, B, C, D, F, G, H, J, CRF01, CRF02, CRF03, CRF05, CRF12, and CRF13) and displaying a viral load range of 200 to >500,000 RNA copies/ml (Table 3). The success rate varied from 91 to 100% depending on the viral load range and from 95 to 100% depending on the specific assay. The total amplification success rate was 97% (including only samples above the cutoff of 1,000 RNA copies/ml recommended for resistance testing in clinical practice).

Production and characterization of replication-competent recombinant viruses from reference strains and clinical samples. At first, the transfection, homologous recombination, and subcultivation procedures were fine-tuned for the X4 strain NL4.3, starting from amplicons covering the *gag*-PR, PR, RT, IN, and *env* regions of the NL4.3 template and the respective

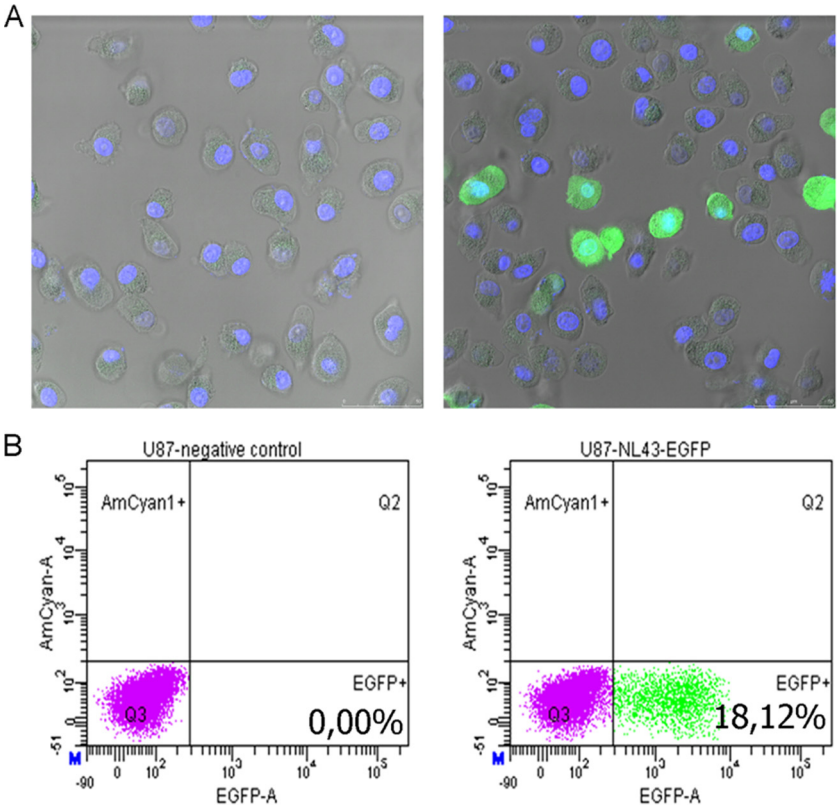

FIG. 2. Discrimination between infected and noninfected cells. U87.CD4.CCR5.CXCR4 cells were infected with HIV-1-NL4.3-EGFP (right) or were mock infected (left). Twenty-four hours later, cells were washed with PBS, trypsinized, and fixed in 2% paraformaldehyde for 10 min. (A) Afterwards, nuclei were stained for 5 min with DAPI (4′,6-diamidino-2-phenylindole). Subsequently, cells were washed three times with PBS, resuspended in PBS, and visualized by fluorescence microscopy. (B) After fixation, cells were analyzed by flow cytometry.

TABLE 4. Intra- and interassay reproducibility of HIV-1 susceptibility testing with several inhibitors from different drug classes

Recombinant virus	Inhibitor	IC_{50} (ng/ml)[a]	SD (ng/ml)[b]	CV (%)[c]	$FCIC_{50}$[d]	SD of $FCIC_{50}$[e]	CV of $FCIC_{50}$[f]
RT-NL4.3	DDI	565.4	54	9.6			
	AZT	1.29	0.35	27			
	ABC	392	43	11			
	D4T	4.00	0.58	14			
	TDF	390	27	7			
	3TC	84.8	15	18			
	EFV	1.77	0.25	14			
	NVP	18.5	3.15	17			
RT-NL4.3-Q151 M	AZT				1.48	0.11	7.4
	ABC				2.68	0.46	17
RT-NL4.3-K70R	D4T				0.95	0.15	15
PR-NL4.3	RTV	75.2	18.3	24			
	NFV	1.66	0.29	17			
PR-CS[g]	NFV				9.52	1.15	12
IN-NL4.3	RAL	6.3	1.1	17			
	EVG	0.40	0.06	15			
IN-NL4.3-T66I	EVG				5.22	1.63	31
Env-NL4.3	ENF	85	11	13			
	AMD3100	32	4	13			
	MVC	No inhibition					
Env-NL4.3-V38 M	ENF				5.26	1.57	30
Env-He	ENF	110	16	15			
Env-BaL	ENF	62	30	48			
	MVC	0.92	0.14	15			

[a] Means for one triplicate experiment.
[b] Standard deviations of IC_{50}s for one triplicate experiment.
[c] Coefficients of variation of IC_{50}s for one triplicate experiment.
[d] Mean fold changes in IC_{50} ($FCIC_{50}$) versus wild-type NL4.3 (*env*-gp41 D36G in the case of ENF) for three independent experiments.
[e] Standard deviations of $FCIC_{50}$ for three independent experiments.
[f] Coefficients of variation of $FCIC_{50}$ for three independent experiments.
[g] Recombinant virus generated from a cloned PR of a clinical sample belonging to subtype B that contains I13V, I15V, K20R, E35D, M36I, I54V, L63P, I64V, A71V, N88D and L90M mutations in PR.

XbaI-cut molecular proviral clones, i.e., pNL4.3-ΔgagPR-EGFP, pNL4.3-ΔPR-EGFP, pNL4.3-ΔRT-EGFP, pNL4.3-ΔIN-EGFP, and pNL4.3-Δenv-EGFP. Using optimized conditions (as mentioned in Materials and Methods), virus supernatant could typically be harvested 3 to 5 days after infection of U87.CD4.CCR5.CXCR4 cells (see Fig. 4).

Next, replication-competent viruses containing *env* genes of different R5 subtype B strains (BaL and Ada) and a dual-tropic (R5/X4) HIV-1 strain (HE) were generated by the same methodology. Potential changes in the tropism of the virus (due to growth on cells expressing high levels of both CXCR4 and CCR5) were controlled by infecting U87.CD4.CXCR4 cells and U87.CD4.CCR5 cells with the respective recombinant viruses, and no changes in tropism were observed (as monitored by fluorescence microscopy), even after cultivation on U87.CD4.CCR5.CXCR4 cells for more than 2 weeks (data not shown).

Finally, 22 recombinant viruses with recombination within the different regions were generated starting from several clinical HIV-1 samples. Irrespective of the viral load (range, 2,000 to 120,000 RNA copies/ml), subtype (A, B, C, D, G, J, and CRF02_AG included), and drug resistance profile, the procedure was successful in generating replication-competent recombinant viruses within a similar time frame to that for the reference strains. For the samples that were tested, no major selection biases occurred, as observed by population sequencing of the harvested recombinant strains. Sequences from all viruses that were generated using the PCR product from a single cloned sequence were identical to the original clone from which they were derived. For a few recombinant viruses generated directly from amplified plasma samples, population mixtures present in the plasma sample were no longer present in the recombinant virus stock. All major resistance-associated mutations were unaltered, and the observed changes in mixtures at positions other than major resistance positions did not change the resistance level according to the Rega interpretation system (46; data not shown). Differences in *gag* between the original isolate and the recombinant virus were E62KE, A81TA, QL108L, KR411K, and NS441S for a subtype G sample, KR28R, IM31I, ND47N, and IML104IM for a CRF02_AG sample, and T239TA and F463FV for a subtype J sample. In PR, the following changes were observed: PS39PAS in a subtype D sample and VL33V in a CRF02_AG sample. For IN recombinants, the following changes were observed: IM50I and M281MV for a subtype A sample and T124TA for a subtype C strain.

Determination of optimal time point for quantification of EGFP-expressing cells as a marker for viral titer. Taking into account practical considerations and the aim to develop a single-cycle assay, it was necessary to investigate whether infection of U87.CD4.CCR5.CXCR4 cells could accurately be quantified at 24 h postinfection based upon EGFP expression. Both fluorescence microscopy and flow cytometry allowed for detection of EGFP expression at this time point (Fig. 2). Moreover, a linear relationship was observed between volume of viral input and the percentage of EGFP-expressing cells (as determined by flow cytometry).

Susceptibility testing with PIs. In using expression of recombinant virus-encoded EGFP as a marker for viral production

TABLE 5. Accuracy of susceptibility testing of mutant HIV-1 recombinant viruses toward PR, RT, IN, and entry inhibitors

Recombined region	Mutation(s)[a]	Inhibitor	Fold change in IC_{50}[b]	Reference[c]
gag-PR	I13V, I15V, K20R, E35D, M36I, I54V, L63P, I64V, A71V, N88D, L90M*	NFV	7.75	25
PR	I13V, I15V, K20R, E35D, M36I, I54V, L63P, I64V, A71V, N88D, L90M*	NFV	9.46	25
RT	Q151M	AZT	1.60	34
		ABC	2.76	34
		TDF	0.85	44
	K70R	AZT	1.71	30
		ABC	0.95	30
		TDF	0.85	55
	K65R	AZT	1.13	40
		ABC	5.17	40
		TDF	2.41	44
	A62V, S68G, V75I, F77L, F116Y, Q151M	AZT	79.07	29
		ABC	11.66	29
		TDF	2.11	29
	M41L, D67N, K70R, V179I, M184V, Y188L, T215Y**	AZT	10.10	20
		ABC	23.26	20
		TDF	2.02	31
		3TC	>1,100	20
		EFV	111.80	25
		NVP	>100	25
IN	T66I	RAL	0.98	18
		EVG	4.29	18
env	gp41, D36G, V38M	ENF	5.88	22
		AMD3100	1.15	39
	Env-He	AMD3100	No inhibition	35
		MVC	No inhibition	35
	Env-He-gp41 V38M	ENF	2.56	39
	Env-BaL	AMD3100	No inhibition	35
	Env-BaL-gp41 V38M	ENF	4.19	39

[a] Mutations were generated by site-directed mutagenesis, except for the two strains that originated from a clonal sequence from a patient sample (subtype B) (*) and one strain that originated from a clonal sequence from another patient sample (subtype C) (**).
[b] Relative to the wild-type recombinant virus recombined in the respective region (*gag*-PR-p83-2, PR-p83-2, IN-p83-2, *env*-p83-10-gp41-D36G, *env*-He, or *env*-BaL) (triplicate experiment).
[c] Published reference in which comparable results were obtained.

(5), drug susceptibility testing needs to be adjusted to determine PI susceptibility profiles. This is because PIs act by inactivating the viral PR produced in the first replication cycle, rendering the produced virus noninfectious, and thus the infectivity can be measured only in a second replication cycle. Therefore, the viral production after a first round of infection in the presence of a dilution series of PI was quantified in a subsequent viral titration experiment. The optimal time point for transferring the supernatant from the first round to the second round was determined based upon several experiments in which p24 Ag production and virus production were determined for different reference strains and clinical samples (data not shown). Typically, production of virus did not start before 30 to 32 h postinfection, ensuring that transfer of supernatant at 40 to 42 h to the subsequent viral titration procedure generated sufficient virus for reproducible measurements and that only infectious particles produced by cells that were initially infected were detected. Additional experiments revealed that correction for the percentage of infected cells during the first cycle was not necessary to measure the IC_{50} for PIs (data not shown).

Reproducibility and accuracy of drug resistance testing. In order to assess the ability of the novel assay to determine susceptibility, the susceptibility of wild-type NL4.3 virus toward different inhibitors was first determined (Table 4). Since NL4.3 is CXCR4 tropic, it is not inhibited by MVC. Therefore, MVC was tested on a recombinant virus containing the envelope of the R5 strain BaL, and inhibition was indeed observed. For ENF, susceptibility was tested for three different recombinant viruses that harbored the envelope of either an X4 (NL4.3), an R5 (BaL), or an R5/X4 (HE) strain. The coefficients of variation ranged from 7 to 27%, with an outlier of 48% for testing of susceptibility of BaL against ENF.

Next, recombinant viruses containing specific mutations were generated using plasmids created by site-directed mutagenesis or cloned patient samples with known resistance mutations as templates for PCR. The change in drug susceptibility relative to that of the wild type was determined (Table 5). Representative examples of susceptibility curves are shown in Fig. 3. All measurements of altered susceptibility were consistent with the scientific literature in terms of magnitude and specificity. For example, the T66I mutation in the IN gene is known to confer resistance to EVG but not to RAL (18). When several resistance-associated mutations were included in the recombinant virus, an increase in the degree of change in susceptibility was observed (e.g., Q151M complex mutation versus single Q151M mutation). Lastly, inclusion of the V38M mutation in gp41 of X4, R5, or R5/X4 strains resulted in a decreased susceptibility relative to that of recombinant viruses carrying the respective wild-type envelopes, and no evidence of cross-resistance to the CXCR4 receptor antagonist AMD3100 was observed.

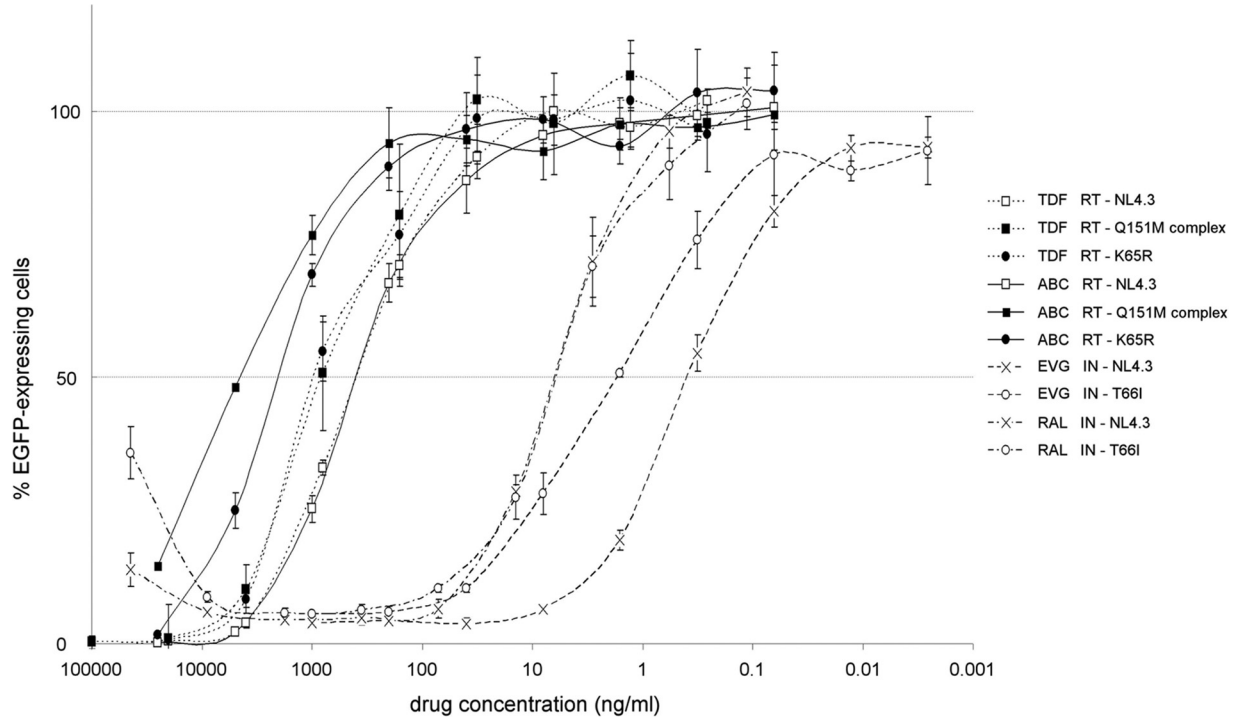

FIG. 3. Representative examples of susceptibility curves for different inhibitors and recombinant viruses. Means and standard deviations of percentages of infection relative to the positive control are shown (data for triplicate experiments are shown). Susceptibility curves are shown for wild-type virus (open squares with dashed line) and RT recombinant virus with A62V, S68G, V75I, F77L, F116Y, and Q151M mutations (filled squares with dashed line) or K65R mutation (filled circles with dashed line) against TDF; for wild-type virus (open squares with solid line) and RT recombinant virus with A62V, S68G, V75I, F77L, F116Y, and Q151M mutations (filled squares with solid line) or K65R mutation (filled circles with sold line) against abacavir; for wild-type virus (× with dashed line) and IN recombinant virus with T66I mutation (open circles with dashed line) against EVG; and for wild-type virus (× with dotted and dashed line) and IN recombinant virus with T66I mutation (filled circles with dotted and dashed line) against RAL.

Assay reproducibility for at least one inhibitor of each drug class was evaluated by three independent determinations of the change in IC_{50} for mutant viruses relative to the wild type (Table 4). The coefficients of variation ranged from 7 to 31%, with the highest variability for EVG and ENF.

DISCUSSION

The use of highly active antiretroviral therapy in the management of HIV disease has resulted in a decrease in HIV-related morbidity and mortality (27). Nevertheless, therapy failure does occur often, with drug resistance as a consequence. The composition of subsequent regimens is guided mostly by genotypic drug resistance testing in the current clinical setting. In cases of multiple failures with broad-drug-class cross-resistance or of failures with new drugs for which genotype-clinical response correlations have not yet been established or are limited, phenotypic drug resistance testing can help in clinical decision-making. Apart from this, phenotypic testing also provides a means to investigate qualitative and quantitative effects of novel mutations or combinations of mutations on drug susceptibilities and is thus a useful tool to improve genotypic interpretation systems.

For practical reasons, such as hands-on time and reproducibility issues, phenotyping is done mostly by recombinant virus assays. Despite the use of different testing strategies, the available assays show good correlations, although care should be taken in extrapolating values near the cutoffs of the different formats (36, 52). The addition of ENF and MVC to the existing range of drugs in clinical practice prompted the need for assays applicable to viruses capable of using either coreceptor. In this respect, the assay described here is a single-cycle format, using U87.CD4.CXCR4.CCR5 cells, based on homologous recombination between patient-derived amplicons and molecular proviral clones (Fig. 4). The proviral clones were tagged with the EGFP reporter gene, and relevant viral genomic regions were removed (p17 codon 7 to PR codon 98 in pNL4.3-ΔgagPR-EGFP, PR codons 1 to 99 in pNL4.3-ΔPR-EGFP, RT codons 1 to 560 in pNL4.3-ΔRT-EGFP, IN codons 1 to 288 in pNL4.3-ΔIN-EGFP, and gp120 codon 34 to gp41 codon 237 in pNL4.3-Δenv-EGFP). Only minor modifications to the described technology would be required to enable susceptibility testing for drugs addressing novel interaction points within the viral replication cycle.

Our assay was optimized for low viral loads and all group M subtypes. Although limited information is available on potential artifacts due to forced intersubtype recombination, intersubtype recombination does occur often in vivo, and recombinant viruses obtained in vitro are clearly viable. Additionally, one in vitro study suggested that using a subtype C backbone as opposed to a subtype B backbone did not significantly alter drug susceptibility measurements (4). The optimized experi-

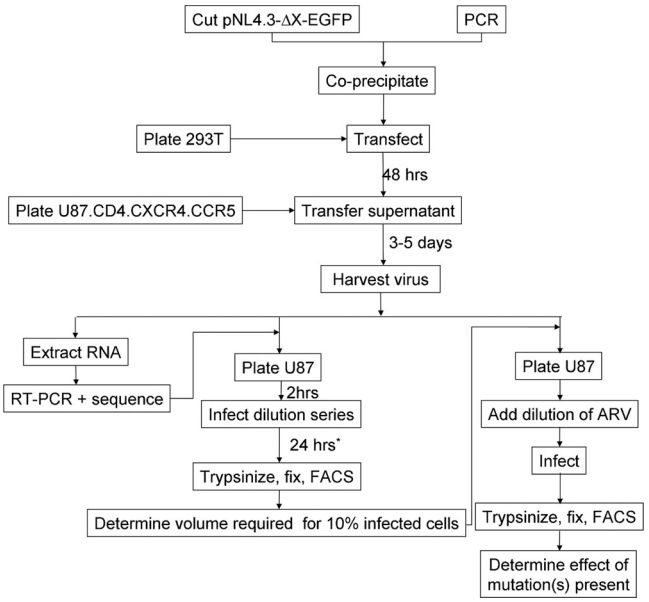

FIG. 4. Schematic overview of recombinant virus assay. *, for testing of susceptibility to PIs, the titration procedure as well as the IC_{50} determination should be modified as indicated in the text. FACS, fluorescence-activated cell sorting; ARV, antiretroviral.

mental conditions enabled the investigation of patient samples regardless of viral load or subtype. The overall extraction and amplification success rate for a set of clinical samples that belonged to the genetic forms A-J, CRF01-03, CRF05, and CRF12-13 within group M was 97%, even if taking only samples above the clinically useful cutoff of 1,000 RNA copies/ml into account. One concern is how well the initial viral population within the patient is represented within the recombinant virus population, as RNA extraction, amplification, and in vitro cultivation represent potential bottlenecks. To address these issues as much as possible, primers were carefully chosen to target the most conserved sites surrounding the viral regions of interest, and cultures were limited in time. No major selection bias occurred, as observed by sequencing comparison between original plasma samples and recombinant virus stocks.

Drug susceptibility values are based on discrimination between infected and noninfected cells by measuring EGFP expression on a single-cell level through flow cytometry. Standard deviations and coefficients of variation were within the ranges found for other recombinant virus assays, and changes were consistent with the scientific literature in terms of magnitude and specificity (15, 17, 31, 34). The single-cycle format ensures very little sequence evolution and selection bias against less replication-competent variants and ensures that potential effects of mutations present in the recombinant virus on interaction with calmodulin or other cellular proteins involved in apoptosis will have no influence on the assay readout (26). An additional value lies in the potential to study recently described resistance mechanisms. Since almost the complete envelope sequence is recombined (starting from codon 34 and stretching to amino acid 237 of gp41), the impacts of mutations within different sections of gp120 on ENF and MVC susceptibilities can be investigated (10, 21, 38, 54; J. Heera, M. Saag, P. Ive, J. Whitcomb, M. Lewis, L. McFadyen, J. Goodrich, H. Mayer, E. van der Ryst, and M. Westby, oral presentation 40LB, 15th Conference on Retroviruses and Opportunistic Infections, Boston, MA, 2008). The recombination of the entire RT sequence enables the measurement of the influence of mutations within the connection and RNase H domains of RT on RT inhibitor susceptibilities (41, 56). Lastly, the potential to make both PR and *gag*-PR recombinants allows for investigation of the impacts of both cleavage site and non-cleavage-site mutations within *gag* on sensitivity to PIs (12, 24, 28, 32).

In conclusion, we have optimized a single-cycle recombinant drug susceptibility assay that allows an accurate and reproducible measurement of susceptibility to all currently approved HIV-1 drugs for patient samples belonging to the major genetic forms within group M. This assay could help to decipher the different pathways leading to drug resistance and could thus improve existing genotypic interpretation systems.

ACKNOWLEDGMENTS

We are thankful to Kristien Erven and Liesbet De Dier for performing p24 Ag enzyme-linked immunosorbent assays.

This work was supported by the AIDS Reference Laboratory of Leuven, which receives support from the Belgian Ministry of Social Affairs through a fund within the Health Insurance System, by the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (grants G.0266.04 and G.485.08), by the Centers of Excellence of the Katholieke Universiteit Leuven (krediet EF/05/015), and by IUAP grant P6/41. Kris Covens was funded by a Ph.D. grant of the Institute for the Promotion of Innovation through Sciences and Technology in Flanders (IWT).

The molecular clones pNL4.3-EGFP and pNL4.3-Δenv-EGFP were provided by M. Quiñones-Mateu of The Cleveland Clinic Foundation (Cleveland, OH). All rights, title, and interest in these materials are owned by The Cleveland Clinic Foundation.

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